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BIOCONVERSION OF PLANT BIOMASS TO ETHANOL

Annual Report and Revised Research Plan
for the period
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ABSTRACT

The objective of this research is to demonstrate on a laboratory scale the technical feasibility of the direct microbial conversion of pretreated wood to ethanol. During the first year of this contract, we investigated the feasibility of biologically delignifying wood with C. prunosum and directly fermenting the pretreated wood to ethanol with a mixed culture. Bench-top fermentations of a thermophilic bacillus growing on glucose and of a mixed culture of thermophilic sporocytophaga (US) and a thermophilic bacillus growing on microcrystalline and amorphous cellulose were evaluated for growth and ethanol production. In the mixed culture fermentation of amorphous and microcrystalline cellulose, the specific rate of substrate depletion was calculated to be 0.087 hr^{-1} and 0.0346 hr^{-1} , respectively. However, defining the growth requirements of C. prunosum and sporocytophaga (US) proved more difficult than originally anticipated. In order to achieve the program objectives within the contract period, a revised research plan was developed based upon chemical pretreatment and the direct fermentation of pretreated hardwood to ethanol.

In place of the biological delignification pretreatment step, we have substituted a chemically supplemented steam pretreatment step to partially delignify wood and to enhance its

accessibility to microbial utilization. Clostridium thermocellum, which ferments cellulose directly to ethanol and acetic acid, has replaced the mixed culture fermentation stage for ethanol production. Research on the production of ethanol from xylose by the thermophilic bacillus ZB-B2 is retained as one means of utilizing the hemicellulose fraction of hardwood. Work on the genetic improvement of the ethanol yields of both cultures by suppressing acetic acid production is also retained.

The rationale, experimental approach, and economic considerations of this revised research plan are also presented.

BIOCONVERSION OF PLANT BIOMASS TO ETHANOL

PART I - ANNUAL REPORT

I. INTRODUCTION

Our analysis of the key technical and economical barriers to the commercial utilization of plant biomass drew attention to the need for developing small scale, decentralized, and low cost processing technology for converting lignocellulosic materials into liquid fuels. The existing and potential biomass productivity of many farming (dairy and grain), lumbering, food processing and animal feeding operations exceeds their annual energy consumption. On site consumption of liquid fuels is not only practical but also provides a logical approach to the introduction of a technology that avoids many of the institutional problems associated with large scale systems. It provides a mechanism for minimizing the price and ensuring the supply of raw materials.

An all-biological process for transforming terrestrial plant biomass into liquid fuels represents a novel and attractive approach to a bioconversion technology that is responsive to many of the recognized barriers to commercial development.

Goals and Objectives

The goal of this research program is the development of a bioconversion process for producing ethanol from lignocellulosic plant biomass. The research is aimed directly at demonstrating on a laboratory scale, the technical feasibility and practicality of a two-stage process for ethanol production from wood. Figure 1 schematically presents the major process elements.

Shredded plant biomass is fed at high solids concentration to

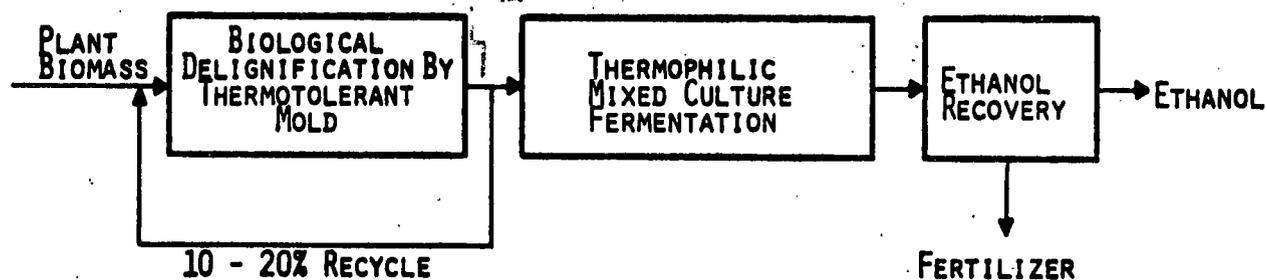


FIGURE 1. A Process for the All-Biological Conversion of Plant Biomass to Ethanol

a bioreactor (see Figure 2) in which sufficient lignin solubilization occurs to make the cellulose accessible for subsequent saccharification. Approximately 10 to 20% of this material is retained for inoculating incoming biomass, and the remainder is discharged into a thermophilic mixed culture fermentor. Cellulose saccharification is accomplished with a thermophilic sporocytophaga, while a compatible thermophilic bacillus simul-

taneously converts the soluble sugars resulting from the saccharification to ethanol. Ethanol recovery is achieved either by conventional batch distillation of the broth or by continuous removal using the modified vacuform process shown in Figure 3.

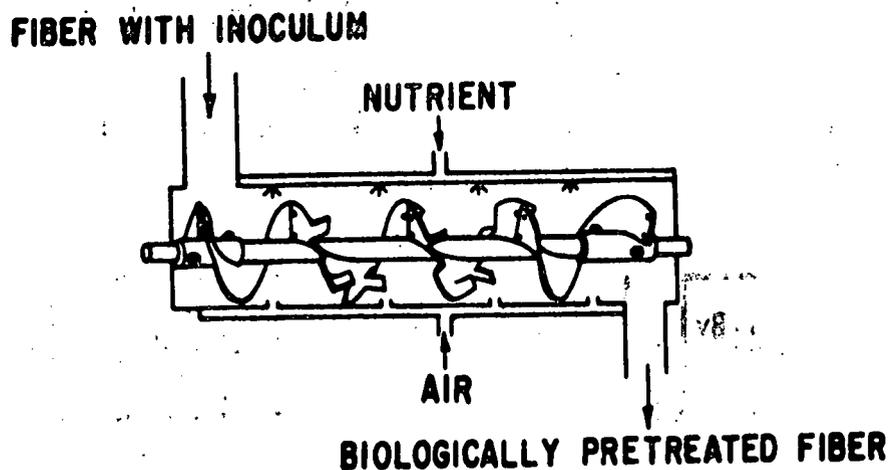


FIGURE 2. Screw Conveyor with Cut Flights on the Shaft for Continuous High Solids Biological Pretreatment

The experimental work presented in Part I of this report summarizes the research conducted during the first year's effort. The discussion is organized around the principal research task; biodelignification, bioreactor design, mixed culture microbiology, and bench-top fermentation.

In Part II of this report, a revised research plan is presented which is based on our experimental findings, recent literature citations, and the accelerated interests of the

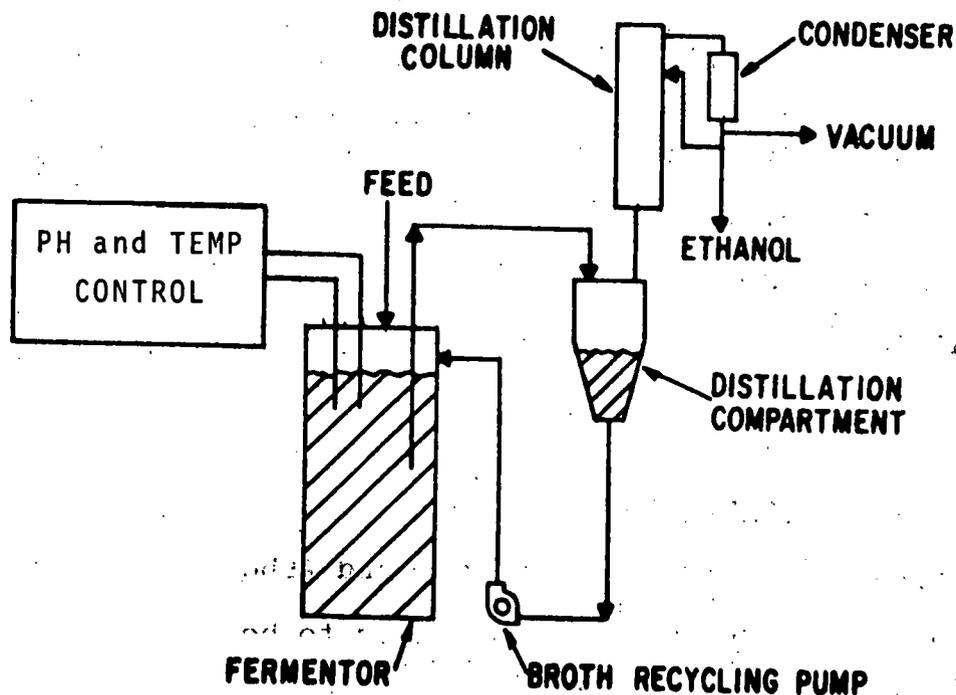


FIGURE 3. Modified Vacuform Process for the Continuous Removal of Ethanol from the Thermophilic Ethanol Fermentation

Department of Energy's Fuels from Biomass program in the early pilot demonstration of practical conversion technology.

II. BIODELIGNIFICATION PRETREATMENT

The objective of this research is the development of a biological pretreatment process that will selectively or preferentially remove or solubilize sufficient lignin to make the remaining cellulosic material accessible for subsequent microbial fermentation to ethanol.

A. Culture Development

Chrysosporium pruinorum (Gilman and Abbott) Carmichael, ATCC 24782, was used in all experiments to be reported and was obtained from the American Type Culture Collection, Rockville, Maryland. This organism has recently been identified as the imperfect state of the fungus Phanerochaete chrysosporium by Burdsall and Eslin.⁽¹⁾ This organism was selected from 21 species of thermophilic and thermotolerant fungi examined for their ability to rapidly degrade a lignocellulosic substrate.⁽²⁾ Cultivation conditions that favor rapid degradation of lignin and cellulose (washed cattle manure feedlot fibers) by Chrysosporium pruinorum have been described.⁽³⁾

B. Nutritional Studies

The minimum nutritional requirements of C. pruinorum (M-10) for growth were determined using 1% glucose and a supplemented

mineral medium (SM) which is given in Table I. Supplying mineral nutrients (mainly nitrogen) to a bioreactor operating on a high solid to liquid ratio (greater than 10% solids) in amounts adequate to support growth and delignification poses a potential problem if the required amount of mineral is greater than its solubility in water; and if high salt concentrations inhibit growth. To resolve the issue, we examined the growth characteristics of M-10 on 1% glucose in the presence of excess nutrient salts. Growth inhibition (dry weight basis) occurs above concentrations of about 2.5 times the minimum nutritional requirement level (Table II).

Further work on the nutritional salt tolerances of C. pruinosum (M-10) demonstrated that the major supplemented mineral (SM) constituents (KH_2PO_4 , Na_2HPO_4 , and $(\text{NH}_4)_2\text{SO}_4$) are responsible for the observed growth inhibition above the 2.5 excess level (Table II). The concentration of the carbon source (1.0% glucose) was kept constant while various ingredients were added in five-fold excess concentration, with the remaining SM components constant at 1x. Dry weight cellular mass yields after three days incubation at 38°C in shake flasks were used as the measure of growth inhibition (Table III).

The inorganic nitrogen source $(\text{NH}_4)_2\text{SO}_4$ is shown to have the most deleterious effect at the 5x level, while trace elements appear to stimulate the growth of M-10.

TABLE I

Supplemented Mineral Medium (SM)

<u>Components</u>	<u>Concentration</u>
$(\text{NH}_4)_2\text{SO}_4$	5.0 g
KH_2PO_4	6.04 g
Na_2HPO_4	0.85 g
Trace Elements Solution	10 ml
Agar	15.0 g
Deionized Distilled Water	960 ml
Thiamine HCl (filter sterilized)	1 mg/ml
pH 5.0	
40 ml of 25% (w/v) glucose solution	

Trace Elements Solution

<u>Components</u>	<u>Concentration</u>
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	5.0 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.5 g
CaCl_2	0.5 g
Versenol	5.0 g
Deionized Distilled Water	250.0 ml

TABLE II

Effect of Nutrient Concentration on Growth ofC. pruinosum

<u>Nutrient Concentration</u>	<u>Dry Weight(mg)</u>
1.0x	20.1
2.5x	13.1
5.0x	2.7
10.0x	2.2

TABLE III

C. pruinosum Nutritional Salt Tolerance

<u>SM Component(s) at 5x</u>	<u>Dry Weight(mg)</u>
$\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$	8.4
$(\text{NH}_4)_2\text{SO}_4$	3.6
$(\text{NH}_4)_2\text{SO}_4 + \text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$	3.6
Trace Elements	24.2
Thiamine-HCl	22.3
Control (all 1x)	15.3

If one assumes a 20 to 30% sawdust fiber feed concentration in the biodelignification reactor and also that no more than 25% of the available sugar (50% of sawdust) will be consumed for growth and delignification, then the total amount of sugar utilized will be on the order of 2.5 g/100 ml of feed (20 grams fiber x 0.5 cellulose x 0.25 = 2.5 g). The 1x level of nutrient concentration is adequate to support M-10 growth on glucose. Since the minimum required nutrient concentration is consistent with the maximum expected sugar utilization, we may anticipate that even a two-fold excess nutrient feed concentration may be employed in the bioreactor without significant effect on the growth of M-10.

The effect of various organic nitrogen sources individually and in combination with stimulatory concentrations of trace elements and thiamine hydrochloride on the growth of C. pruinosa are shown in Table IV.

All organic nitrogen sources examined except urea enhanced the growth of M-10. Based on these studies, the original growth medium (Table I) was modified to include 0.5% Bacto-peptone and the concentration of trace elements was increased five fold.

C. C. pruinosa Growth on Wood

Conditions previously found to support good growth of C. pruinosa (M-10) on washed cattle manure fibers were employed

TABLE IV
Effect of SM Medium Modifications on
Growth of *C. pruinosum*

<u>Medium Modifications</u>	<u>Dry Weight(mg)</u>
Trace Elements-5x	100.8
Thiamine HCl-5x	99.9
Trace Elements-5x, Thiamine HCl-5x	101.1
Peptone-0.5%	269.2
Trace Elements-5x, Peptone-0.5%	542.1
Thiamine HCl-5x, Peptone-0.5%	318.8
Trace Elements-5x, Thiamine HCl-5x, Peptone-0.5%	537.8
Urea-0.3%	55.0
Proteose-peptone-0.1%	250.7
Proteose-peptone-0.5%	273.1
Tryptone-0.1%	232.5
Tryptone-0.5%	284.6
Control - all 1x	100.6

to examine its growth on Ponderosa Pine sawdust. Dried sterilized sawdust (~200 mg) was placed on a membrane filter which was layered on a 200 ml bed of supplemented mineral (SM) agar medium. The membrane was used to facilitate sample handling. The center of the sawdust was inoculated with a spore suspension of C. pruinorum. Despite numerous attempts, we were unable to observe growth of C. pruinorum on Ponderosa Pine.

Additions of yeast extract and/or peptone to the medium, each at a concentration of 0.1 and 0.5%, resulted in good growth of M-10 on the agar surface but not on the sawdust. Similarly, Ponderosa Pine which was ball-milled for 18 hours in a porcelain jar, also failed to support the growth of M-10. To test the possibility that Ponderosa Pine contained C. pruinorum growth inhibitors, a manure fiber/pine fiber gradient plate was prepared and inoculated with M-10 (Figure 4).

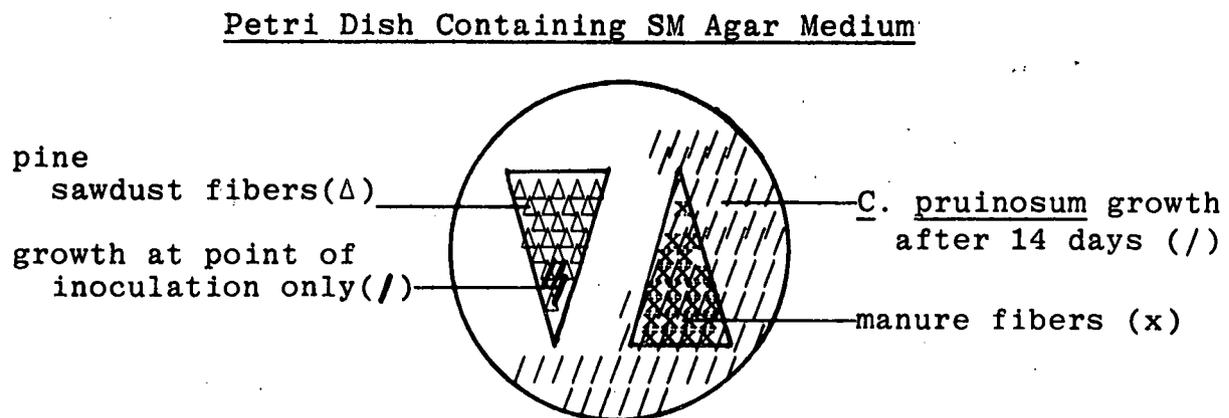


FIGURE 4. Fiber Gradient Plate

After the first seven days, C. pruinorum growth was observed on the manure fiber and only on the agar medium to the right on the manure fiber. These results were interpreted to indicate that a diffusible substance in the pine sawdust was interfering with the growth of M-10. Several attempts at solvent-extraction of this substance proved unsuccessful. Ten grams of pine sawdust fibers were placed in 100 ml acetone and agitated at room temperature overnight. The acetone was decanted and replaced with 100 ml of absolute ethanol and agitated for an additional nine hours at room temperature. The ethanol was decanted, and the residual fibers were rinsed four times with water, filtered, washed again with 1 liter of distilled water, and dried at 60°C overnight. Even after this rigorous washing procedure, M-10 failed to grow on these solvent-extracted fibers.

Since the inhibitor appeared to diffuse in agar, we examined distilled water extracts of the pine sawdust for growth inhibition. Pine sawdust wash water was used as a partial diluent in preparing SM broth. When inoculated with M-10, C. pruinorum grew as it typically does in the broth, which suggests that if the pine does contain an inhibitor, it is not readily soluble in water.

Several unsuccessful attempts were also made to use a manure fiber infusion and a water soluble extract to stimulate

growth on pine sawdust fibers. In one case, 3 ml of filtered aqueous extract, prepared by agitating 5 g of manure fiber with 100 ml of distilled water, was added to 200 ml of the SM agar medium. In the second case, 2 ml of sterile manure fiber extract was added directly to 200 mg of pine fibers on an SM agar plate. To prepare an infusion, 1.8 g of manure fibers in 1800 ml of distilled water was heated with agitation at 75°C for 1½ hours and at 100°C for 30 minutes. The suspension was filtered and combined with 200 ml of 10x SM to give 2 liters of SM agar medium. No growth on pine was obtained using either the aqueous manure fiber extract or the infusion. Neither a large inoculum nor the use of a vegetative cell inoculum showed any growth on pine sawdust, and inocula grown in the presence of cellulose as opposed to dextrose behaved similarly.

Various other woods were also examined, e.g. ash, maple, and birch. Growth as judged by the appearance of white filamentous hyphae was observed on maple and birch, but only after four weeks on ash. Growth on maple is not as rapid as on manure fiber but appeared sufficient to permit the studies to go forward.

In the interests of integrating these studies with other work tasks, we elected to discontinue further work on pine sawdust and to concentrate on maple. C. pruinatum strains adapted to growth on pine could no doubt be developed but not without impeding other aspects of the proposed research.

Previous attempts to reproducibly grow M-10 on high solids, in moist environments were successful only on petri dishes which contained an agar mineral medium overlaid with lignocellulosic fibers supported on a porous membrane for quantitative assays of cell mass and substrate degradation. Good visible growth of M-10 in erlemmeyer flasks containing 200 mg quantities of maple fiber moistened with 1.0 ml of modified SM medium broth was observed between four and seven days after inoculation with 0.1 ml of spores and incubation at 38°C. Incubation was conducted in a humid atmosphere, and the fibers were moistened every three to four days with 0.1 - 0.2 ml of broth. The conditions also proved satisfactory for growth of M-10 on manure fibers in flasks in the absence of agar. Based on these results, quantitative flask studies of M-10 growth and delignification were initiated on maple fibers.

D. Cellulose Accessibility

The objective of this study is to correlate the amount of M-10 growth and delignification with the accessibility of the residual cellulose as measured by saccharification with a T. viride enzyme preparation. The experimental design (Table V) was patterned after earlier studies of M-10 grown on manure fibers on petri dishes (Figure 5) and incorporated a temperature shift while enzymatic lignin and cellulose degradation continued (Figure 6).

TABLE V

Experimental Design for Cellulose AccessibilityDelignification of Maple by C. pruinorum

- I. $A + B \xrightarrow{\text{not incubated}} + C \xrightarrow{\text{incubated at } 55^{\circ}\text{C, 1 hr}} SS_{T_0}$
- II. $A + B \xrightarrow{\text{incubated at } 38^{\circ}\text{C, "x" days}} SS_1$
- III. $A + B \xrightarrow{\text{incubated at } 38^{\circ}\text{C, "x" days}} \xrightarrow{\text{incubated at } 55^{\circ}\text{C, 1 hr}} SS_2$
- IV. $A + B \xrightarrow{\text{incubated at } 38^{\circ}\text{C, "x" days}} + C \xrightarrow{\text{incubated at } 55^{\circ}\text{C, 1 hr}} SS_3$
- V. $A + B \xrightarrow{\text{incubated at } 38^{\circ}\text{C, "x" days}} \xrightarrow{\text{incubated at } 55^{\circ}\text{C, 3 days}} SS_4$

$$\text{True SS} = SS_3 - SS_1 - SS_2$$

SS_4 = Accessibility of cellulose after temperature shift.

A = Maple Fibers (200 mg)

B = C. pruinorum (spores & vegetative cell inocula)

C = Trichoderma viride enzyme

SS = Soluble Sugars as determined by Anthrone method

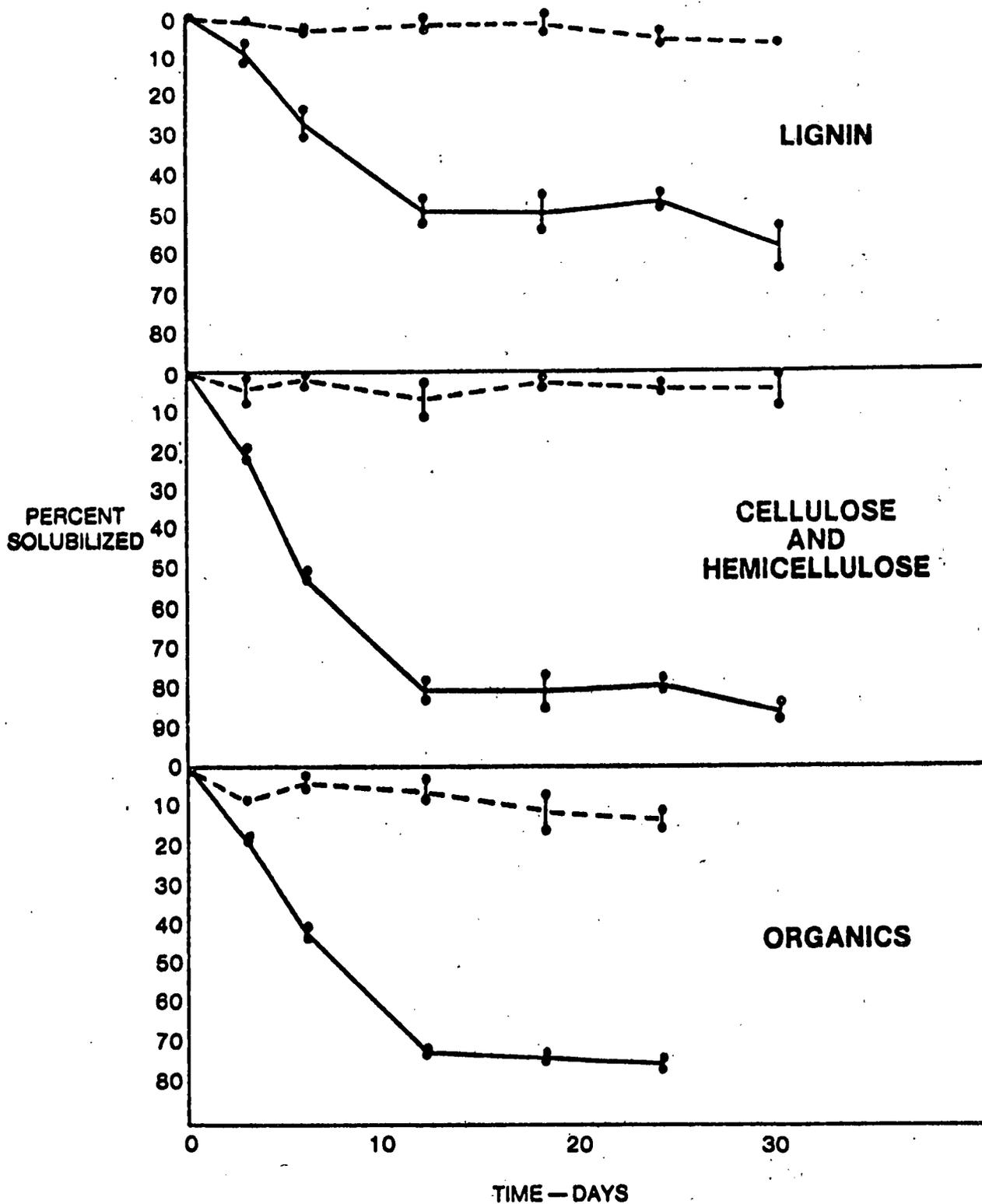


FIGURE 5. Lignin and Cellulose Degradation by *C. pruinosa* Growing on a Damp Substrate

—, Inoculated Culture
 ----, Uninoculated Culture

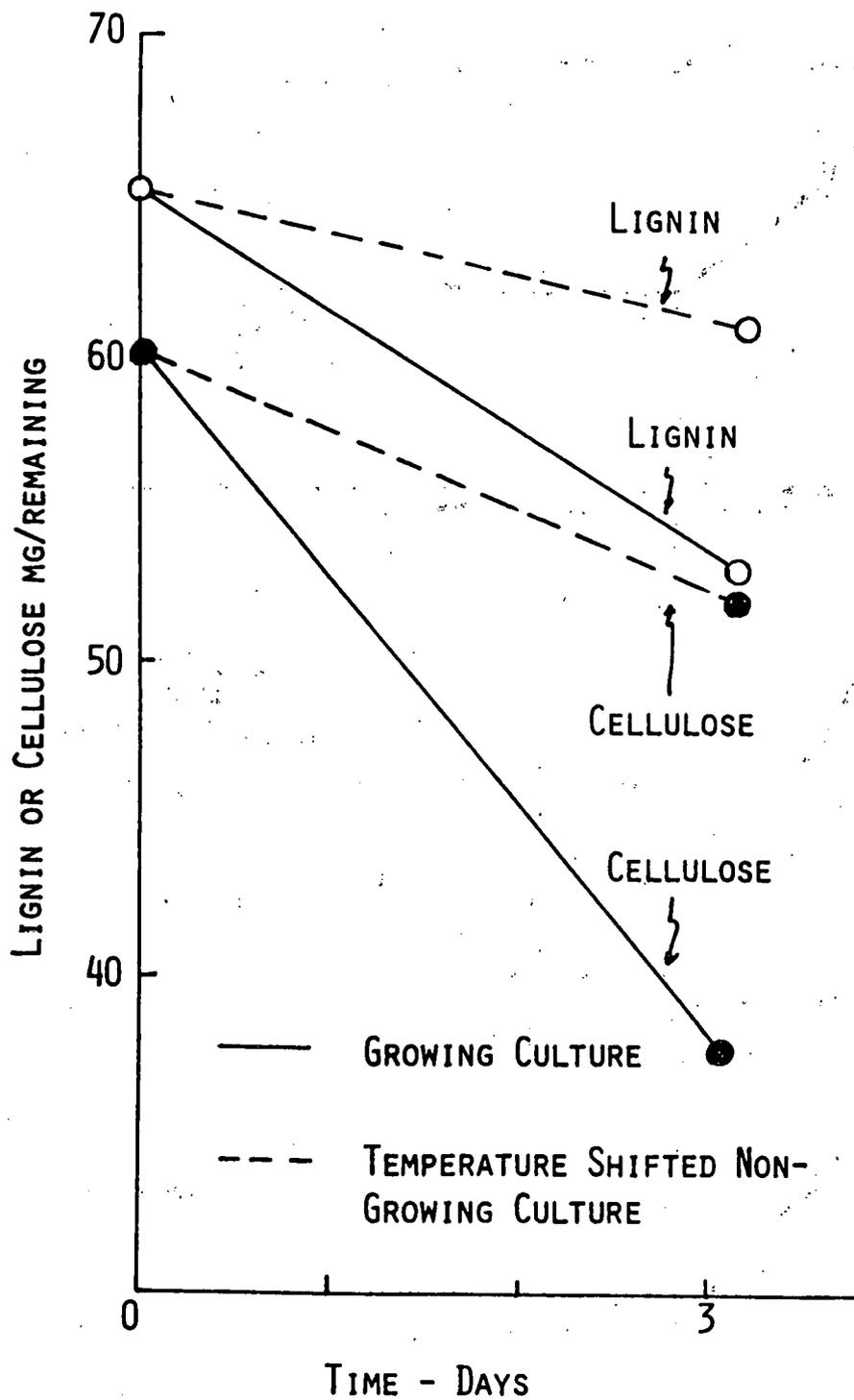


FIGURE 6. Lignin and Cellulose Degradation by Growing and Temperature-Shifted (Non-Growing) Cultures of *C. prunosum*

Using vegetative cells, as well as spores, 200 mg of maple fibers containing 1.0 ml of modified SM broth was inoculated and incubated at 38°C under humid conditions for various times. Under these conditions, which were identical to earlier successful flask studies, C. pruinorum visible growth could not be observed, and soluble sugar production was negligible. Variations in the environmental and inoculation conditions were examined--some of which are shown in Table VI--in an attempt to obtain growth on maple fibers but with no success.

TABLE VI

Attempts to Grow C. pruinorum on Maple in Flask

<u>Variable Conditions</u>	<u>Growth</u>
Incubator #1 (38°C)	-
Spore Inoculum (9 days old), foam plug	-
Vegetative Cell Inoculum (3 days old), foam plug	-
Spore Inoculum (9 days old), cotton plug	-
Humidity (~30-100%)	-
Spore Inoculum (9 days old), foam plug; manure fiber only (control)	+

Visual growth of M-10 on manure fibers in flask under comparable conditions was observed in a subsequent experiment (Table VII) but growth was accompanied by only about 20% cellulose and no lignin degradation.

TABLE VII

Growth of *C. pruinosum* on Manure Fibers in Flask

Growth Conditions:

Modified SM Broth (1.0 ml)

200 mg Manure Fibers

Spore Inoculum (9 days old)

25 ml Erlenmeyer Flask (foam plug)

Stationary Incubation, 38°C, High Relative Humidity

Addition of 0.1 ml Modified SM Every 48 Hours

Harvested after 8 Days Growth:

Cellulose Remaining - 55 mg

Lignin Remaining - 57 mg

The experimental results were reviewed by Dr. Steven L. Rosenberg (Department of Chemistry, University of California at Berkeley) who indicated that he, too, was unable to repeat his original M-10 growth and delignification results on manure fibers (see Figures 5 and 6).

In an attempt to resolve some of the problems with obtaining reproducible growth conditions for M-10, we evaluated the growth of C. pruinosa in flasks under similar conditions on four carbon substrates: glucose, cellobiose, Avicel, and Whatman #1 filter paper. Visual growth and dry weight yields declined in the order given, and the results varied in repeat experiments. These experiments suggested that at least part of the variation was arising from cultural characteristics, i.e. physiological state, age, or morphology.

When incubated in flask, C. pruinosa was observed to grow in the form of mycelial balls of various size and numbers. Four-day-old cells were harvested and gently homogenized in an attempt to prepare a more uniform inoculum of hyphal fragments. The cells prepared in this manner were used to inoculate flasks containing 1 and 5% glucose and various combinations of trace elements and peptone added to SM (Table VIII).

Homogenization of the cells prior to inoculation apparently improved the uniformity of the inoculum and the reproducibility of growth under the conditions studied. Glucose consumption was independent of the initial glucose concentration and varied only moderately with trace elements and peptone additions to SM. The first six experiments indicate that after 1% glucose is consumed, that further growth and glucose consumption is precluded under the experimental conditions. In runs seven and eight, a

TABLE VIII

Effect of Substrate Concentration and Nutrients on Growth of C. pruinosum

<u>Medium</u>	<u>Dry Weight (mg)</u>	<u>Glucose Remaining (mg/ml)</u>		<u>Total Glucose Consumed (mg/ml)</u>
		<u>24 hrs</u>	<u>96 hrs</u>	
SM - 1% glucose	372.6	9.0	0	10
SM - 1% glucose 5 x Trace Elements	399.1	9.5	0	10
SM - 1% glucose 0.5% Peptone	463.9	9.5	0.25	9.75
SM - 1% glucose 5 x Trace Elements 0.5% Peptone	372.1	10.5	1.0	9
SM - 5% glucose	486.7	44.0	39.0	11
SM - 5% glucose 5 x Trace Elements 0.5% Peptone	562.8	47.0	41.0	9.0
SM - 5% glucose 5 x Trace Elements	1.1368	44.0	41.5	8.5
SM - 5% glucose 0.5% Peptone	1.3386	47.0	40.5	9.5

visible slime was associated with the harvested cell mass. Because the cell mass from these two runs was dried under conditions identical to the other runs, the former probably contained residual water, which would account for the abnormally high cell yield. Unfortunately, the cultures were discarded before the need for reweighing was apparent.

E. Mutation Protocol

Parallel development work on an effective mutation protocol for C. pruinosum was initiated early in the year in anticipation of the desirability of isolating M-10 mutants with enhanced enzyme production or activity and/or which are unable to utilize cellulose-derived soluble sugars. The experimental objective is to select plating conditions which reproducibly yield 50 - 100 colonies per plate. Oxgall and Rose Bengal, which are known hyphal inhibitors, were investigated for their ability to restrict the spreading plate growth of C. pruinosum.

Adjusting the inoculum level and the concentration of one or both of these agents proved difficult. At a given inoculum size, reproducible plate growth was difficult to achieve with either inhibitor. In order to obtain clearing zones and discrete colonies, we investigated several experimental parameters: agar/cellulose monolayers vs bi-layers; microcrystalline, attrited microcrystalline, and acid-swollen cellulose substrates;

various Oxgall and Rose Bengal concentrations; and inoculum size. Zones of clearance, due to the presence of cellulase, on monolayer plates using 5% attrited microcrystalline cellulose as the carbon source, were clearly discernable with both inhibitory agents. At high inhibitor concentrations, (>0.5% Oxgall and >5.0% Rose Bengal) no M-10 growth was observed. At lower inhibitor concentrations, variation of the inoculum size from 10^{-1} to 10^{-6} dilutions of a standardized inoculum resulted in, respectively, the appearance of excessive colony plate counts or single nondiscrete colony formation with no hyphal inhibition.

The following procedure was used to standardize the inoculum of C. pruinosum. A PDA plate (Potato Dextrose Agar) was inoculated in the form of a spread plate with C. pruinosum, incubated at 38°C for eight to ten days. The spores were harvested from the plate with 10 ml sterile distilled water by rubbing the surface gently with a glass spreading rod. The 10 ml of spore concentrate was vortexed in a tube containing sterile glass beads (4 mm) to break up any clumps. The spore concentrate usually read 1% LT @ 525 nm on a B&L Spectronic 20. A 10^{-1} dilution was always adjusted to read 45 - 40% LT; this inoculum was considered standardized for experiments.

Concentrations of Oxgall between 0.025% and 0.05% and Rose

Bengal employed at 2.5 mg% or 5.0 mg% gave the most reproducible plating; however, the optimum inoculum concentration was not determined.

Conclusion

On the basis of these experimental findings, it is apparent that considerably more basic research on determining suitable environmental cultivation conditions for C. pruinosum is required before further developmental studies of biological pretreatment can be justified. Fundamental studies of C. pruinosum growth, physiology, and nutritional and environmental requirements fall outside the objectives of this contract. Further studies of this organism will be discontinued. An alternative pretreatment approach has been developed and will be discussed in Part II of this report.

III. BIOLOGICAL REACTOR

Solid State Bioreactor Design and Construction

The proposed all-biological ethanol-from-wood fermentation process relies heavily upon the effectiveness of the first stage biological pretreatment unit operation to solubilize sufficient lignin to make the residual cellulose readily available for enzymatic saccharification. In the design and construction of a high solids or solid state bioreactor, the most crucial design parameters are the nutrient, air, and moisture distribution systems. Previous studies with C. prunosum demonstrated that the ability of this organism to grow and solubilize lignin is particularly dependent upon the moisture content of the environment and the nutrient supply rate. In view of these considerations and the need to handle a concentrated slurry feed, we elected to employ a screw conveyor with cut flights on the shaft for the continuous biological pretreatment unit operation (Figure 2). The conveyor cut flights provide slow substrate mixing, while the mist spray and pressurized air inlet controls distribute and maintain optimum moisture and oxygen levels. The C. prunosum nutrient salt tolerance studies reported earlier indicated that nutrient concentrations two times higher than required for growth can be

added to the bioreactor without significant deleterious effect on growth. The construction effort on a 3 foot by 6 inch ID screw conveyor with cut and folded flights housed in a tubular trough was arrested soon after the difficulties with achieving reproducible growth of M-10 were recognized.

IV. MIXED CULTURE MICROBIOLOGY

The mixed culture fermentation of cellulose to ethanol is conducted by employing a thermophilic sporocytophaga (US) for rapidly saccharifying cellulose to soluble sugars which are readily converted by a compatible thermophilic bacillus co-culture into ethanol. Mixed culture compatibility is favored primarily by the dependence of each culture upon growth factors supplied by the other and by the more rapid growth of the bacillus. Differences in the response of each culture to initial and altered pH, temperature, oxygen, tension, and agitation rate is used to control the growth rate of both cultures and the rates of saccharification and ethanol production during the fermentation.

A. Sporocytophaga (US)

During the previous studies on the production of microbial protein from lignocellulosic materials, a very active cellulolytic anaerobe was isolated and identified as the first reported thermophilic sporocytophaga.⁽⁴⁾ The culture grows between 50°C and 65°C and over the pH range from 7.0 to 8.5. It produces large terminal spores which are heat stable at 96°C for 20 to 30 minutes. It will not grow on the surface of agar plates,

but will grow readily in shallow or deep broth culture. During growth on cellulose, it produces a bright yellow-orange pigment but not when grown on glucose or cellobiose. The cellulase activity appears to be primarily cell-wall bound with a pH optimum of about 6.0. When grown in the presence of a thermophilic bacillus, only mineral salts and cellulose are required for growth. Under these conditions, the specific rate of cellulose utilization, in uncontrolled test tube fermentations was calculated to be about 0.12 hr^{-1} which corresponds to a volumetric efficiency of about 2.5 g/1-hr.

B. Thermophilic Bacilli

A number of thermophilic bacilli were initially examined for growth compatibility with sporocytophaga US and for ethanol production. All of the cultures examined produced acidic metabolites along with varying amounts of ethanol. In the absence of a direct method of screening for ethanol production, plates containing an acid indicator were employed to select cultures with minimal acid production.

A number of thermophilic cultures, which had previously looked promising, were selected for ethanol screening on phenol red plates. These cultures were taken from TSA stock slants and grown in mineral broth with 0.1% yeast extract and either 0.5% glucose or 0.5% cellobiose. After incubation at 55°C , they

were diluted out to 10^{-4} , and 0.1 ml of the 10^{-4} dilution was used as an inoculum for agar plates containing mineral broth, 0.1% yeast extract, 0.5% glucose or cellobiose, and 3 μ g/ml phenol red. The plates were placed in a dessicator, flushed with N_2 , and incubated at $55^\circ C$ for several days. Individual colonies were picked off of the agar plates on to TSA slants and stored for future use. The best cultures from the phenol red plates were inoculated into mineral broth with 0.1% yeast extract and 0.5% glucose or cellobiose. After growth at $55^\circ C$, they were deproteinized and measured for ethanol production. The cultures producing the highest ethanol concentrations are shown in Table IX.

In order to be assured that the cultures are not vitamin limited, ten of these cultures were selected for growth on mineral media containing 0.5% yeast extract and 0.5% glucose or cellobiose. Ethanol assays were run, and the results are given in Table X. The observed ethanol concentrations correspond to about 35% of the theoretical value.

The dependence of ethanol yields upon yeast extract concentration for two of the cultures (ZB-B2, XB-G2) that produced the most ethanol was examined and compared (Table XI) with the two cultures (OK_2 and OD_1) that previously gave the highest ethanol yields at low phosphate concentrations (important because of costs and compatibility with sporocytophaga

TABLE IX

Ethanol Production by Thermophilic Bacilli

<u>Substrate Cellobiose</u>		<u>Glucose</u>	
<u>Culture</u>	<u>mg/ml ETOH</u>	<u>Culture</u>	<u>mg/ml ETOH</u>
XG 1	0.38	OW ₂ 1	0.32
UE 3	0.39	OW ₂ 2	0.41
OL ₂ 3	0.34	XB 1	0.39
XB 1	0.37	XB 2	0.36
XB 2	0.45	XC 2	0.39
ZB 1	0.41	ZB 1	0.43
ZB 2	0.45	ZG 3	0.36
		ZG 4	0.37

TABLE X
Ethanol Production by Selected
Thermophilic Bacilli

<u>Culture</u>	<u>Substrate*</u>	<u>ETOH Produced (mg/ml)</u>
UE	G	0.56
	B	0.70
XB _G	G	0.68
	B	0.57
XB _G	G	0.76
	B	0.85
ZB _G	G	0.30
	B	0.85
ZB _G	G	0.81
	B	0.87
OW	G	0.63
	B	0.78
XB _B	G	0.81
	B	0.71
XB _B	G	0.85
	B	0.78
XC	G	0.73
	B	0.82
ZB	G	0.71
	B	0.76

* G = Glucose
 B = Cellobiose

TABLE XI

Ethanol and Acid Production from Cellobiose
by Thermophilic Bacilli

<u>Culture</u>	<u>Yeast Extract mg/ml</u>	<u>Cellobiose Utilized mM</u>	<u>Ethanol Produced mM</u>	<u>MEQ Acid Produced</u>	<u>% of* Maximum Yield</u>
ZB-B2	0.25	0.13	0.19	0.30	39
	0.50	0.13	0.21	0.30	44
	1.00	0.13	0.22	0.33	45
XB-G2	0.25	0.13	0.12	0.33	25
	0.50	0.13	0.24	0.33	50
	1.00	0.13	0.20	0.36	41
OK 2	0.25	0.14	0.12	----	23
OD 1	0.25	0.14	0.10	----	21

*Maximum yield = 46% of carbohydrate w/w

Grown anaerobically at 55°C without shaking.

Media: Cellobiose, 5.0 g, $(\text{NH}_4)_2\text{SO}_4$, 1.0 g, Na_2HPO_4 , 5.3 g; KH_2PO_4 , 4.0 g; NaCl , 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.008 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.02 g; CaCl_2 , 0.02 g; Versenol, 0.2 g per 1000 ml adjusted to pH 7.5.

which grows best at 0.07 molar phosphate). The acidic end-products were not identified but simply titrated with 0.1N NaOH. Yeast extract was supplied as a source of growth factors; however, at the higher concentrations, it must be considered as a source of carbon and energy as well. Changing the ratio of cellobiose to yeast extract would introduce additional complications since these test tube experiments were not pH controlled. In order to relate the yield of ethanol directly to cellobiose, experiments were performed to identify the culture with the simplest growth requirements.

C. Growth Requirements of Thermophilic Bacilli

A conventional Latin square method in which the growth factors given in Table XII, removed one at a time, was used to assess the growth requirements of four cultures. The results indicate that OK₂ and OD₁ either require less common growth factors (i.e. polypeptides or nucleic acids) or have need for a more complex assortment than presented in this experiment. Culture ZB-B2, on the other hand, when grown on mineral medium with cellobiose as the sole carbon and energy source, require thiamine, riboflavin, biotin, or one or more of the three nucleotide bases. The observed growth in the absence of biotin and the bases may suggest that these specific requirements are accessory rather than essential. A similar interpretation can

TABLE XII

Thermophilic Bacillus Growth Requirements

<u>Growth Factors*</u>									<u>Culture Growth**</u>											
1	2	3	4	5	6	7	8	9	<u>ZB-B2</u> (hrs)			<u>XB-G2</u> (hrs)			<u>OK₂</u> (hrs)			<u>OD₁</u> (hrs)		
<u>A,G,U</u>	<u>Thi</u>	<u>Rf</u>	<u>CaP</u>	<u>B₆</u>	<u>NA</u>	<u>Biot</u>	<u>FA</u>	<u>AA</u>	<u>24</u>	<u>48</u>	<u>96</u>	<u>24</u>	<u>48</u>	<u>96</u>	<u>24</u>	<u>48</u>	<u>96</u>	<u>24</u>	<u>48</u>	<u>96</u>
-	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0
+	+	+	+	+	+	+	+	+	2+	3+	3+	3+	3+	3+	+	+	+	+	+	+
-	+	+	+	+	+	+	+	+	0	0	2+	0	+	+	0	+	+	0	+	+
+	-	+	+	+	+	+	+	+	0	0	0	0	+	+	0	+	+	0	+	+
+	+	-	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	+
+	+	+	-	+	+	+	+	+	2+	3+	3+	2+	2+	2+	+	+	+	+	+	+
+	+	+	+	-	+	+	+	+	2+	3+	3+	2+	3+	3+	+	+	2+	+	+	+
+	+	+	+	+	-	+	+	+	2+	3+	3+	2+	3+	3+	0	+	+	0	+	+
+	+	+	+	+	+	-	+	+	0	0	2+	0	0	0	0	+	+	0	+	+
+	+	+	+	+	+	+	-	+	2+	3+	3+	2+	3+	3+	0	+	+	0	0	+
+	+	+	+	+	+	+	+	-	2+	3+	3+	0	2+	2+	0	+	+	0	0	+

34

*1) Adenine Sulfate, Guanine HCl, Uracil, 5.0 µg/ml each; 2) Thiamine HCl, 1.5 µg/ml; 3) Riboflavin, 0.5 µg/ml; 4) Calcium Pantothenate, 1.0 µg/ml; 5) Pyridoxal HCl, 2.0 µg/ml; 6) Nicotinic Acid, 5.0 µg/ml; 7) Biotin, 0.8 µg/ml; 8) Folic Acid, 0.1 µg/ml; 9) Amino Acids (vitamin free), 10.0 µg/ml. All factors except the bases; Adenine, Guanine and Uracil were sterilized by filtration and added to the heat sterilized mineral medium.

**Growth was estimated as visual turbidity when compared with growth in the test tube with all growth factors.

be advanced for culture XB-G2 except that it appears to have additional requirements for one or more amino acids.

D. Mutation Protocol

The co-production of acetic acid by the thermophilic bacilli is limiting the attainable ethanol yield to about 50% of that achieved by commercial yeast homofermentors. A mutation effort was initiated to develop a bacillus strain capable of yeast-like conversion of glucose to ethanol. For our initial experiments, N-methyl, N'-nitro, N-nitrosoguanidine (MNNG) was used as the mutagenic agent.

The thermophilic bacillus NW was grown for 16 hours at 55°C in mineral broth containing 0.5% yeast extract and 0.5% cellobiose. Test tubes were inoculated with 0.1 ml of a 1/100 dilution of a 24 hour culture grown on the same medium. The cells were centrifuged, washed with one volume of M/15 phosphate buffer, and resuspended in an equal volume of the same buffer. The mutagenic agent MNNG was added to test tubes containing 10 ml of a suspension of NW cells in concentrations of 100, 250, 500, 750, and 1000 µg per test tube. The cultures were incubated at 55°C for 30 minutes with shaking. After incubation, the cultures were diluted and streaked on mineral agar plates containing 0.5% yeast extract and 0.5% cellobiose.

Figure 7 shows the killing curve obtained with the mutagenic agent MNNG. From these results, we concluded that a

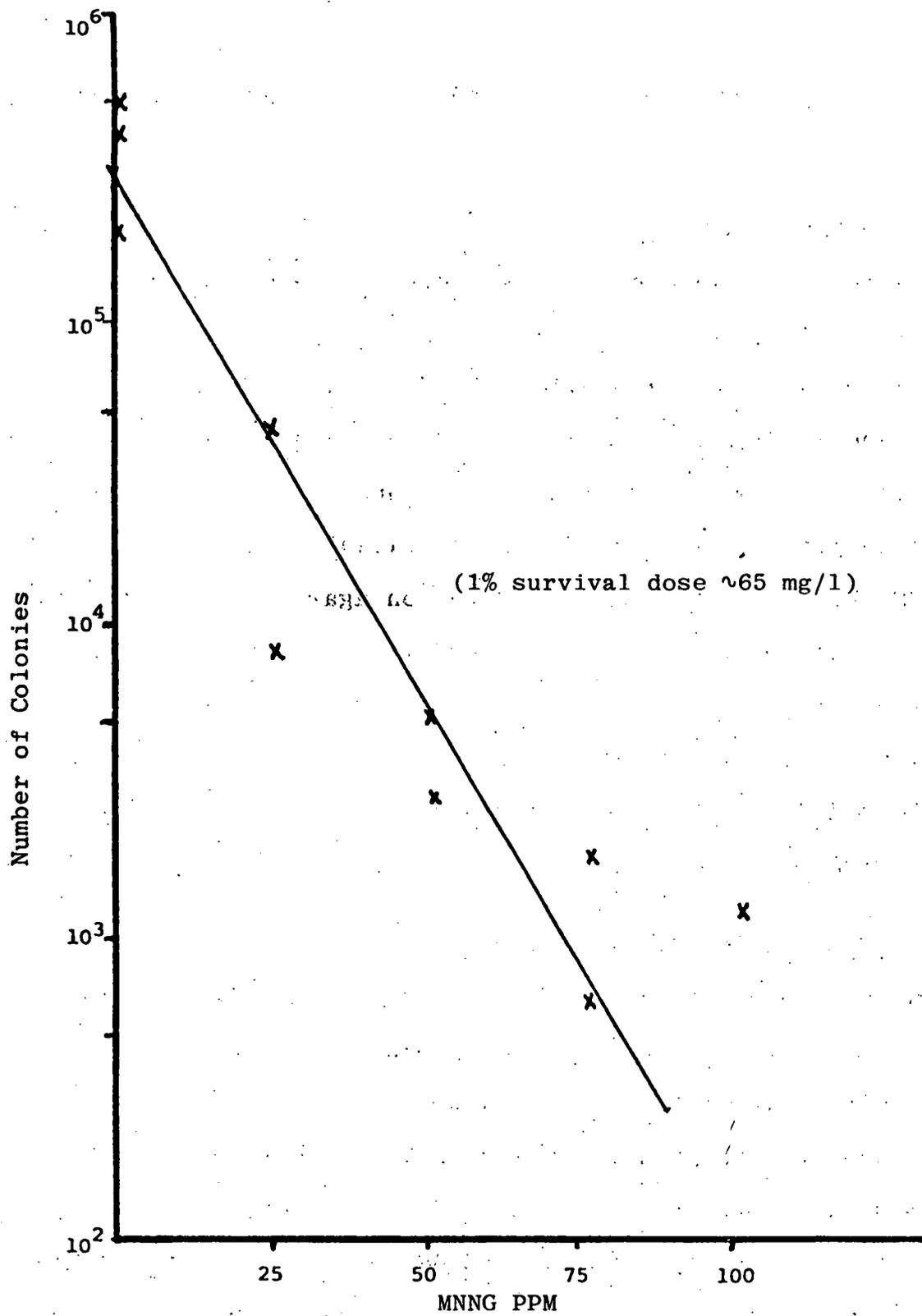


FIGURE 7. Survival Curve of Thermophilic Bacillus NW

dose of 65 mg/l for 30 minutes at 55°C is adequate to obtain a 99% kill.

E. Mutant Screening

The metabolic end-products for glucose fermentation by the thermophilic bacilli examined to date are primarily ethanol, acetic acid, and CO₂. Desirable mutants will display enhanced ethanol and limited acid production. In the absence of a visual detection scheme for ethanol production on plates, reduced acid production is employed to initially screen mutants.

MNNG treated cultures are plated on agar plates containing peptone, yeast extract, glucose, and bromthymol blue indicator. The plates are incubated for 24 - 48 hours. Colonies showing no or limited color change are compared with untreated controls and selected for subsequent test tube fermentation and gas chromatographic ethanol and acetic acid determinations.

F. Ethanol Tolerance of Thermophilic Bacillus ZB-B2

The growth of ZB-B2 in the presence of varying concentrations of alcohol was studied to determine the point at which significant growth inhibition occurs.

The results of growth studies on 1% glucose in the presence of from 0 to 8.0% ethanol are plotted in Figure 8 as a function of the optical density after 24 and 42 hours of growth. Batch culture growth in the presence of ethanol is delayed. However,

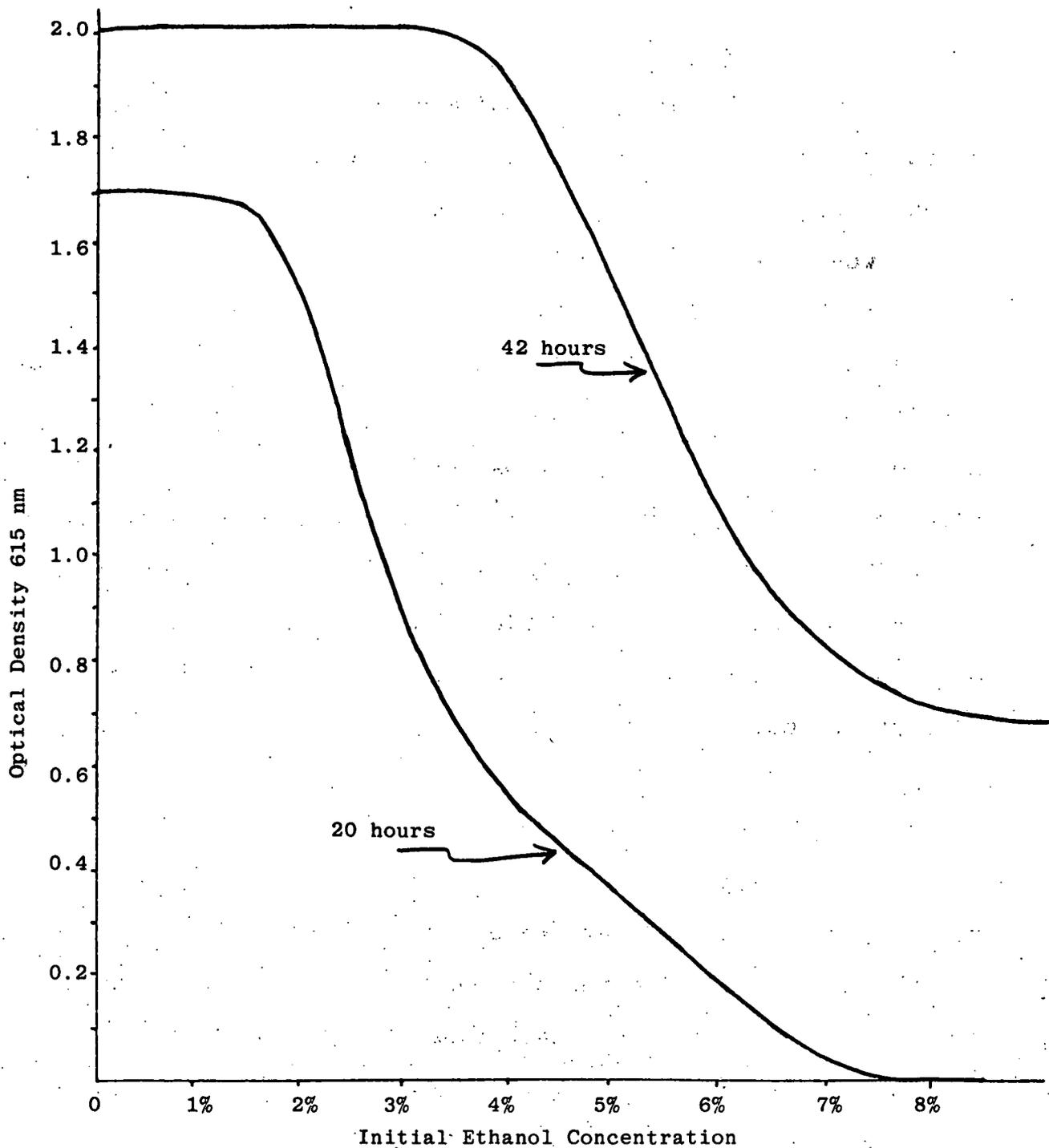


FIGURE 8. Effect of Initial Ethanol Concentration on the Growth of Thermophilic Bacillus ZB-B2

in continuous culture, ethanol concentrations up to 4% can readily be tolerated.

G. Growth of Thermophilic Bacillus on Xylose

The hemicellulose content of many biomass substrates constitutes a significant fraction of the total biomass. If this fraction can be converted to ethanol, the overall conversion yields would be enhanced.

The ability of the thermophilic strain ZB-B2 to grow on xylose and a mixture of xylose and glucose was examined in test tube fermentations. The results of growth studies on 0.5% xylose, a mixture of 0.25% xylose and 0.25% glucose, and 0.5% glucose alone in mineral media containing 0.5% yeast extract and 0.2% tryptone are shown in Figures 9, 10, and 11, respectively. Optical density (turbidity at 615 nm) determinations, sugar utilization, and ethanol and acetic acid production are also presented.

There was a long lag before initiation of growth on xylose (Figure 8). The apparent biphasic growth at 8 to 10 hours, which is not observed on glucose or the mixed sugars, is probably due to initial growth on yeast extract and tryptone. The shortest lag period and the most rapid growth occurred on the mixed sugars. In replicate runs, the ratio of ethanol to acetic acid production after 23 hours averaged 0.75 on glucose, 0.5 on xylose, and 0.75 on the mixed sugars.

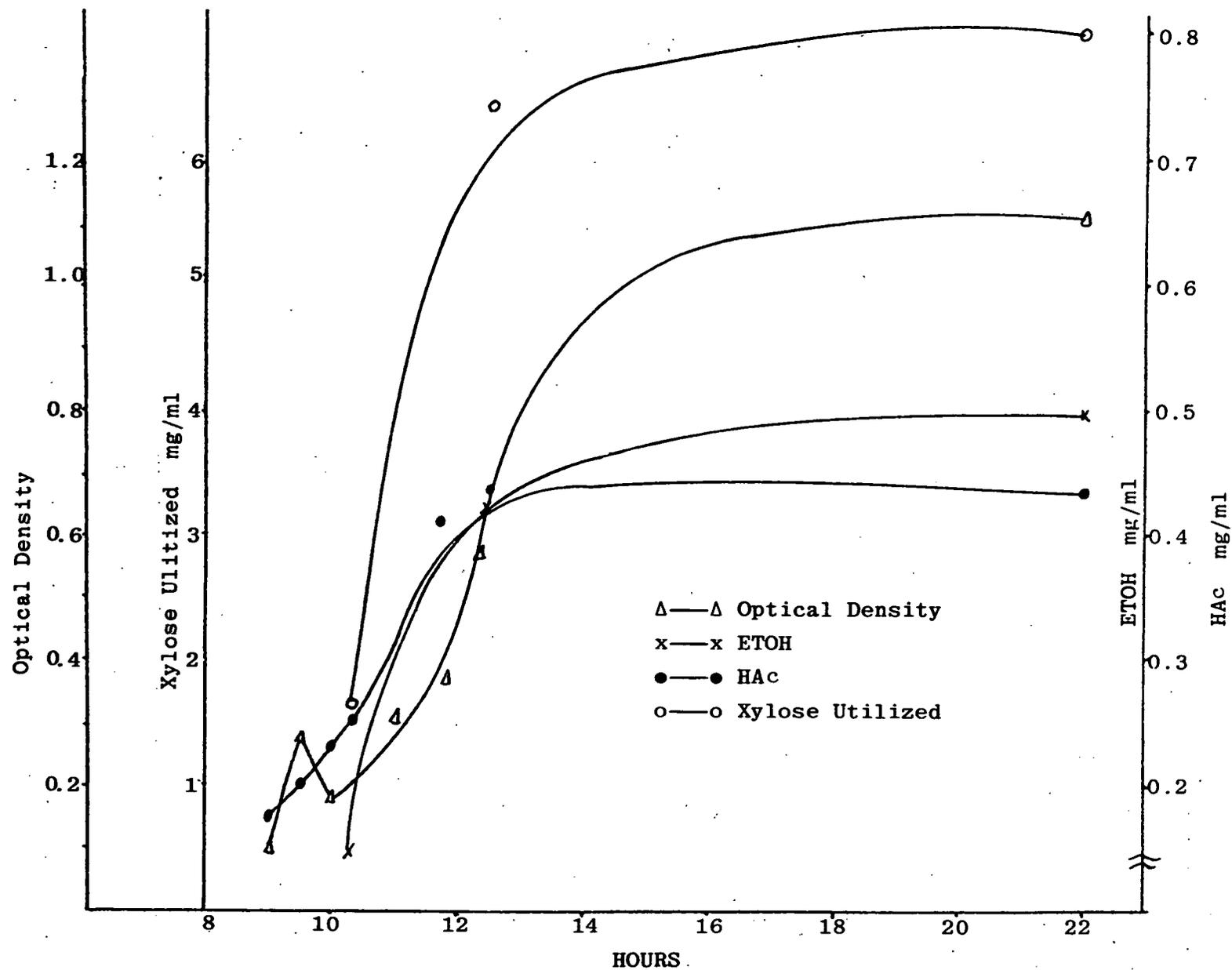


FIGURE 9. Growth of ZB-B2 on 0.5% Xylose in Medium #3

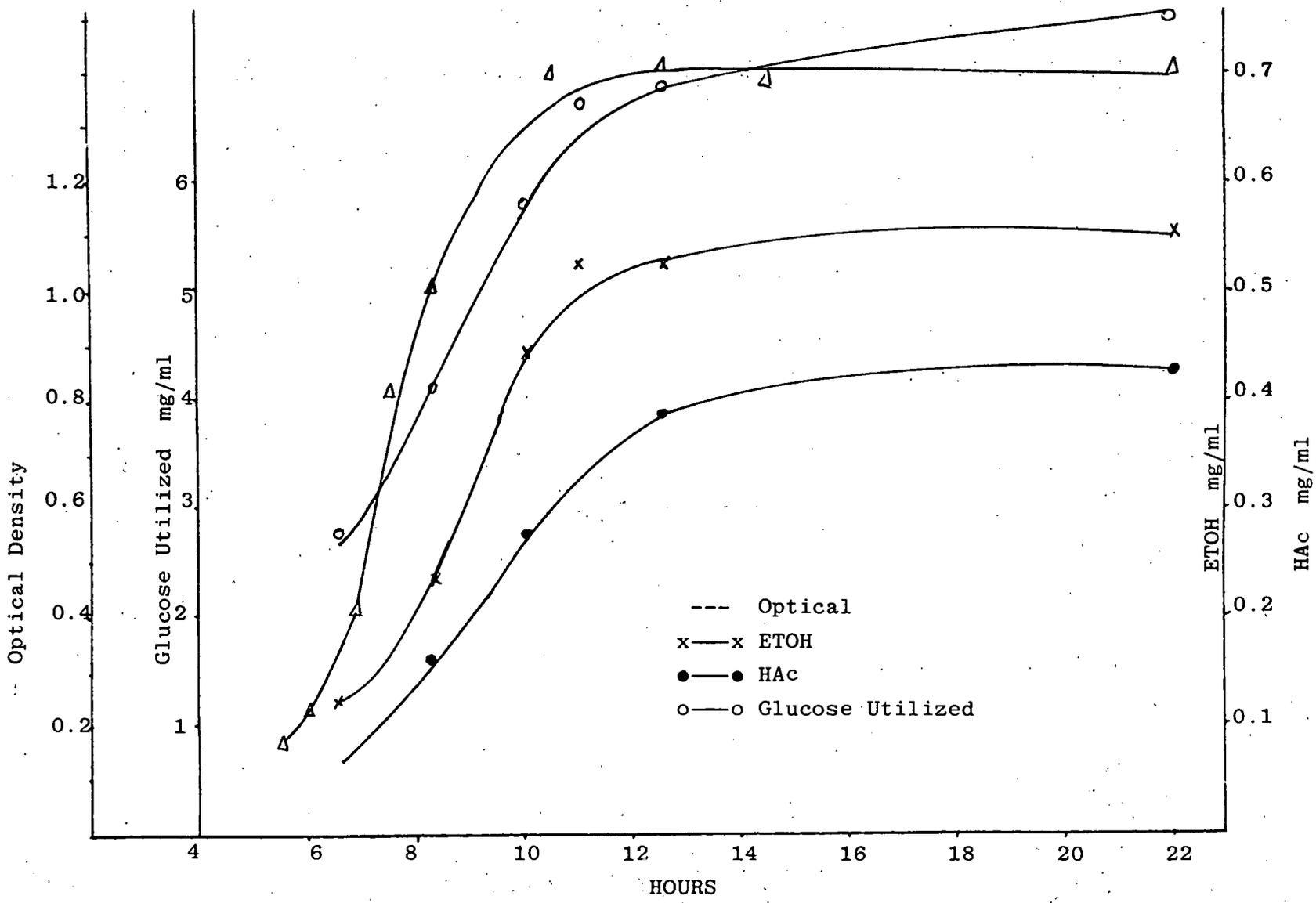


FIGURE 10. Growth of ZB-B2 on 0.5% Glucose in Medium #3

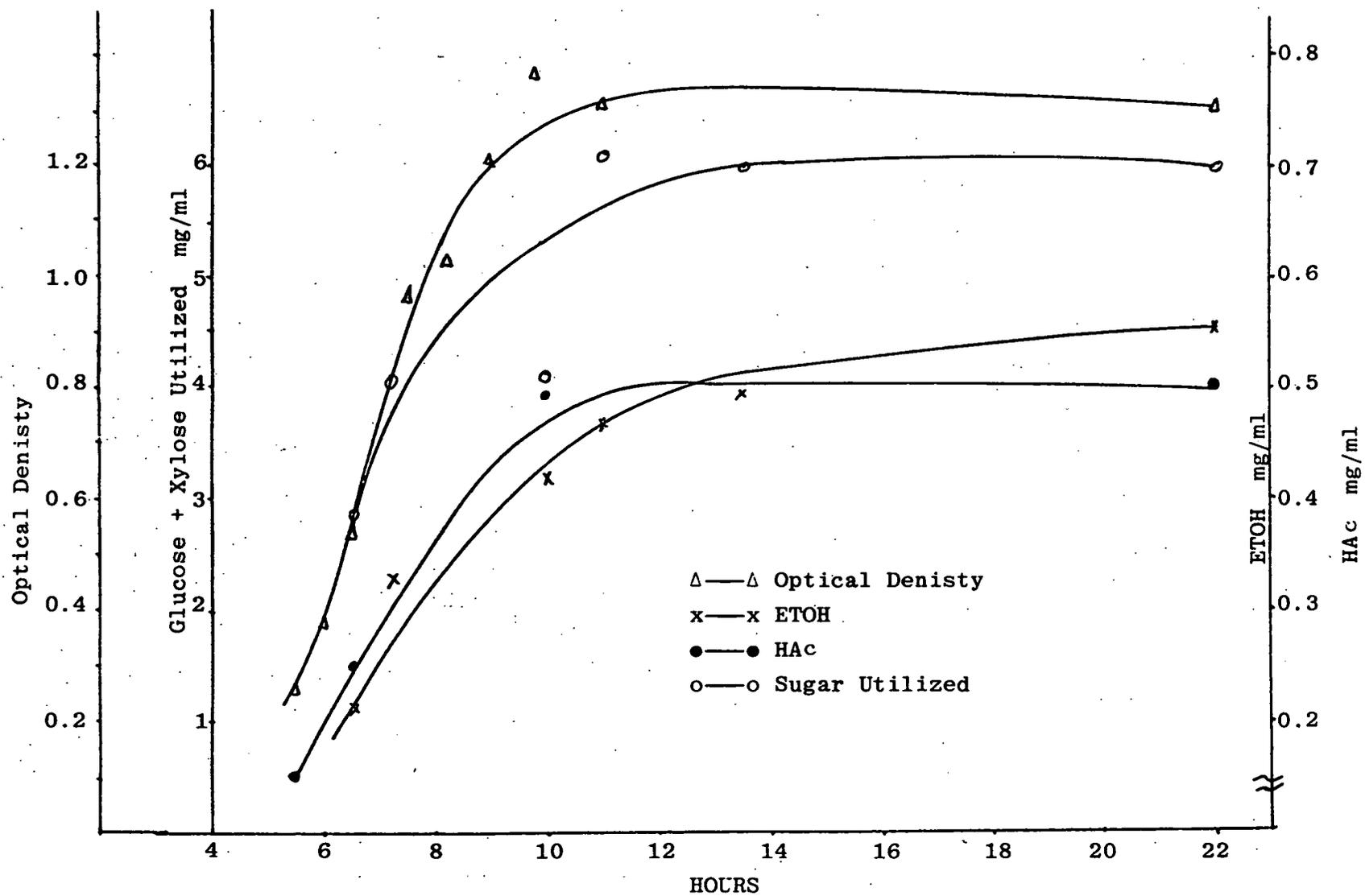


FIGURE 11. Growth of ZB-B2 on 0.25% Xylose + 0.25% Glucose in Medium #3.

H. Purification Studies on Sporocytophaga Strain US

Defining the growth requirements and product yields of sporocytophaga have proven more difficult than originally anticipated. It now appears that sporocytophaga requires for growth some product derivable from the bacillus co-culture that is not found in common yeast extract, peptone, or casein preparations. The growth of sporocytophaga in mixed culture is always preceded by the initial development of modest bacillus growth; however, additions of sonicated or heat-killed bacilli are ineffective in initiating the growth of sporocytophaga. While we have not yet determined what function the bacillus performs in initiating the growth of sporocytophaga, it is very probable that the former supplies a specific nucleic acid or peptide.

Some of the myxobacteria as well as the cytophaga and the recently discovered Bdellovibrio bacteriovorus have been found to require living host bacteria.⁽⁵⁾ In some cases, heat-killed cells will substitute, but in others, only living cells support good growth.

The anaerobic roll tube method of Hungate⁽⁶⁾ and the modified serum bottle technique of Miller and Wolin⁽⁷⁾ have been used to isolate individual anaerobic colonies. In addition, the Gas PakTM anaerobic system⁽⁸⁾ for growing cultures on plates and in test tubes has been used without success.

The complex growth requirements of sporocytophaga strain US suggest that the effort required to purify this culture is beyond the scope of the current contract and that additional purification studies would unduly retard progress on the primary objectives. Preliminary experiments on an alternative cellulolytic anaerobe (see Section IV) are sufficiently encouraging to justify discontinuing further work on sporocytophaga.

V. BENCH-TOP FERMENTATION

In this section, we review the results obtained when a mixed culture of sporocytophaga (US) and the thermophilic bacillus (NW) are grown on microcrystalline and amorphous cellulose and compared to the batch and continuous fermentation of NW grown on glucose. A kinetic study of Clostridium thermocellum growing on microcrystalline cellulose is also reported.

A. Materials and Methods

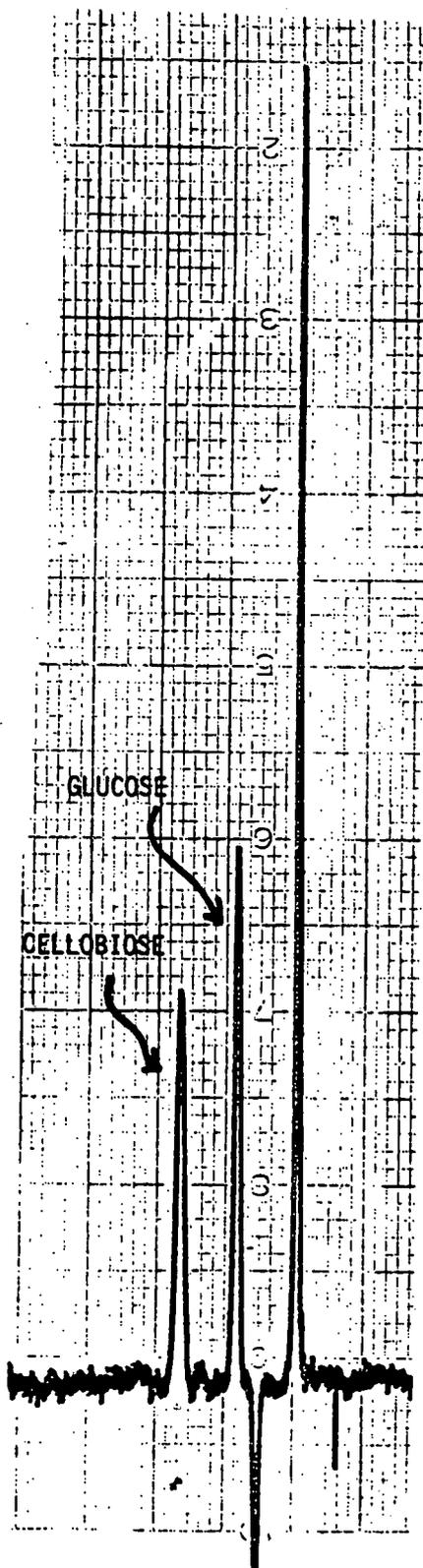
Amorphous cellulose was prepared according to Walseth.⁽⁹⁾ Microcrystalline cellulose (PH 105, 19 μ) was dissolved in cold 85% H_3PO_4 and then reprecipitated in cold water. The precipitated swollen cellulose was continuously washed with distilled water until the wash water pH was neutral. The swollen cellulose was resuspended in distilled water and stored in the refrigerator. The swollen cellulose was used directly in the fermentor runs without drying.

Microcrystalline cellulose. PH 105 Avicel was purchased from the FMC Corporation. The average particle size is 19 μ . Less than 1% of this grade Avicel was retained on a 450 mesh screen.

Cellobiose. B-D (+) cellobiose was obtained from the Sigma Chemical Company.

Soluble sugar. Total soluble sugar was routinely measured as glucose by the anthrone method (dissolution in 72% H₂SO₄ and spectrophotometric determination at 610 mμ after reaction with anthrone reagent) on a Technicon model AA-1 autoanalyzer using glucose as a standard. Cellobiose and glucose assays were performed on a Waters Associates' model 244, high pressure liquid chromatograph system, which consisted of a solvent programmer and UV absorbance and differential refractometer detectors. Samples were injected onto a Waters Associates' μ-Bonapak/carbohydrate column (4 mm x 30 cm) eluted with a constant (4 ml/min) solvent (acetonitrile/water: 75/25) flow rate detected by refractive index. Figure 12 shows a typical high pressure liquid chromatograph of glucose and cellobiose with a retention time of 6 and 9.8 minutes, respectively. An excellent linear correlation was obtained for glucose concentrations ranging from 0.5% to 8% (Figure 13). A good linear correlation of the cellobiose concentration was also observed between 1% and 6%. Using this solvent system, the lowest detectable amounts of glucose and cellobiose are 15 and 20 μg, respectively.

Analysis of volatile acids via gas chromatography. A glass column packed with the porous Chromosorb 101 was found to efficiently separate the acids commonly observed in anaerobic fermentation broths. Figure 14 shows the chromatographic



Column: Bondapak/carbohydrate
Flow: 4 ml/min
Solvent: CH₃CN/H₂O (75/25)
RI = X4
Glucose: 0.05%
Cellobiose: 0.05%
Injection Volume: 150 µl

FIGURE 12. HPLC Chromatograph of Glucose and Cellobiose

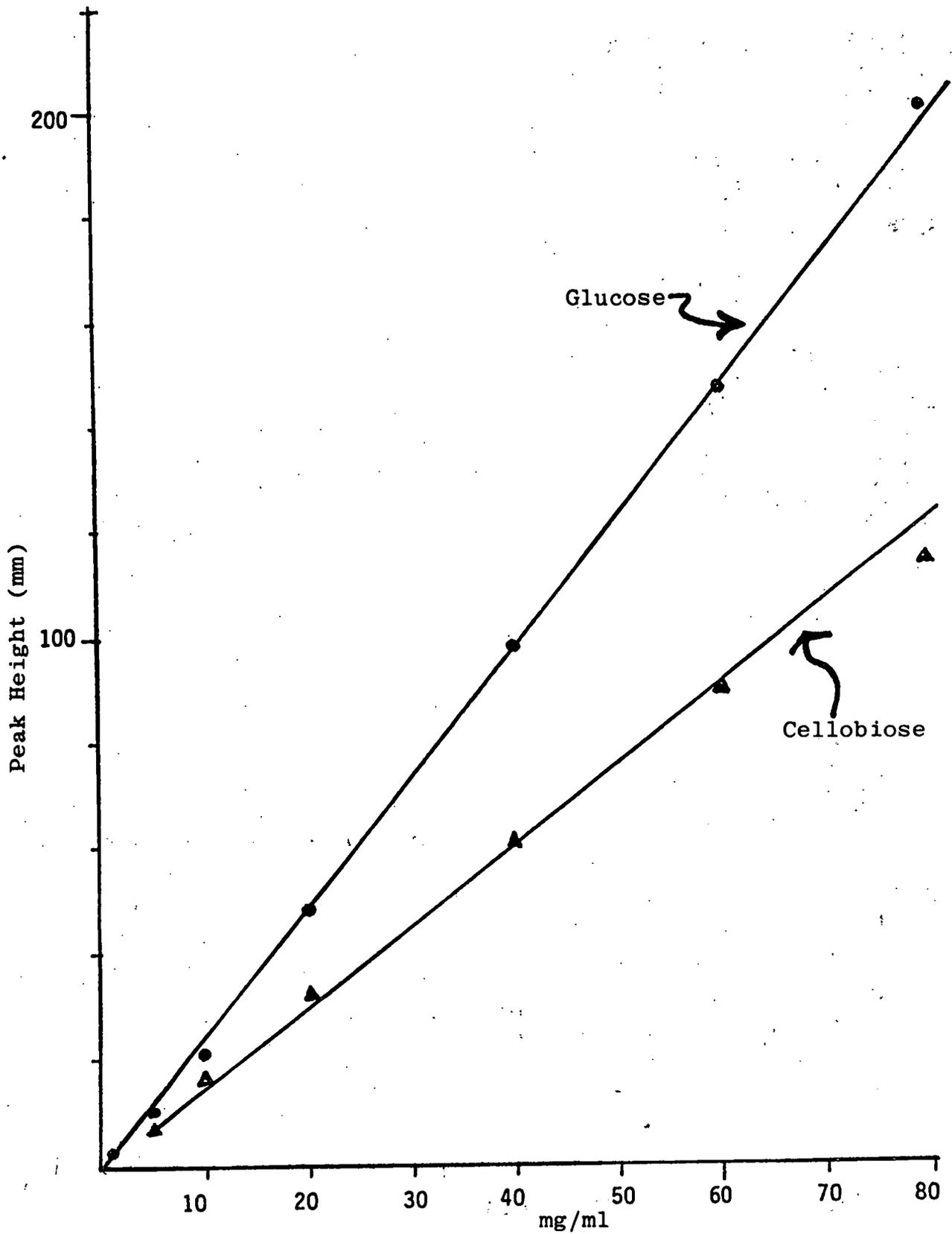


FIGURE 13. Standard Curves and High Pressure Liquid Chromatograph Analysis of Glucose and Cellobiose

TEMP1 150 195
 TIME1 0.0
 RATE 20.00
 TEMP2 210
 TIME2 15.0
 INJ TEMP 200 200
 FID TEMP 300 300
 AUX TEMP 300 300
 OVEN MAX 300

CHT SPD 1.00
 ATTN 2↑ 7
 FID SGNL A
 SLP SENS 1.00
 AREA REJ 101
 FLOW A 12
 FLOW B 0
 OPTN 0
 0.1 AREA REJ -
 0.1 ATTN 2↑ 20
 1.0 ATTN 2↑ 10

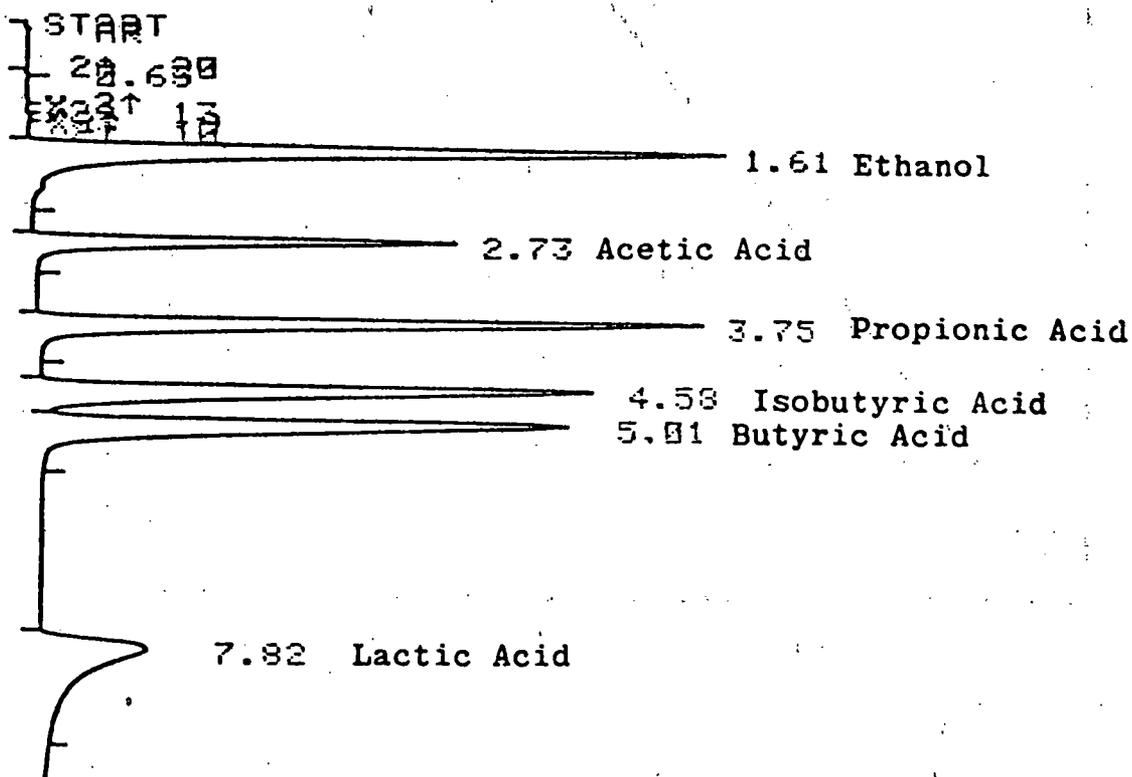


FIGURE 14. Gas Chromatographic Analysis of Fermentation End-Products /

separation obtained with a mixture of acids and ethanol prepared from authentic samples.

Symmetrical peaks were observed for acetic, propionic, isobutyric, and butyric acids. However, lactic acid showed substantial tailing.

A good linear correlation was obtained for acetic acid concentrations up to 0.1 mg/ml (Figure 15).

For the analysis of fermentation broth, the clear fermentor broth (1 ml) was acidified with 0.5 ml of 3N HCl solution. The solution was then injected directly into the column with a syringe. If precipitation occurred after the acidification, the precipitate was removed by centrifugation prior to injection to G.C.

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

Cellular protein. Cellular protein was prepared by sonicating the cell pellet in 0.1 N NaOH solution for three minutes in an ice-water bath. The clear supernatant from centrifugation was analyzed for cellular protein.

T.C.A. precipitable protein. Cell-free filtrate was precipitated overnight with 5% trichloroacetic acid (T.C.A). The precipitate was redissolved in 0.1 N NaOH solution for protein analysis.

Cellulase activity. The cellulase activity was measured

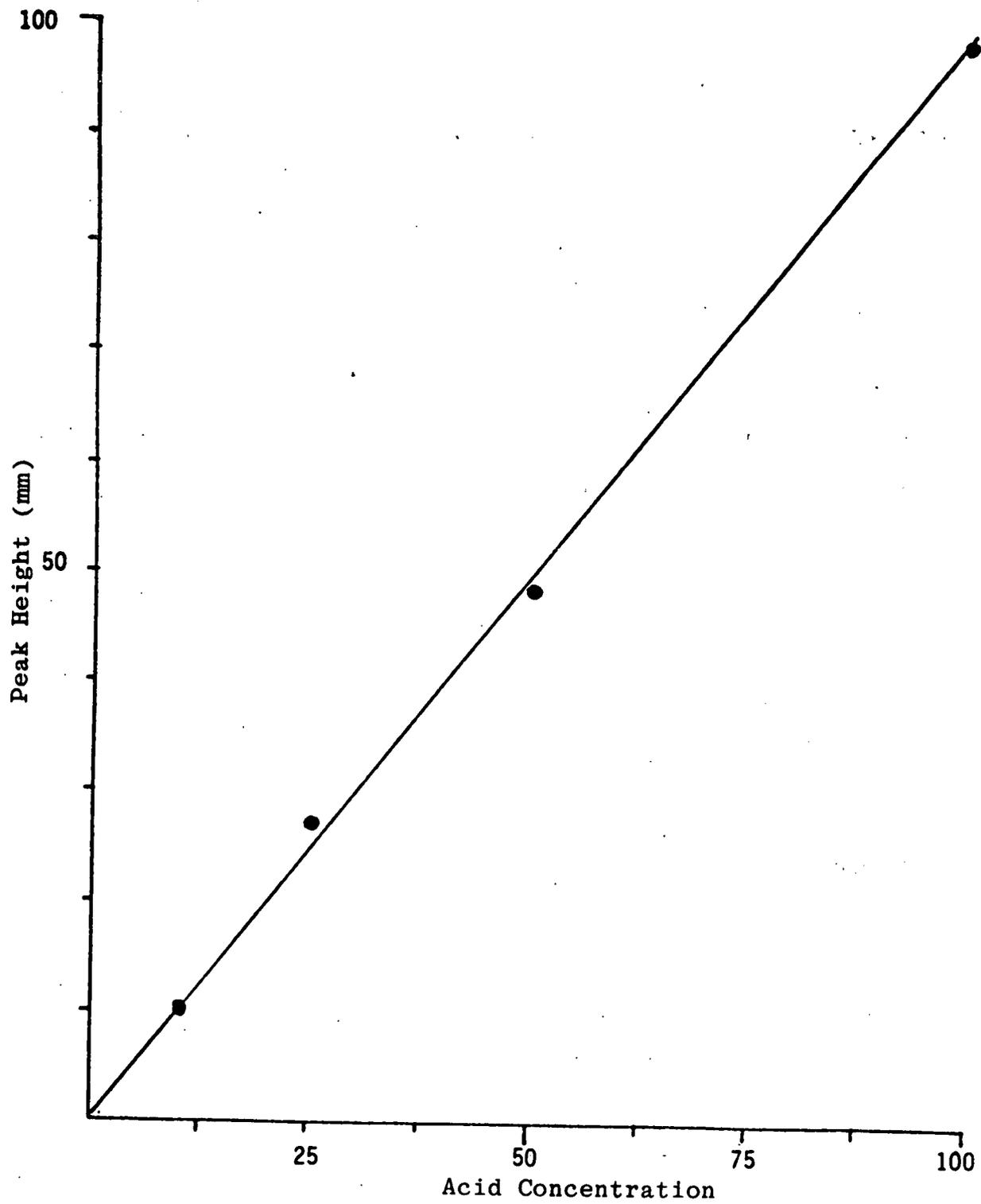


FIGURE 15. Acetic Acid Standard Curve
(from GLC analysis)

as mg/ml of glucose equivalents in the solution by incubating a mixture of 1 ml of enzyme solution (clear supernatant from fermentor broth) and 1 ml of 20% microcrystalline cellulose (Avicel PH 105) in pH 7.5 buffer solution at 55°C for three hours. The mixture was quenched by addition of 8 ml of 0.1 N H₂SO₄. The clear supernatant was analyzed for soluble sugar by the Anthrone method.

Fermentations were conducted in 1-liter flasks at a constant pH of 7.4 and a temperature of 55°C. The desired amount of substrate was suspended in 800 ml of basal medium and sterilized in the fermentation flask for 15 minutes at 125°C. In the case of glucose, the substrate was sterilized separately. The fermentor was inoculated with a culture which was grown in the same medium as in the fermentor. The pH was maintained at 7.4 by the continuous addition of 3N NaOH solution, by means of a peristaltic pump, regulated by an automatic pH controller. Anaerobic conditions were maintained by continuously passing nitrogen gas above the medium. As the fermentation progressed, aliquots were withdrawn aseptically and analyzed for cellulose, protein, soluble sugar, ethanol, and volatile acids.

Unless otherwise indicated, the composition of the growth medium per 1000 ml was: (NH₄)₂SO₄, 5.0 g; K₂HPO₄, 10.4 g; KH₂PO₄, 0.9 g; NaCl, 1.0 g; yeast extract, 2.0 g; and trace metals: MgSO₄ · 7H₂O, 0.2 g; FeSO₄, 20.0 mg; MnSO₄ · 4H₂O, 20.0 mg; CaCl₂, 20.0 mg; ZnSO₄, 8.0 mg; Versenol, 0.2 g.

In the case of swollen cellulose, the concentration of cellulose was 6 g/liter of distilled water.

B. Growth of NW on Glucose

The kinetic profile of NW growth, glucose uptake, and ethanol and acetic acid production are shown in Figure 16. The fermentor was inoculated with a 1% suspension of an 18 hour old culture. The long lag time was probably due to the use of a stationary phase inoculum. The specific growth rate was calculated to be 0.59 hr^{-1} (generation time 70 minutes) based upon turbidity measurements at 615 m μ . Ethanol and acetic acid were the major metabolites produced during the fermentation. The production of ethanol and acetic acid paralleled the increase in cell mass. The rate of ethanol production was slower than the rate of acetic acid production and reached a maximum value at the same time as maximum cell density was attained. However, acetic acid production was found to increase even after the cessation of culture growth.

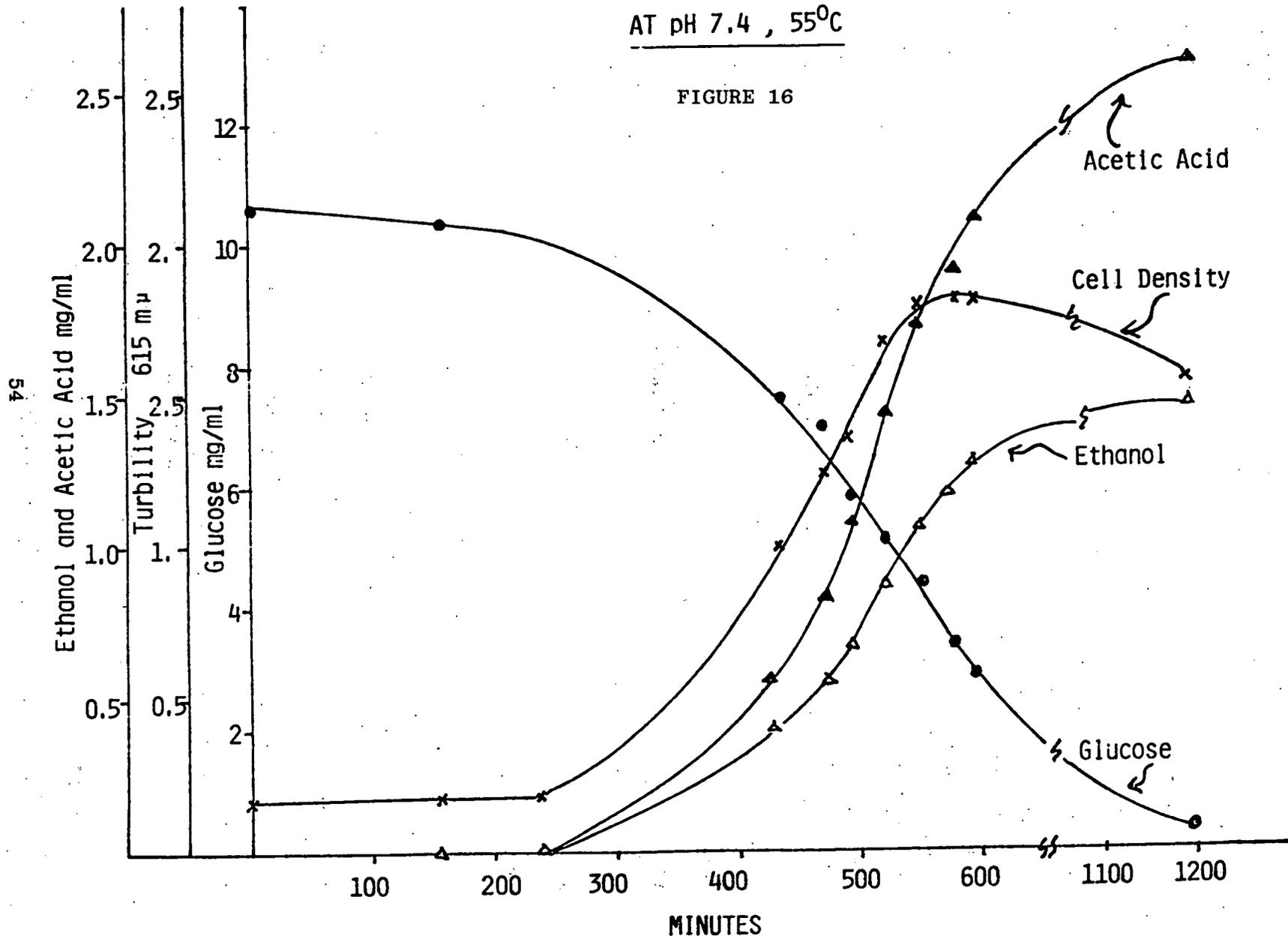
C. Mixed Culture Growth on Amorphous Cellulose

The specific substrate depletion rate of US-NW growing on amorphous cellulose (Figure 17) was calculated to be 0.087 hr^{-1} . Soluble sugar accumulation did not occur during the fermentation, and its relative concentration remained small. As with the NW glucose fermentation, ethanol and acetic acids were the primary

GROWTH OF THERMOPHILIC BACILLI ON 1% GLUCOSE

AT pH 7.4 , 55°C

FIGURE 16

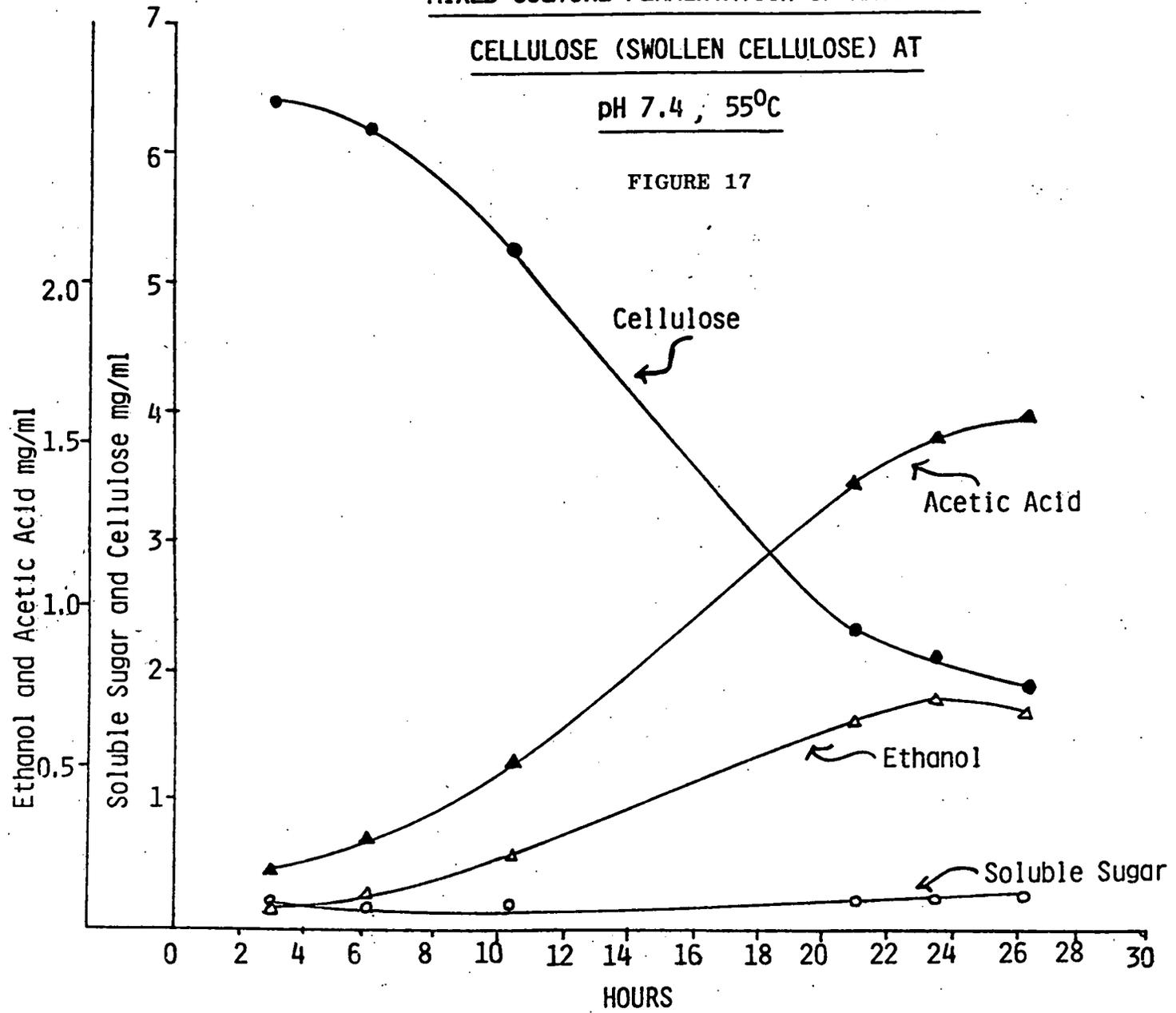


MIXED CULTURE FERMENTATION OF AMORPHOUS

CELLULOSE (SWOLLEN CELLULOSE) AT

pH 7.4 ; 55°C

FIGURE 17



metabolic products. Again, acetic acid production was faster than ethanol production and the former continued after ethanol production ceased. The yield of ethanol was about 24% of theoretical.

D. Mixed Culture Growth on Microcrystalline Cellulose

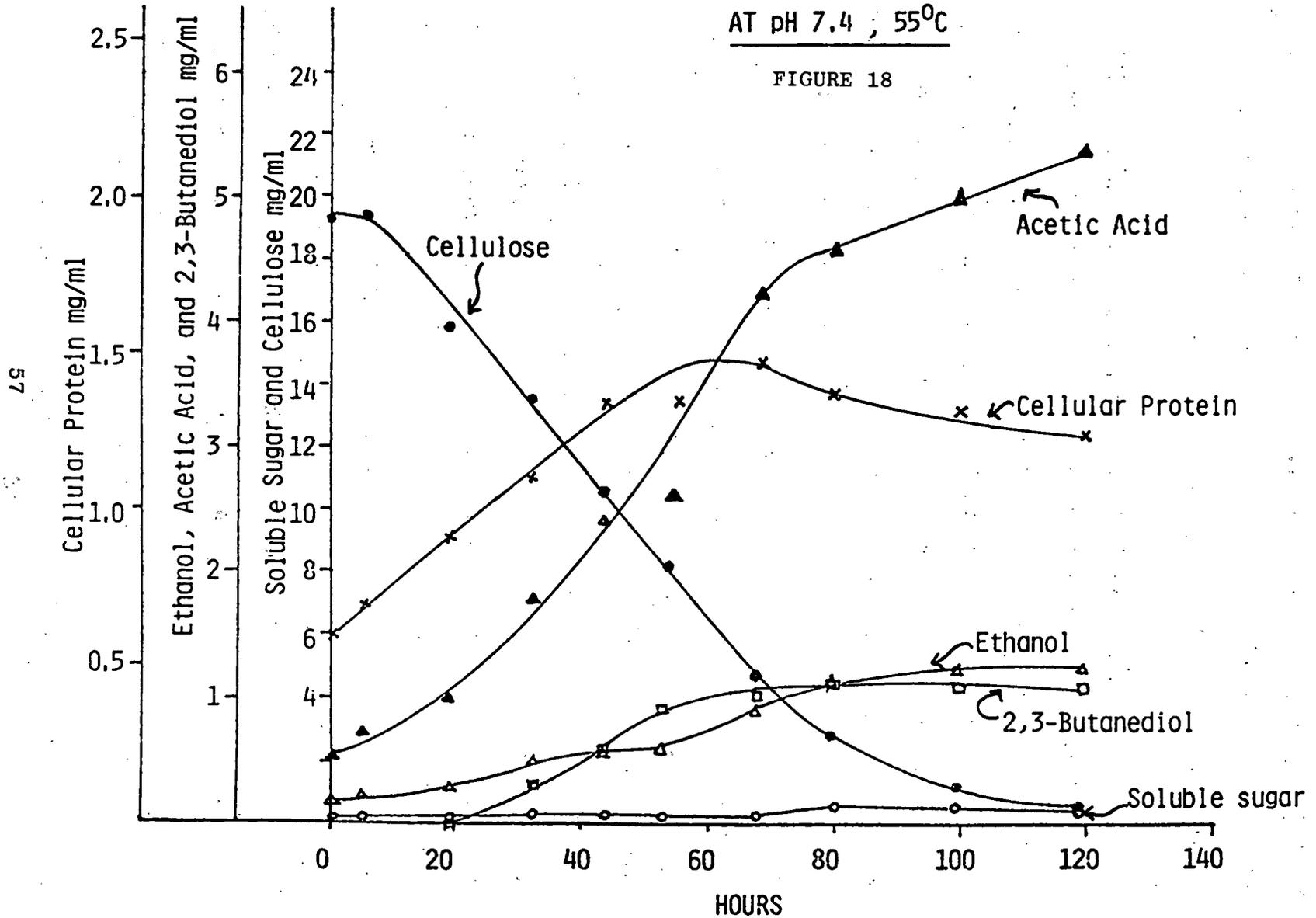
The specific substrate depletion rate of US-NW growing on microcrystalline cellulose (Figure 18) was calculated to be 0.0346 hr^{-1} . No soluble sugar accumulation occurred during fermentation, and as observed in the two previous fermentor runs, acetic acid production was faster and persisted longer than ethanol production. However, 2,3 butanediol was found to be an additional metabolite, occurring in yields and rates of production which were comparable to ethanol. Ethanol appeared earlier than 2,3 butanediol; however, the observed yield of ethanol was below that (14-19%) found during growth on swollen cellulose. Extracellular cellulase was observed in the broth; however, for meaningful assessments, the substrate-bound activity (which was not measured in the fermentor run) must also be taken into account.

The ethanol production and cell growth from NW growing on glucose fell off even when substantial glucose was still present. Since glucose starvation is unlikely under these conditions, the most likely interpretation is either ethanol or acetic acid

MIXED CULTURE FERMENTATION OF MICROCRYSTALLINE CELLULOSE

AT pH 7.4 ; 55°C

FIGURE 18



inhibition. Since acetic acid production persisted after ethanol production stopped, NW may in the vegetative state convert ethanol to acetic acid to obtain maintenance energy. However, growth factor depletion normally supplied by US in mixed-culture growth cannot be entirely ruled out. Mixed-culture growth on amorphous cellulose displayed similar ethanol yields; however, the substrate depletion rate was lower, and the ratio of ethanol to acetic acid fell from 0.9 to 0.6 when compared to the NW glucose fermentation. Unlike the latter case, glucose levels relative to cell mass were low when log phase cell growth stopped, which suggests that glucose starvation arrested growth. Mixed-culture growth on microcrystalline cellulose displayed still lower yields of ethanol, and the ethanol to acetic acid ratio dropped to 0.3. Soluble sugar concentration remained negligible throughout the fermentation. The appearance of 2,3 butanediol (Figure 19) toward the latter stage of fermentation was unexpected; however, since the fermentation time is considerably longer on microcrystalline cellulose, either a change in culture physiology leading to a different mix of products or a contaminant could explain the appearance of this product. The change in metabolic pathway with culture age is the more likely explanation if the 2,3 butanediol pathway is more energy conserving. Ethanol, acetic acid, and 2,3 butanediol account for about 85% of the cellulose

TEMP1 110
 TIME1 0.0
 RATE 20.00
 TEMP2 210
 TIME2 7.0
 INJ TEMP 250 250
 FID TEMP 300 300
 AUX TEMP 300 300
 OVEN MAX 300

CHT SPD 0.50
 ATTN 21 10
 FID SGNL A
 SLP SENS 0.50
 AREA REJ 100
 FLOW A 89
 FLOW B 34
 OPTN 0

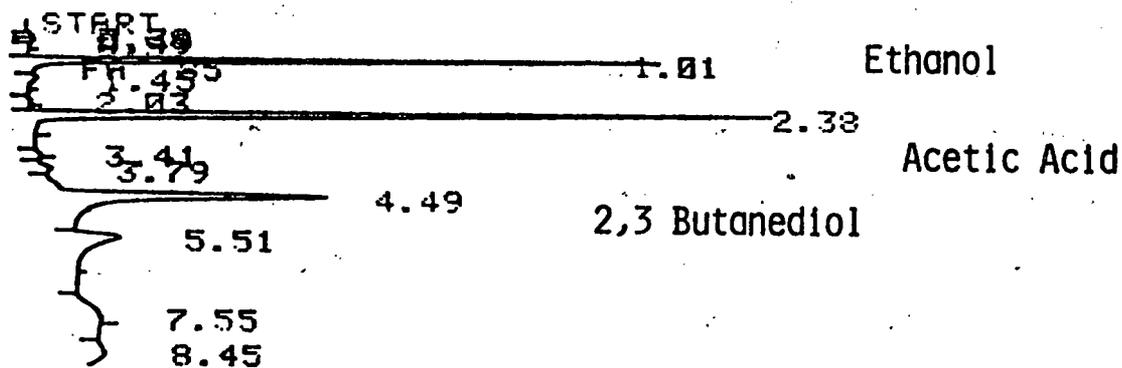


FIGURE 19. Fermentation Broth, Gas Liquid Chromatogram

utilized assuming the production of 50% CO₂. If lactic acid is produced as observed in the test tube experiments, its concentration in the 1-liter fermentor runs is negligible.

The observed ethanol yield and rate of production vary with the carbon substrate as does the molar ratio of ethanol and acetic acid. The relatively slow rate of microcrystalline cellulose degradation appears limited by the accessibility of this substrate to enzymatic saccharification, which may in turn lead to an alteration in the culture physiology and to the production of 2,3 butanediol, at the expense of ethanol. The formation of acetic acid from ethanol by resting bacilli cells may also be occurring in the latter stage of fermentation. Ethanol yields in relatively uncontrolled test tube fermentations, are higher than observed in 1-liter pH controlled fermentations suggesting that other parameters such as agitation, dissolved oxygen, and CO₂ tension may also influence the yield of ethanol.

In order to understand and improve the ethanol yield in bench-top fermentation, the continuous fermentation of thermophilic bacilli growing on soluble sugar was investigated.

E. Continuous Fermentation of Thermophilic Bacillus on Glucose

The continuous fermentation was studied in a 1-liter

fermentor at constant pH of 7.5 and temperature of 55°C. The pH was held constant by a pH controller and by automatic addition of 3N NaOH solution. The fermentor was filled with 700 ml of fermentation broth and autoclaved. The glucose solution was autoclaved separately to avoid caramelization. After the addition of the sterilized glucose solution to the fermentor, the fermentor was inoculated with a 1%, 16 hour-old inoculum which was previously grown on the same medium as in the fermentor. At the end of the batch growth, the medium feed pump was turned on. The medium was continuously fed to the fermentor from a 20-liter reservoir. The rate of medium addition was calibrated by a buret which was attached to the system as shown in Figure 20. A fermentor working volume of 800 ml was maintained by a constant leveling probe which activated the outflow pump when the fermentor volume exceeded 800 ml. After more than three fermentor volumes (i.e. 2.5 liters) of the broth passed through the fermentor, 5 ml samples of broth were collected in timed intervals and the cell mass was measured. When the cell mass remained constant over a period of 5 to 6 fermentor volumes, a steady state was assumed. Subsequently, the flow rate of medium addition was changed and a new steady state was established.

The results of continuous fermentation of thermophilic bacillus growing on glucose are shown in Figure 21. The cell

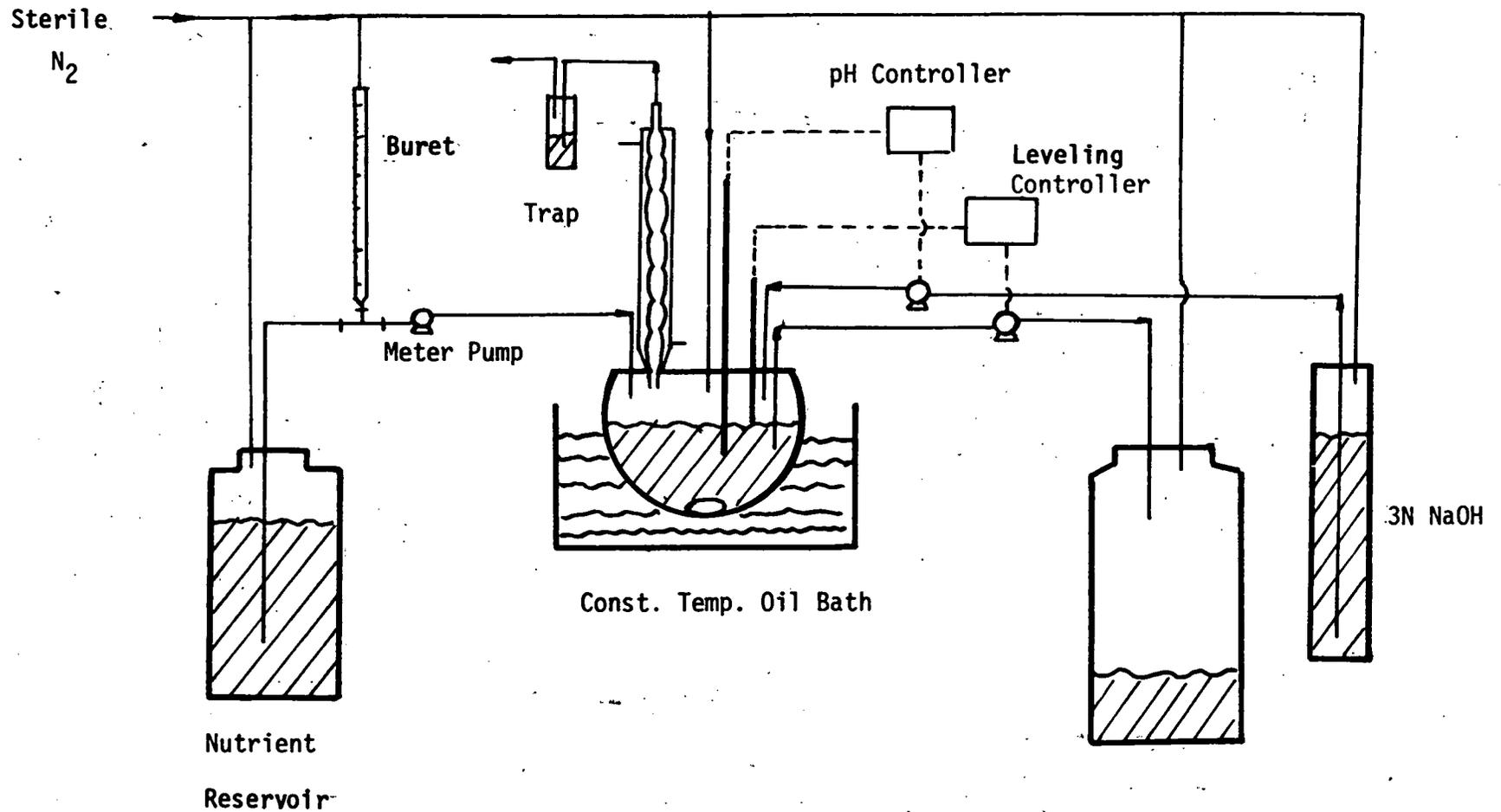


FIGURE 20. Continuous Fermentation of Thermophilic Bacillus Growing on Glucose

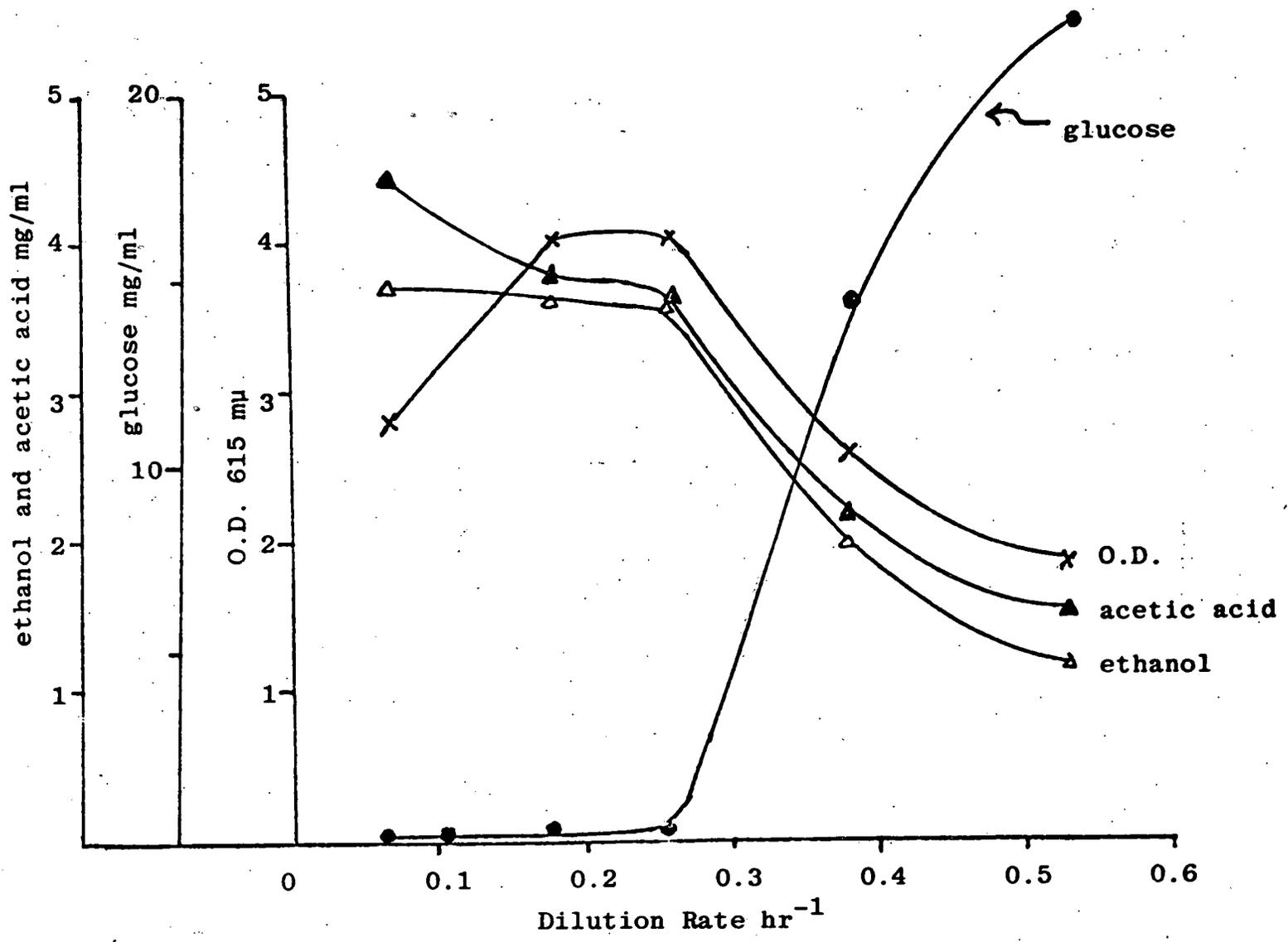


FIGURE 21. Growth of Thermophilic Bacillus on Glucose in Continuous Fermentation

mass, acetic acid, ethanol, and remaining glucose are plotted against the dilution rate. The cell density measured as optical density at 615 m μ was found to increase as the dilution rate increased from 0.075 hr⁻¹ to 0.18 hr⁻¹, and a washout of cell mass occurred at the dilution rate of 0.26 hr⁻¹. This value is much lower than the calculated critical dilution rate based on the growth rate in a batch fermentation. In the batch fermentation, the maximum specific growth rate, according to the increase in optical density, is calculated to be 0.69 hr⁻¹. It is expected that the critical dilution rate in the continuous fermentation should fall around this value. At the present time, we are unable to explain this discrepancy. The lower value of cell mass observed at a dilution rate of 0.075 hr⁻¹ is most likely due to the lysis of the culture. Matshé and Andrews⁽¹⁰⁾ reported that higher maintenance energy was needed for microorganisms growing at higher temperature. The maintenance of energy coefficients for thermophilic bacterium (Bacillus sp) at 56°C and 60°C are 0.069 and 0.103 hr⁻¹, respectively. The 0.075 hr⁻¹ dilution rate used in this study is too slow to supply adequate carbohydrate for culture growth. Therefore, it appears that the culture was starving at the 0.075 hr⁻¹ dilution rate and underwent lysis which resulted in a lower value of cell mass.

The profile of ethanol and acetic acid production as a

function of the dilution rate is similar to that of cell mass. The decrease of cell mass resulted in decreased ethanol and acetic acid production. A higher concentration of acetic acid occurred at a dilution rate of 0.074 hr^{-1} and is probably due to the conversion of ethanol to acetic acid by the culture. The molar ratio of ethanol to acetic acid in the fermentor broth as a function of the dilution rate is shown in Figure 22. As the dilution rate increases, the ethanol to acetic acid molar ratio increased and reaches a maximum value at the wash-out dilution rate. However, at lower dilution rates, the molar ratio approached unity. These results suggest that the metabolite distribution depends on the physiology of the culture. At low dilution rates, the cells utilize ethanol as an energy source to supplement the limiting supply of carbon from glucose. These results agree with the results obtained in batch fermentation, where the ethanol to acetic acid ratio decreases as growth approaches the stationary phase.

F. Kinetics of *Clostridium thermocellum* Growing on
Microcrystalline Cellulose

Shortly after we initiated our studies on the conversion of plant biomass to ethanol, Professor Zeikus of the University of Wisconsin, visited our laboratory and reported on his studies of *Clostridium thermocellum*. This cellulolytic anaerobe was

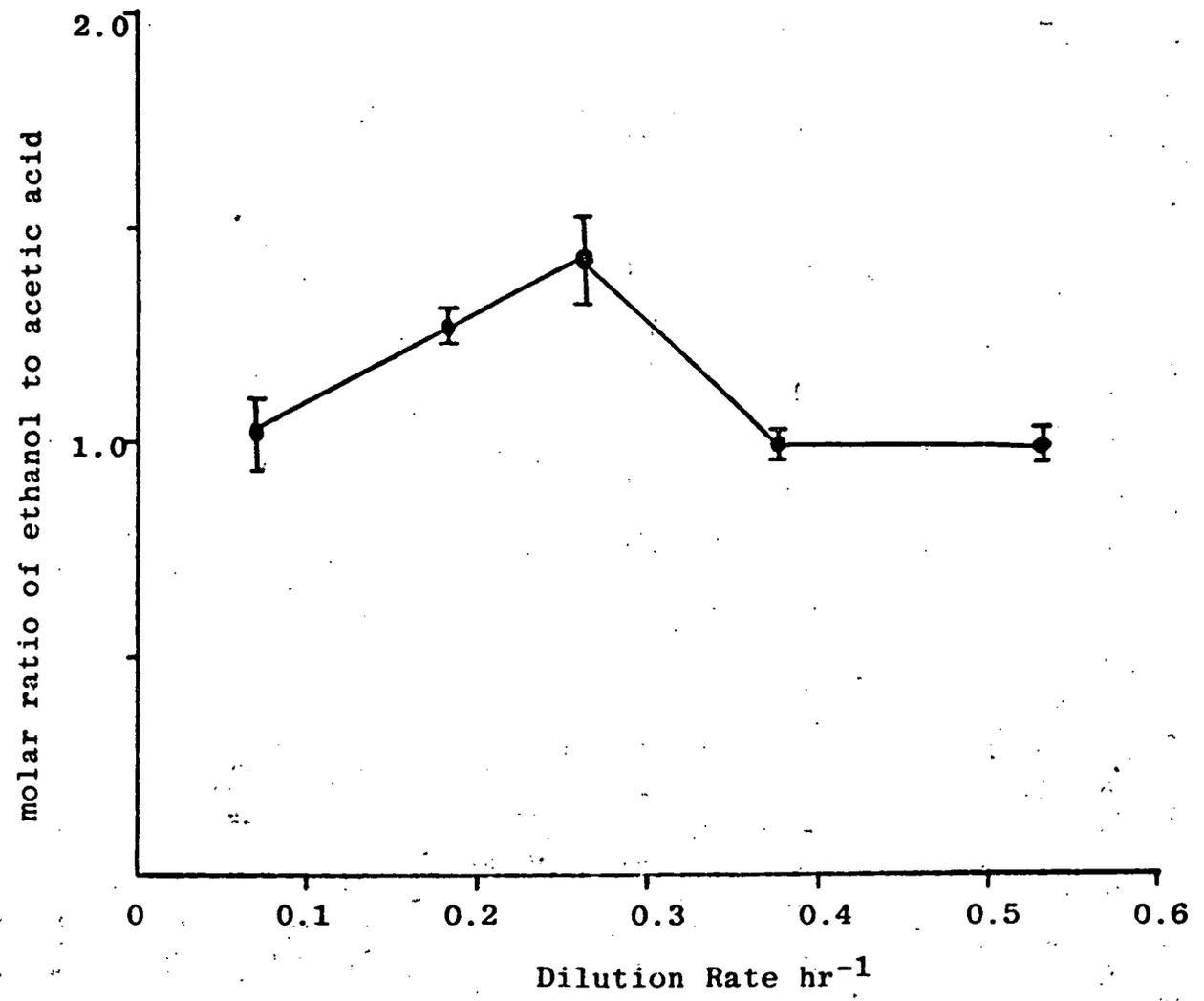


FIGURE 22. Continuous Fermentation of Thermophilic Bacillus (Glucose)

shown to produce, in flask experiments, ethanol and acetic acid as the major metabolites from growth on glucose.⁽¹¹⁾ Since this appeared to be the first report of thermophilic cellulolytic organism which ferments cellulose directly to ethanol, we examined its performance under controlled fermentor conditions.

The microorganism used in this study was Clostridium thermocellum strain LQ8, provided by Professor Zeikus. The composition of the growth medium was essentially the same as reported in the literature⁽¹¹⁾ and is shown in Table XIII.

TABLE XIII

Composition of Medium for Clostridium thermocellum

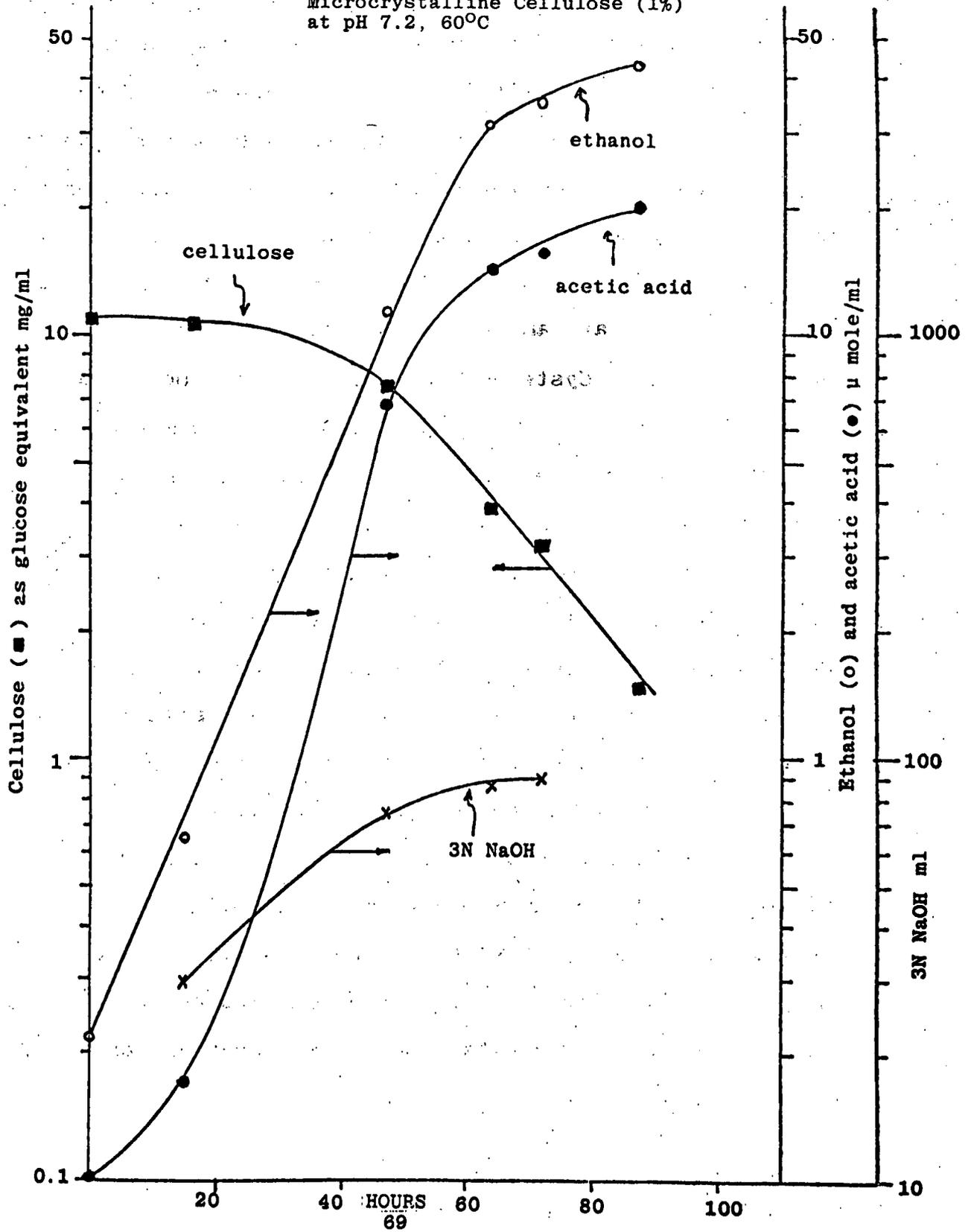
<u>Component</u>	<u>Per Liter</u>
Yeast Extract	2.0 g
(NH ₄) ₂ SO ₄	1.3 g
KH ₂ PO ₄	1.5 g
K ₂ HPO ₄	2.25 g
MgCl ₂ · 6H ₂ O	1.0 g
CaCl ₂	2.15 g
1% FeSO ₄ Solution	0.125 ml
2.5% Cysteine Hydrochloride	10.0 ml
2.5% Na ₂ S 9H ₂ O	10.0 ml
Microcrystalline Cellulose PH105	10.0 g

The batch fermentation of Clostridium thermocellum was studied in a 1-liter fermentor at constant pH (7.3) and 60°C. The pH was maintained by automatic addition of 3N NaOH solution through a peristaltic pump controlled by a pH controller. A condenser was used to condense ethanol or water vapor that might escape from the fermentor. Anaerobic conditions were maintained by purging N₂ gas continuously through the system.

The fermentor was filled with about 800 ml of broth containing appropriate amounts of microcrystalline cellulose, and was autoclaved. Cysteine hydrochloride and sodium sulfide solutions were sterilized separately. The fermentor was first started by adding the cysteine hydrochloride and sodium sulfide solution, and then a 5% inoculum which was previously grown on the same medium as in the fermentor for at least 20 hours. The culture was slowly stirred by a magnetic stirrer. Samples (20 ml) were taken from the fermentor aseptically at timed intervals and analyzed for cellulose, soluble sugar, ethanol, acetic acid, cellular protein, trichloroacetic acid precipitable protein, and cellulase activity in the broth.

Figure 23 shows the kinetic profile of the growth of C. thermocellum LQ8 on microcrystalline cellulose at pH 7.2. In this experiment, only the metabolite (ethanol and acetic acid) and remaining cellulose were analyzed during the course of fermentation. The rate of cellulose degradation was found

FIGURE 23. Growth of *C. thermocellum* LQ8 on Microcrystalline Cellulose (1%) at pH 7.2, 60°C

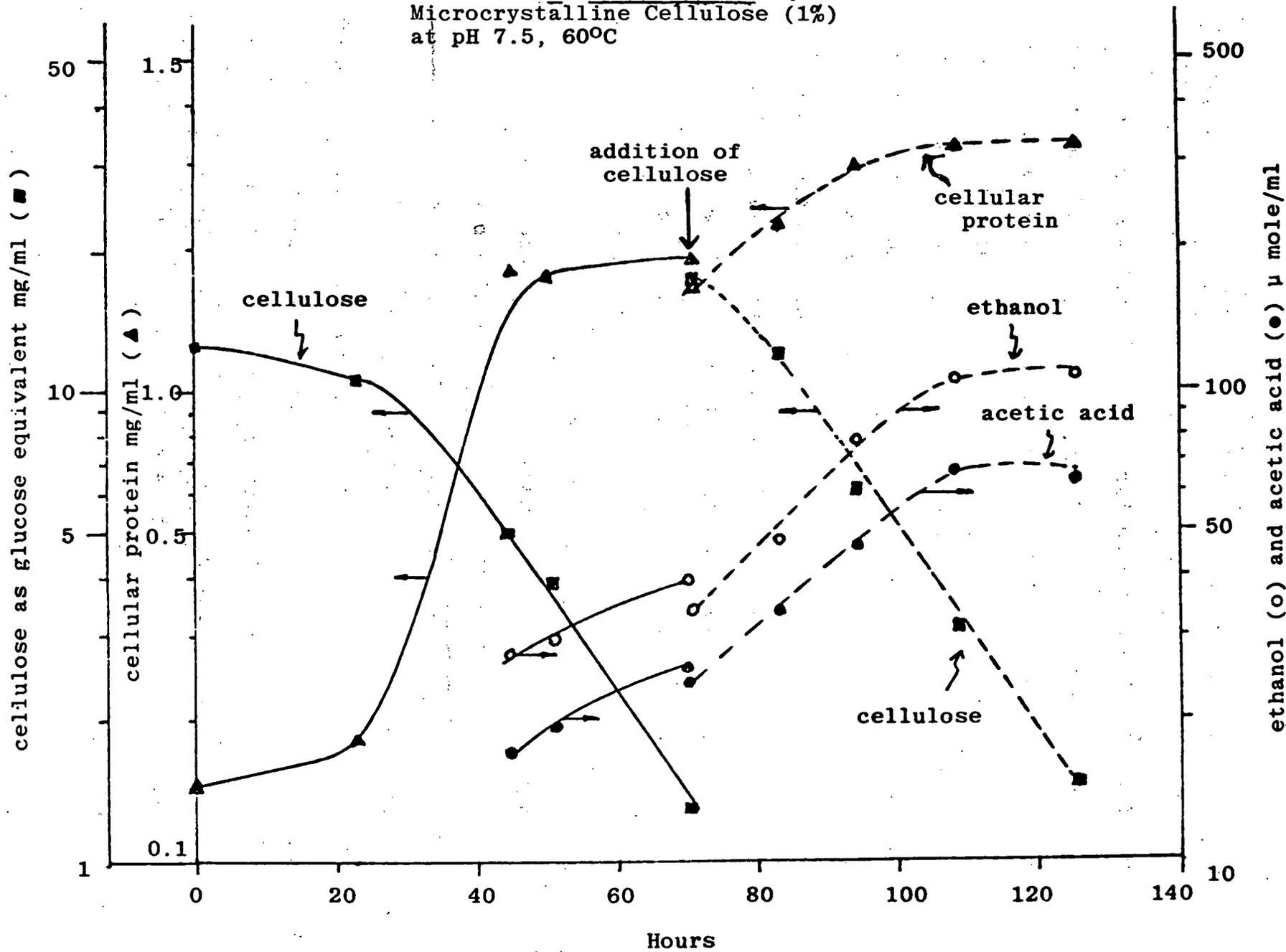


to be a first order after a lag period of about 40 hours. The slope of first order rate constant was calculated to be 0.043 hr^{-1} . The observed lag time is most likely due to the lack of adequate initial cellulase activity at the beginning of the fermentation for hydrolyzing the cellulose in the fermentor. However, as the fermentation proceeded, the culture produced more cellulase that increased cellulose saccharification during the later stage fermentation. The production of ethanol and acetic acid seems to be associated with culture growth. The ethanol and acetic acid production are exponential in the early stage of fermentation and falls off just prior to the complete depletion of the cellulose. The molar ratio of ethanol to acetic acid is calculated to be 2 to 1, and the overall yield of ethanol is about 36% of the theoretical yield of ethanol from the cellulose. The low yield of ethanol is due to the production of acetic acid.

Figure 24 shows the kinetic profile of a batch fermentation in which sterilized cellulose was replenished in the fermentor after the original cellulose was almost depleted as shown by the arrow on top of the figure. The initial cellulose degradation was found to be similar to that observed in the previous experiment. However, replenished cellulose was degraded by first order kinetics without a lag time. The observed difference in lag time between the initial degradation

FIGURE 24. Growth of *C. thermocellum* LQ8 on Microcrystalline Cellulose (1%) at pH 7.5, 60°C

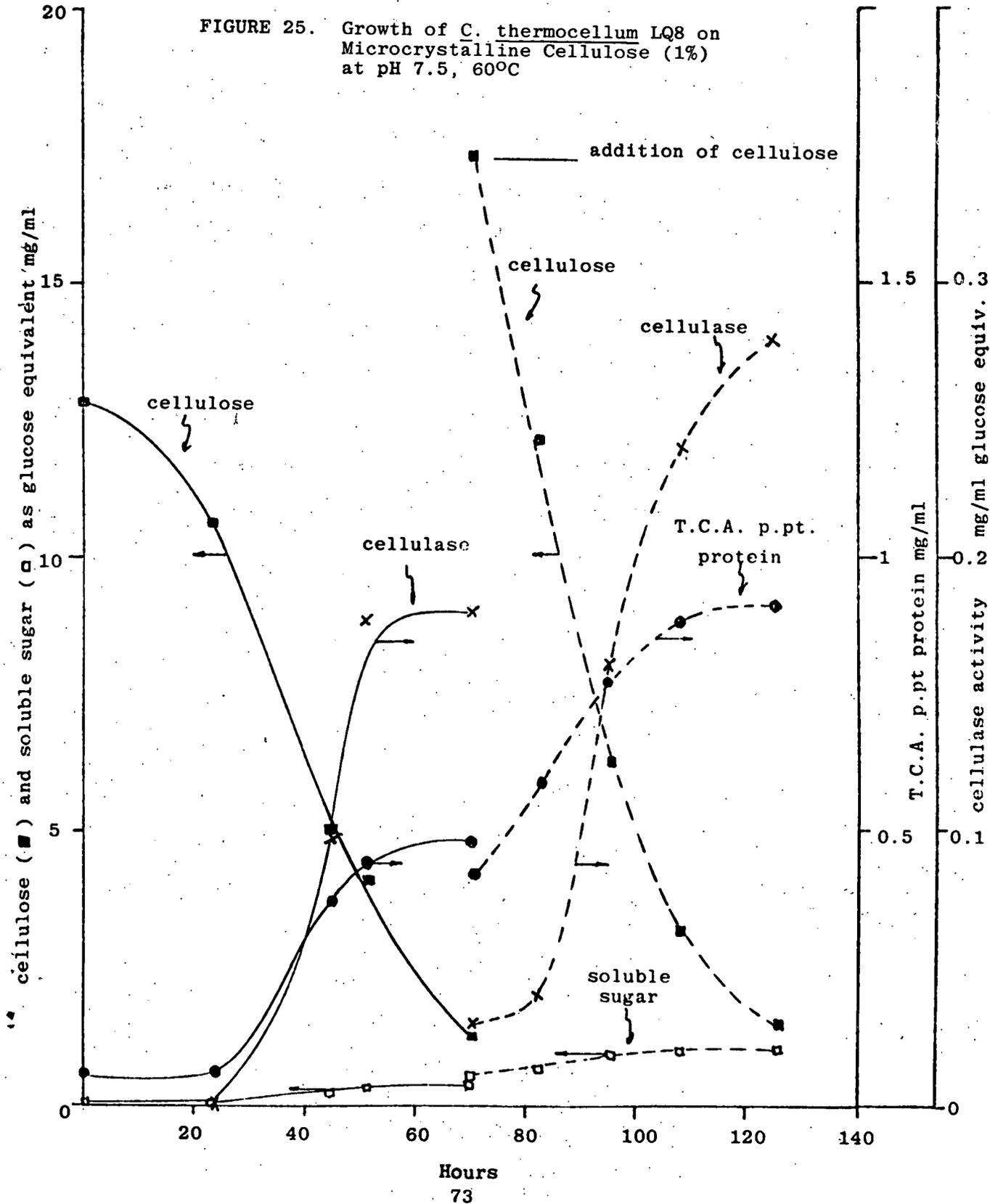
17



and that associated with the replenished cellulose is due to the cellulase concentration in the fermentor as shown in Figure 25. At the end of the initial cellulose fermentation, there was a high cellulase activity (0.18 mg/ml glucose equivalent) in the broth. As soon as the fresh cellulose was added to the fermentor, the cellulose immediately absorbed most of the cellulase and was quickly degraded. As a result, the cellulase activity decreased sharply from 0.18 mg/ml glucose equivalent in the broth, and the cellulose was degraded with no lag. Since there was ample cellulase in the broth, the slow rate of cellulose degradation is controlled by the accessibility of the cellulose to the cellulase. The first order rate constant of the cellulose degradation was calculated to be 0.056 hr^{-1} .

The ethanol and acetic acid production was found to parallel the increase of cellular protein as shown in Figure 24. This suggests that metabolite production of C. thermocellum is closely associated with culture growth. The molar ratio of ethanol to acetic acid was 2.1 to 1. Since no appreciable amount of soluble sugar was observed in the broth, the cellulose was used by the microorganism for growth and metabolite production. The lack of soluble sugar accumulation is in sharp contrast to the results reported by Professor Cooney, et al. (12) The overall yield of ethanol is calculated to be about 37% of theoretic yield. The observed low yield of ethanol

FIGURE 25. Growth of *C. thermocellum* LQ8 on Microcrystalline Cellulose (1%) at pH 7.5, 60°C



is attributed to the production of acetic acid. In order to obtain a higher yield of ethanol, a mutant which does not produce acetic acid is required since the manipulation of fermentation parameters is not likely to alter the ratio of the ethanol to acetic acid ratio.

VI. SUMMARY

Based on the experimental findings of the first year's research, the objectives of this research program cannot be achieved within the remaining contract period, without modifying the originally proposed experimental tasks.

An alternative pretreatment process must be developed in order to circumvent the experimental difficulties of erratic growth of C. pruinosum.

The availability and performance of C. thermocellum LQ8 in bench-top fermentation provides an alternative route to developing a whole cell direct fermentation of cellulose to ethanol. To overcome the latter's low ethanol yield requires genetic improvement to suppress acetic acid production.

The efficient utilization of the hemicellulose fraction of plant biomass by thermophilic bucellus ZBB2 will require similar genetic improvements to reach higher ethanol yields.

A revised program plan based on our recent experimental work and a literature survey of suitable alternative pretreatment processes is presented in Part II of this report.

PART II - REVISED RESEARCH PLAN

VII. INTRODUCTION

Alternative experimental routes for converting wood into ethanol have been reexamined in the light of recent experimental findings, the unfavorable process economics of enzymatic saccharification, and the increasing imperatives for a pilot fermentation facility in the DOE Fuels from Biomass Program. (13)

A revised research plan based on a review of the wood pretreatment literature and our recent experimental results is presented. The principal modifications are the substitution of a chemically supplemented steam pretreatment of wood for biological delignification and the use of Clostridium thermocellum in place of the mixed culture for ethanol production. Research on genetic improvement and the modified vacuum-ferm are retained.

The modified research plan, rationale, and new experimental tasks are discussed after the literature survey of wood pretreatment schemes.

VIII. WOOD PRETREATMENT LITERATURE REVIEW

Literature reports of wood pretreatment methods are more extensive than the uses and applications of wood products. The susceptibility of pretreated wood to bioconversion, its production costs, and market value set natural limitations on the feasibility of any given pretreatment scheme. The principal characteristics of wood that have been advanced for explaining its resistance to chemical, enzymatic and microbiological conversion to liquid fuels are: the content and degree of polymerization (DP) of lignin, the crystallinity and DP of wood cellulose, the extent of lignin-carbohydrate association, the porosity, and the particle size.⁽¹⁴⁻¹⁸⁾ A given pretreatment will invariably effect more than one of these properties to an extent which is very dependent on the wood species and the treatment conditions. However, before reviewing the effects of various wood treatments on its susceptibility to conversion, a brief review of the structure and composition of wood is given to provide a common basis for interpreting the action of a given chemical or physical treatment.

A. Composition and Structure of Wood

The chemical composition of some North American wood species, that dictate the theoretically recoverable carbohydrate are given in Table XIV.⁽¹⁹⁾

TABLE XIV

Chemical Composition of Some North American Species⁽¹⁹⁾
 (Extractive-Free Wood Basis)

Species	Analysis				Estimation									Gross composition			
	Ash %	Lig- nin, %	Acety- l, %	Uronic acid anhy- dride, %	Glucan, %	Galac- tan, %	Man- nan, %	Ara- ban, %	Xy- lan, %	Cellu- lose, %	Non- cellulosic glucan? %	Gluco- mannan, (acetate) %	Arabino- galac- tan, %	4-O-methyl- glucurono (arabino- xylan (acetate) %	Cellu- lose, %	Hemicel- luloses, %	Lig- nin, %
Trembling aspen <i>Populus tremuloides</i>	0.2	16.3	3.4	3.3	57.3	0.8	2.3	0.4	16.0	53	3	4	1	23	53	31	16
White elm <i>Ulmus americana</i>	0.3	23.6	3.9	3.6	53.2	0.9	2.4	0.6	11.5	49	2	4	2	19	49	27	24
Beech <i>Fagus grandifolia</i>	0.4	22.1	3.9	4.8	47.5	1.2	2.1	0.5	17.5	42	4	4	2	25	42	36	22
White birch <i>Betula papyrifera</i>	0.2	18.9	4.4	4.6	44.7	0.6	1.5	0.5	24.6	41	2	3	1	34	41	40	19
Yellow birch <i>Betula lutea</i>	0.3	21.3	3.3	4.2	46.7	0.9	3.6	0.6	20.1	40	3	7	1	28	40	39	21
Red maple <i>Acer rubrum</i>	0.2	24.0	3.8	3.5	46.6	0.6	3.5	0.5	17.3	41	2	7	1	25	41	35	24
Sugar maple <i>Acer saccharum</i>	0.3	22.7	2.9	4.4	51.7	—	2.3	0.8	14.8	—	—	4	1	22	—	—	—
Hardwoods, average										45	3	5	1	25	45	34	21
Balsam fir <i>Abies balsamea</i>	0.2	29.4	1.5	3.4	46.8	1.0	12.4	0.5	4.8	44	0	18	1	8	44	27	29
Eastern white cedar <i>Thuja occidentalis</i>	0.2	30.7	1.1	4.2	45.2	1.5	8.3	1.3	7.5	44	0	11	2	12	44	25	31
Eastern hemlock <i>Tsuga canadensis</i>	0.2	32.5	1.7	3.3	45.3	1.2	11.2	0.6	4.0	42	0	17	1	7	42	26	33
Jack pine <i>Pinus banksiana</i>	0.2	28.6	1.2	3.9	45.6	1.4	10.6	1.4	7.1	41	0	16	2	12	41	30	29
White spruce <i>Picea glauca</i>	0.3	27.1	1.3	3.6	46.5	1.2	11.6	1.6	6.8	44	0	17	2	10	44	29	27
Tamarack <i>Larix laricina</i>	0.2	28.6	1.5	2.9	46.1	2.3	13.1	1.0	4.3	43	0	18	3	7	43	28	29
Softwoods, average										43	0	16	2	9	43	28	29

The structure and physical properties of wood are determined for the most part by the physical and chemical relationship of the three principal wood components, cellulose, hemicellulose, and lignin. The more common subdivisions of these components are illustrated in Figure 26 based on a diagram first given by Norman. (20)

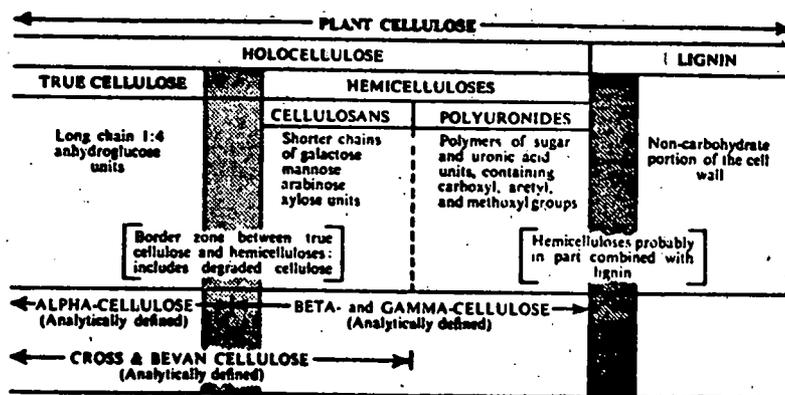
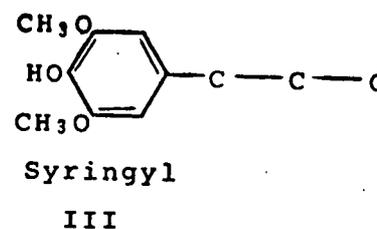
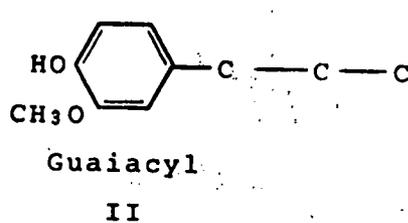
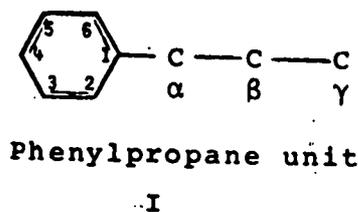
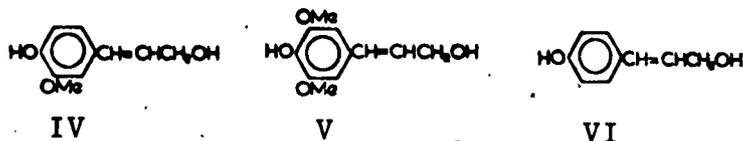


FIGURE 26. Classification of Plant Cellulose (20)

Lignin, an amorphous heterogeneous polymer composed of substituted phenyl propane units (I) joined together through a variety of linkages is distinguished primarily by the aromatic ring substitution (Guaiacyl II or Syringyl III) and nature of the propane substituents and bonding.

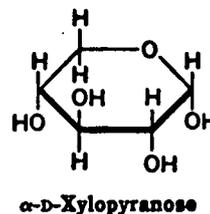
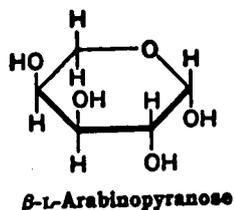
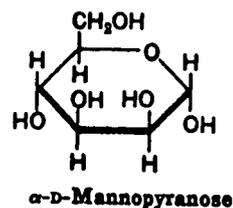
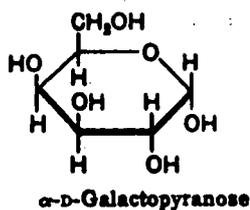


It is important to remember that lignin is a generic term which describes the variable polymeric arrangements derived from three principal precursors: trans-coniferyl (IV), trans-sinapyl (V), and trans-p-coumaryl (VI) alcohols.

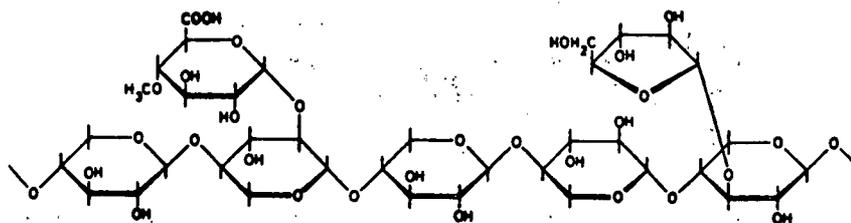


The relative proportion and sequence in which these monomers are incorporated into a lignin core molecule and the degree of polymerization determined the different physical and chemical characteristics of lignin which vary with the wood species and even within wood cells. The "guaiacyl lignins" are characteristic of softwoods, and the guaiacyl-syringyl lignins make up the majority of hardwood and grass lignins. (21)

Wood hemicellulose consists of cellulosans and polyuronides. The cellulosans are composed of relatively short chains of galactose, mannose, arabinose, and xylose.



The polyuronides are polymers of sugar and uronic acid units containing carboxyl, acetyl, and methoxyl substituent groups.



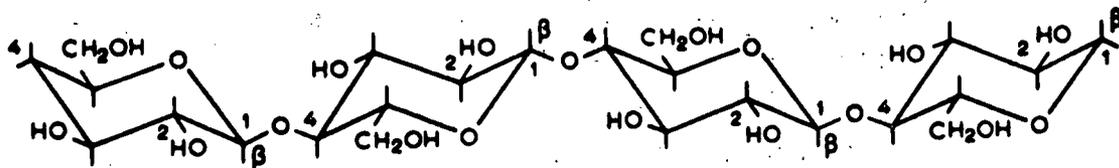
Polyuronides

The relative amounts of the major hemicelluloses in plant material are given in Table XV. (22)

TABLE XV
Proportions of the Major Hemicelluloses in Plant Material
(Percentage of Dry Fiber)

		<i>Xylan</i>	<i>Araban</i>	<i>Mannan</i>	<i>Uronic acids</i>	<i>Total</i>
Softwoods	6-12	Small amounts	4-8	2-5	12-25
Hardwoods	18-26	Small amounts	Very little	3-6	21-34
Esparto grass	18-20	2-4	None	2-4	26-30

Plant celluloses are linear polymers of D-glucose anhydride units linked by β -1,4-D glycosidic bonds. The linear polymer



Conformational Formula (Chair Form) of Cellulose.

chains are arranged in ordered elementary fibrils, which are further aggregated into microfibrils and subsequently, into macrofibrils of varying size. The organization of the adjacent polymeric chains of the elementary fibril and the higher aggregates is such as to form crystalline regions held together by hydrogen bonds. The length of cellulose chains is great relative to the length of crystallites, which means that a given cellulose chain will extend through both highly oriented and disoriented regions as depicted in Figure 27 after Stamm. (23)

The dimensions of the structural elements of cellulose are brought together in Table XVI. (22)

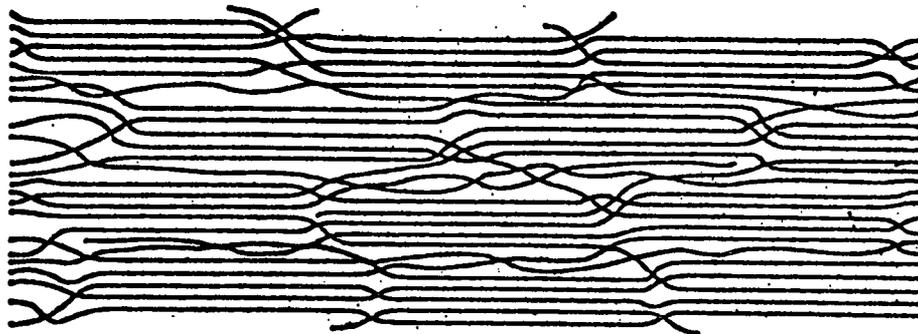


FIGURE 27. Diagrammatic Sketch of Highly Oriented Crystallite Regions and of Disoriented Amorphous Regions of Cellulose, Showing that Cellulose Chains may Extend Through a Number of Crystallites. (23).

TABLE XVI

Size of the Structural Elements of Cellulose (22)

	<i>Length</i> (Å)	<i>Width</i> (Å)
Carbon atom	1.54	1.54
Glucose residue	5.2	7.5
Cellulose molecule (of DP 4000)	20,000	7.5
Crystallite	500	50-100
Microfibril		150-250

1 mm = 10^3 microns (mu) = 10^7 Angström units (Å)
 1 thousandth of an inch = 25.4 mu
 1 millionth of an inch = 254 Å

The two main classes of woods, gymnosperms or conifers (softwoods) and dicotyledons, angiosperms or deciduous woods (hardwoods) differ from one another in composition and structure.

The general properties of these two classes of woods are given in Table XVII. (26a)

TABLE XVII
Comparison of Hardwoods and Softwoods (26a)

	Softwoods	Hardwoods
Major type of fiber	Tracheids	Wood fibers and vessels
Length of fibers, mm.	2.5-3.0 for eastern species 3.5-5.0 for southern and western species	Wood fibers: 0.6-2.0; vessels: shorter and very wide
Lignin, %	25-32	17-26
Cellulose (Cross and Bevan), %	55-61	58-64
Pentosann content, %	8-13	18-25
Density of green wood, lb./cu. ft.	21-26	22-35

Morphologically, wood is composed of various kinds of cells or fibers that perform specialized functions such as food storage, water transport, or impart mechanical strength. The areas of wood fibers may be reduced to two principal regions: the middle lamella, or intercellular region and the cell proper, which is composed of a primary wall, a secondary wall, and the lumen or cell cavity. (24) The arrangement of these various elements are diagramed for a cross sectional area in Figure 28. The middle lamella is composed principally of lignin while the primary and secondary walls contain mostly cellulose and hemicellulose together with some lignin. The distribution of chemical elements in a wood fiber is important. Holocellulose appears to be evenly distributed across

the cell wall, while cellulose is most concentrated around the lumen, and the hemicellulose is most concentrated in the outer regions of the cell wall. (19b)

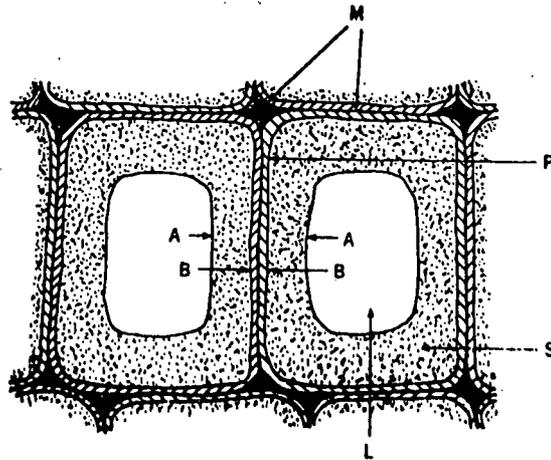


FIGURE 28. The Cell Wall. A, the compound cell wall; B, the compound middle lamella; P, primary wall; S, secondary wall; M, middle lamella; L, lumen. (24)

In soft woods, the greater part of the wood substance consists of longitudinal cells called tracheids which conduct sap and provide mechanical strength. In hardwoods, the cell structure is more diverse and consists of cells of relatively large diameter superimposed to form a continuous tube called a pore or vessel which serve to conduct sap. The comparative fine structure of soft and hardwood fibers, as deduced from electron microscope studies, is schematically presented in Figure 29. (25a) The true middle lamella forms a network which is amorphous, completely surrounds each cell, and is continuous

throughout the wood structure. The compound middle lamella, which comprises the true middle lamella and the adjacent primary walls of the contiguous cells, contains about 70% lignin, 14% pentosans, and only about 4% cellulose. (26b)

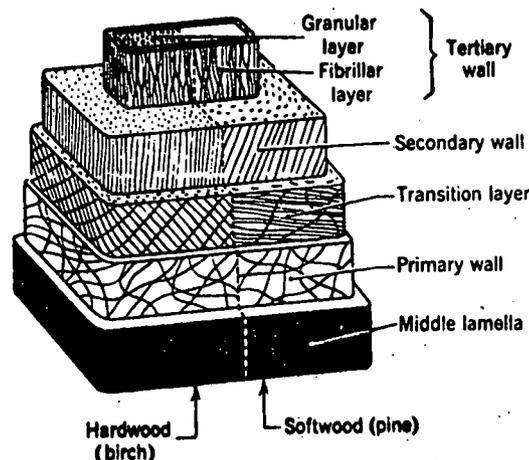


FIGURE 29: Model of Fine Structure (left half) and Softwood (right half) Fibers. (25a)

The relatively thin primary wall forms a fairly inelastic membrane around the outside of the fiber and greatly influences the surface properties. The chemical composition is not well known. Crystalline cellulose is present but a high percentage of noncellulosic matter is also present.

The secondary wall of wood fibers constitutes the major portion of the wall of most cell types. It is a relatively thick elastic layer bordered on the outside by the thin primary wall and on the inside by the lumen. The secondary wall contains cellulose hemicellulose and lignin organized in a

complex matrix of highly oriented fibrils interpenetrated by noncellulosic fiber constituents. The secondary wall is laminated into layers which differ in composition.

The effects of various physical and chemical treatments upon wood and cellulose components are well known from studies performed in the hardboard, pulp and paper, and viscose rayon industries. The primary objective in these processes is to separate the fiber components and to reconstitute whole fibers or to recover the cellulose fibers for conversion to products. The required purity of the cellulosic fibers depends upon the intended product and varies from pure α -cellulose used in rayon to reconstituted lignin, hemicellulose, and cellulose materials used in hardboard manufacture. Unlike the aforementioned applications, where fiber structure and physical properties are important, biomass conversion requires only that the cellulosic components be accessible for chemical or enzymatic conversion to simple sugars. The identification of a physical or chemical fiber separation technology which presents the fibers in a form where they can be readily converted to sugars (in high yield and low cost via acid or enzymatic hydrolysis) is the most formidable technical obstacle to biomass conversion.

B. Wood Pretreatment Processes

1. Pretreatment to enhance acid hydrolysis. Several laboratory studies^(27,28) and pilot wood-acid hydrolysis

operations (29,30,31) have demonstrated that the principal limitations of heterogeneous acid hydrolysis of wood is the poor yield of sugar. This is due to the concurrent acid catalyzed, sugar degradation which occurs under most acidic conditions required to rapidly hydrolyze cellulose (Figure 30). Since homogeneous acid hydrolysis of cellulose is known to proceed rapidly under conditions which result in quantitative recovery of sugar, (32) the major constraint on heterogeneous acid hydrolysis of cellulose is ascribed to the latter's crystallinity. (33)

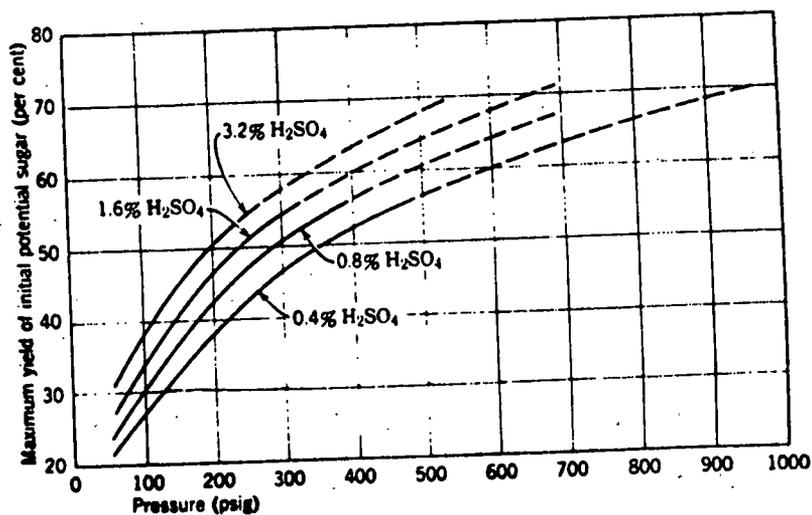
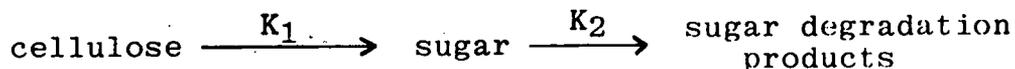


FIGURE 30: Effect of Temperature (pressure) and Acid Concentration on Maximum Sugar Yield from Hemicellulose-free Douglas-fir (33)

The kinetics of acid hydrolysis of wood follows two consecutive first-order reactions:



The maximum sugar yield depends primarily on the ratio K_1/K_2 . The activation energy for sugar degradation is higher than that for hydrolysis of crystalline cellulose, and the latter reaction is favored by short time, high temperature, and increased acid concentrations. Since dilute acid does not penetrate the crystalline regions of cellulose under moderate conditions, the high temperature and pressure conditions must lead either to an alteration of the crystalline regions or to facilitated acid penetration of the crystalline regions. Processing under these extreme conditions is feasible only in short retention time, plug-flow reactors. Under such conditions, the primary constraints are the engineering problems associated with material handling and corrosion.

A reduction in the crystallinity of wood cellulose can be accomplished by the use of concentrated cellulose solvents⁽³⁴⁾ and hydrotrophic solutions⁽³⁵⁾ that can effectively penetrate the crystalline regions of cellulose. Such reagents, the most common of which is sodium hydroxide, act by reversibly swelling the intracrystalline regions of cellulose and complexing with the liberated hydroxyl groups. Reagent concentrations required to effect the intracrystalline swelling of cellulose are high enough to mandate recovery and reuse. The viscosity of swollen cellulose solutions precludes industrial scale operations at consistencies much above 10%. The latter not only limits the volumetric productivity but seriously limits the sugar concentration which can be achieved without resorting to a separate sugar concentration

unit operation. If the handling problems at high consistency could be resolved, the need to separate the swelling agent or solvent from either amorphous cellulose or sugar (if formed during swelling) would still entail a subsequent dilution or neutralization that pose additional penalties for swelling agent recovery. Since many cellulose solvents require relatively dry wood to effect appreciable decrystallization, the cost of substrate drying represents a major obstacle to large scale development. In many of the swelling or dissolution processes proposed for cellulose, the energy cost of recovery becomes comparable to the energy content of the fermentation products. The more recent studies with binary and tertiary cellulose solvent systems appear to suffer similar if not greater disadvantages.⁽³⁶⁾ For acid hydrolysis of wood to be successful, either a readily recoverable cellulose swelling agent must be developed or a new decrystallizing agent effective at low and perhaps expendable concentrations must be invented.

Comminution of wood via hammer⁽³³⁾ two roll,⁽³⁹⁾ or vibratory ball⁽³⁸⁾ milling has been shown to decrease wood cellulose crystallinity without appreciable concurrent sugar degradation. The principal drawback to commercial consideration of the comminuting approach lies in the energy cost (milling time or energy input) required to decrystallize wood cellulose into fine powders.⁽³³⁾

High energy electron irradiation of wood cellulose has been shown to cause depolymerization, reduction in crystallinity, and extensive decomposition at high dose levels. (39) Random depolymerization in the amorphous and crystalline regions was observed, but decomposition of glucose occurred simultaneously with depolymerization. The apparent ratio of the number of glucose units destroyed to the number of depolymerizations was about 5 to 1. The maximum yields of sugar from irradiated wood was, therefore, limited to about 61%. The inherent problem of uniform irradiation makes this approach unattractive.

Free radical iron-catalyzed decomposition of cellulose fibers proceeds rather slowly even in the presence of peroxide. (40) Unfortunately, no major accumulation of soluble breakdown products occurs during the decomposition reaction which indicates that soluble sugars, if formed at all, are degraded much more rapidly than the cellulose polymer.

We conclude from this review of the wood-acid hydrolysis literature that cellulose crystallinity is the principal technical barrier to improved sugar yields and cannot be readily overcome by state-of-the-art treatment methods.

2. Pretreatments to enhance the enzymatic hydrolysis.

Unlike the acid hydrolysis of cotton cellulose which proceeds with an initial rapid rate (amorphous region) and then

at a slower rate of hydrolysis (Figure 31), the enzymatic hydrolysis of cotton by concentrated T. viride cellulase has been shown to proceed to 97% solubilization by a linear first order reaction (Figure 32) with no apparent breaks similar to those observed during acid hydrolysis. The enzymatic saccharification of Avicel cellulose which is the acid resistant residue remaining after boiling cotton cellulose in 6N HCl is also readily hydrolyzed by active cellulase preparations.⁽⁴¹⁾ The ability to solubilize crystalline regions of cellulose is due to the endo- β -1, 4-glucan hydrolase (C_x) component of cellulase that randomly depolymerizes cellulose. The other major enzyme component is a β -1, 4-glucan cellobiosyl hydrolase (C_1) which cleaves cellulose at the non-reducing end to produce cellobiose, which is subsequently hydrolyzed to glucose by β -1, 4-cellobiase. This model for cellulase action is schematically presented in Figure 33 according to Nisizawa, et al.⁽⁴²⁾

The comparison of acid and enzymatic hydrolysis given in Figures 31 and 32 is not equivalent and represents somewhat of an oversimplification. The rate of acid attack on cotton and the amount of the rapidly hydrolyzed fraction varies with the acid concentration⁽⁴¹⁾ as does the rate of enzymatic hydrolysis (lower curves in Figure 32). Part of the complexity stems from the definition of the crystalline and amorphous regions of cellulose which varies depending on the measurement technique (Table XVIII) and is significantly influenced by the accessibility

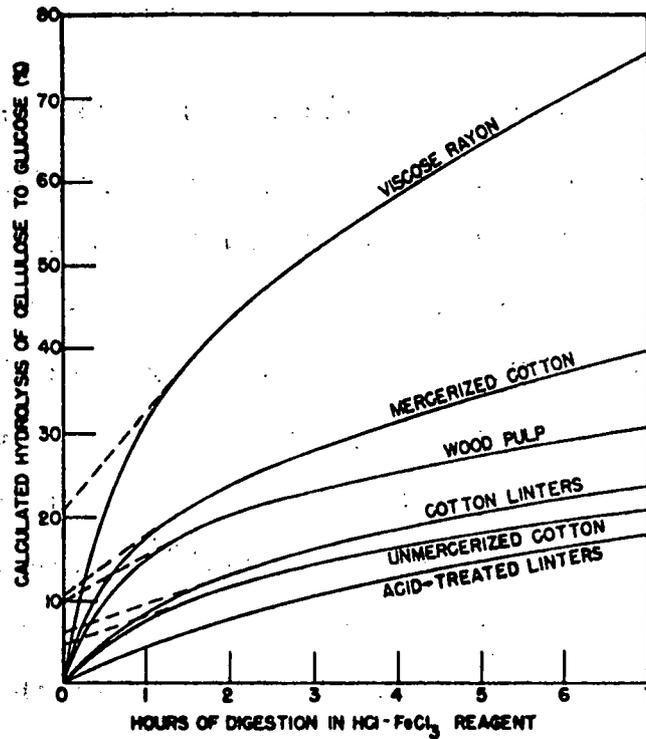


FIGURE 31. Percentage of Cellulose Hydrolyzed vs. Time for Various Cellulosic Materials in Boiling 2.4 N hydrochloric acid-0.6 M ferric chloride. (32)

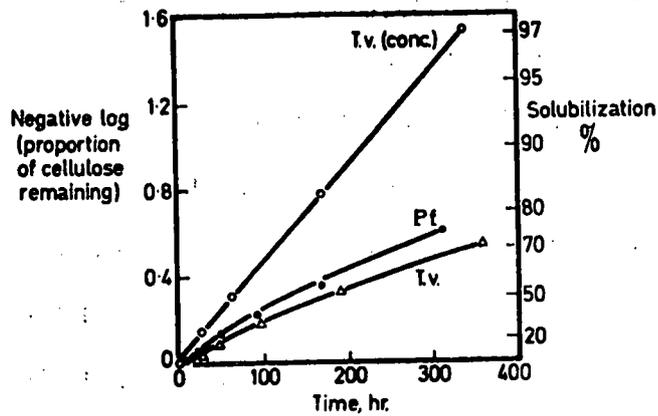


FIGURE 32. Solubilization of Cotton by Cellulase (46)
 T.v. = T. viride
 Pf = P. funiculosum

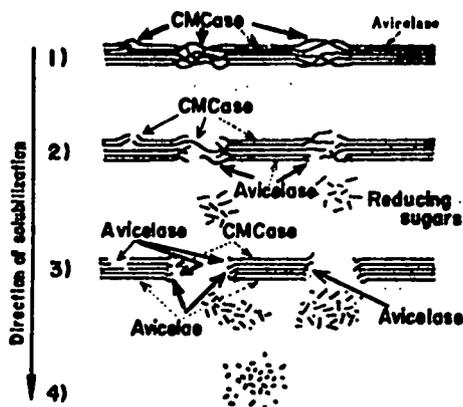


FIGURE 33. Proposed Mechanism of Synergism for the Solubilization of the Cotton Fiber by CMCase and Avicelase. (43)

- very easily
 → slightly easily
 --> difficult

TABLE XVIII
Crystallinity and Accessibility Measurements
on Cellulose (25b)

	Per cent			
	Crystal- linity (X-ray diffraction)	Crystal- linity (D ₂ O exchange)	Non- accessibility (HCl-PeCl ₂)	Non- accessibility (1N HCl)
Wood pulp (hot alkali refined)	70	54	92	
Cotton linters (chemical grade)	70	---	95	88-89
Cotton	69-71	79	91	86
Mercurized cotton	48	---	84	68
Regenerated cellulose (viscose rayon)	38-40	34	73	62-69

of the cellulose to reagent penetration^(25b). A straight forward interpretation is also not possible without taking into account the recrystallization of amorphous cellulose chains which occurs during both acid and enzyme hydrolysis.⁽¹⁴⁾ In addition, it is very likely that the transition from crystalline to amorphous regions in cellulose is not sharp and regions of intermediate order (mesomorphous) have been postulated.⁽⁴⁴⁾

The fall-off in rate of enzymatic saccharification, the extent of which varies with the ratio of substrate to enzyme, has been ascribed to enzyme inactivation, inhibition,⁽⁴⁵⁾ decreased accessibility, and changes in DP during hydrolysis.⁽¹⁴⁾ Nevertheless, it has been established that, under suitable conditions, the synergistic action of an active cellulose preparation can efficiently and effectively hydrolyze "crystalline" cellulose.

Since the cellulose crystallinity is not a serious limitation, to enzymatic saccharification of wood cellulose, other constraints must account for the limited susceptibility of the latter to enzymatic hydrolysis. Cowling⁽¹⁴⁾ has reviewed the physical and chemical impediments to the hydrolysis of lignocellulosic materials.

Accessibility

Several authors^(46,47) have discussed the question of cellulose accessibility to various cellulases in relation to the

morphological properties of different celluloses such as moisture content, porosity, and pore size distribution, before and after physical and chemical treatment. Independent porosity measurements based on solute^(48,49) exclusion techniques, using different molecular weight polyethylene glycols and dextrans to model cellulase have established a strong correlation between accessibility (internal surface area) and reactivity for 40Å and smaller sized molecules. Kinetic evidence for the influence of porosity on enzymatic hydrolysis of a variety of celluloses with different porosities has recently been presented by Griffen, et al.⁽⁵⁰⁾ The authors found the rate of solubilization at substrate saturation (V_{max}) to be equal for a variety of common cellulase assay substrates and nearly constant K_m values ($V_{max}/2$) over a broad range of cellulase activities.

These studies are consistent with the earlier work of Stamm and Millet⁽⁵¹⁾ on the internal surface characteristics of cellulosic materials as measured primarily by gas adsorption. The authors distinguished two groups: one in which the cell wall is not swollen, which have surface areas of about $2 \times 10^3 \text{ cm}^2$ per gram; and swollen cell wall systems, which give surface areas of about $3 \times 10^6 \text{ cm}^2$ per gram.

The prevailing data on accessibility indicates that crystallinity is not a controlling factor in itself, because cellulase enzymes can penetrate the pores of moist wood and hydrolyze the crystalline regions.

Accordingly, one is forced to seek alternative explanations for the resistance of wood to enzymatic saccharification. Considerable and rather convincing evidence has been advanced by Millet and Baker that implicates lignin as the primary impediment to enzymatic hydrolysis of wood cellulose. (15)

Based on rumen in vitro digestibility studies of treated and untreated woods (Table XIX), the authors obtained a moderate correlation with lignin content (Figure 34). However, the variation in the digestibilities of different woods subjected to similar and dissimilar pretreatments suggested that the structure of the wood and the method of pretreatment was equally important (Figure 35).

TABLE XIX

Effect of NaOH Treatment of the In Vitro
Digestibility of Hardwoods^a (15)

<u>Species</u>	<u>Lignin</u> %	<u>Digestibility</u>	
		<u>Untreated</u> %	<u>Treated</u> %
Quaking aspen	20	33	55
Bigtooth aspen	20	31	49
Black ash	20	17	36
American basswood	20	5	55
Paper birch	21	8	38
Yellow birch	21	6	19
American elm	23	8	14
Silver maple	18	20	41
Sugar maple	23	6	28
Red oak	24	3	14
White oak	23	4	20

^aFive grams wood treated 1 hr with 100 ml 1% NaOH, washed and dried.

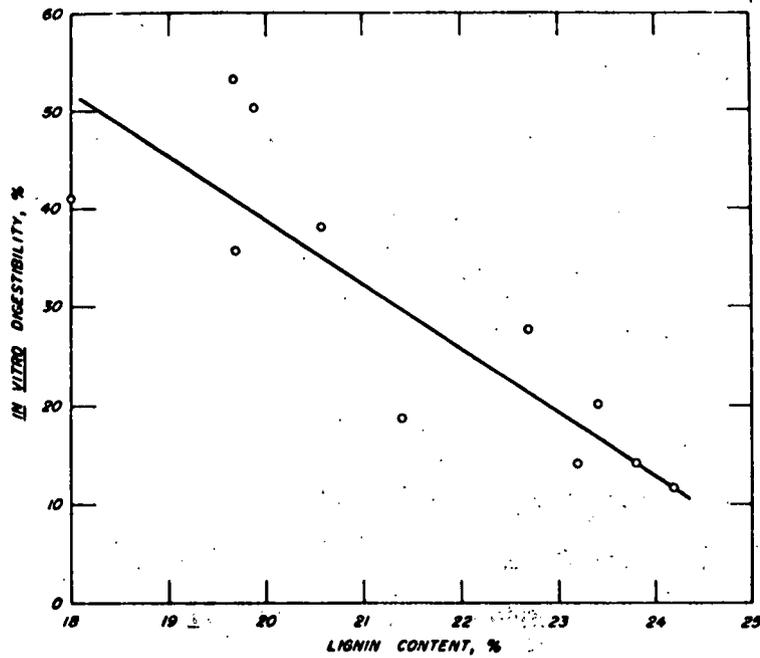


FIGURE 34. Relationship Between Lignin Content and In Vitro Digestibility for NaOH Treated Hardwoods(15)

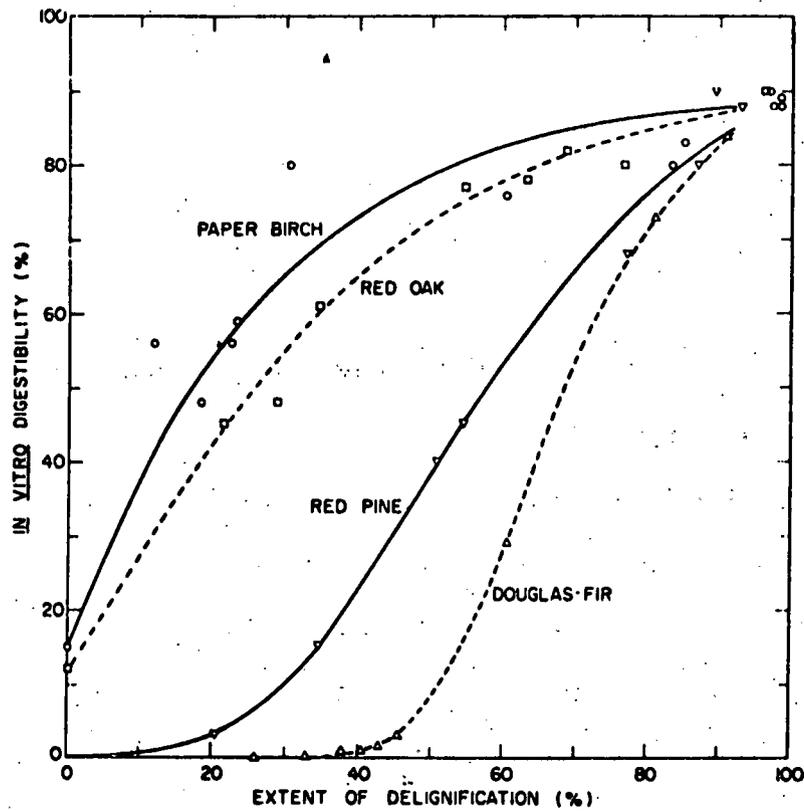


FIGURE 35. Relationship Between the Extent of Delignification and In Vitro Digestion of Kraft Pulps from 4 Wood Species(15)

The most interesting results arose from 48-hour cellulase digestibility studies on SO₂ treated wood. Essentially, quantitative sugar conversion was observed on the four hardwoods examined (Table XX).

TABLE XX

Composition and Cellulase Digestion of Various Woods Before and After SO₂ Treatment⁽¹⁵⁾

Species	Lignin %		Carbohydrate %		Digestibility %	
	Before	After	Before	After	Before	After
Quaking aspen	20	7	70	71	9	63
Yellow birch	23	9	66	67	4	65
Sweetgum	20	5	66	64	2	67
Red oak	26	8	62	60	1	60
Douglas-fir	30	24	65	63	0	46
Ponderosa pine	31	19	59	58	0	50
Alfalfa	17	--	51	--	25	--

The lignin was retained in the pretreated hardwoods but was extensively depolymerized during pretreatment which accounts for the lower Klason lignin values. Quantitative enzymatic conversion of wood carbohydrate cellulose in the presence of lignin has also been reported by Pew⁽⁵²⁾ for ball milled woods. Significantly enhanced wood digestibilities for dilute alkali⁽⁵³⁾ liquid and gaseous ammonia⁽⁵⁴⁾ and steam⁽⁵⁵⁾

and steam exploded⁽⁵⁶⁾ pretreated materials have also been reported. The principal changes in wood structure and accessibility caused by these physical and chemical pretreatments may be interpreted in the light of the earlier review of wood properties and conventional wood pulping chemistry.

Milling. The action of milling on wood is analogous to that produced during mechanical pulping via grinding stones and also to the mechanism termites employ to make wood cellulose available to their symbiotic intestinal microbial population. Defiberization and delamination of the wood fibers occur in response to the localized high shearing forces and hydraulic pressures, and result in reduced particle size, increased new surface area, and reduced crystallinity.^(57,58) At increased milling times, lignin depolymerization occurs as evidenced by increased solvent extractability. However, as indicated earlier, the milling processes are grossly energy inefficient and too costly for use in the production of low value products.

Dilute alkali. Dilute sodium hydroxide solution causes significant swelling (the maximum nonsolvent moisture content or fiber saturated point is doubled) of hardwood fibers and facilitates defiberization. Chemically, this is accompanied by deacetylation and an appreciable increase in the free carboxyl content which suggests cleavage of polyuronic ester bonds associated with the xylan chains.⁽⁵⁹⁾ Softwoods are relatively unresponsive to this treatment.⁽¹⁵⁾

Ammonia. Liquid or gaseous ammonia effect changes in wood, similar to those caused by dilute alkali treatment. Ammonolysis of uronic ester group is the principal chemical reaction. (60)

Steam. The action of high temperature (pressure) on certain woods is well documented. (61,62,63) The effect of pressure is merely to facilitate penetration and can be achieved by boiling water treatment at longer times. Water softens and swells wood fibers at low temperatures and enhances defiberization. As the temperature is raised between 100°C and 150°C, hydrolysis of the acetyl groups and hemicellulose occurs with a decline in pH and degradation of lignin-carbohydrate association. The greatest weight loss occurs between these temperatures, after which weight loss decreases occur in an orderly way, with increasing temperature from 150°C to 180°C. Thermal softening and glass transition temperatures of wood components have been reported by Goring (64) and by Salmén and Back. (65) Beyond 180°C, hydrolysis and degradation occur in this order: hemicellulose, lignin, and cellulose. Condensation of lignin occurs and volatilization becomes extensive. The foregoing effects are most sensitive to time at a given temperature; high temperatures are generally only compatible with short times if extensive degradation is to be avoided.

Steam and dilute acid. Dilute acid hydrolysis of softwood pulp was investigated over a broad range of pressures by

Saeman, et al. (27) However, to achieve sugar conversion yields beyond 50%-60%, required shorter retention times than conventional autoclave equipment permits. Developmental studies on dilute acid hydrolysis are currently in progress at Dartmouth. (13)

Steam explosion. The effect of explosion on steam treated wood is to cause a rapid defiberization to fine particles. The technology is used in the commercial production of particle or hardboard. When used as an animal feed supplement, conditions favoring the coarser particle sizes are selected because fine particles are inefficiently utilized by cattle. Little work beyond conventional rumen fluid digestibility assays (56) appears to have been done on the microbial utilization of steam-exploded particles or on chemical additions to steamed wood chips prior to explosion. Steam explosion is attractive relative to conventional autoclaving because part of the energy input can be recovered in a single operation in the form of fine fibers. Steam treatment also permits rapid processing of green wood (50% moisture) in commercial size equipment without deleterious dilution and affords a high yield recovery of the wood charge.

On the basis of these studies, it is clear that enzymatic saccharification can proceed efficiently even in the presence of lignin. However, disruption of the strong lignin-carbohydrate (66) and some delignification, are apparently required for high conversion yields. (15) What remains to be determined is

the minimum required lignin-carbohydrate alteration and the optimum pretreatment reagent and conditions for producing a substrate suitable for direct microbial conversion to ethanol.

Commercial scale sulfur-based wood pulping has posed considerable environmental problems for the pulp and paper industry and alternative approaches are seriously being examined. Recent studies of fiber separation and delignification (pulping) achieved by dilute sodium hydrozide,⁽⁶⁷⁾ oxygen/alkali,^(68,69,70) dilute ammonia,⁽⁷¹⁾ ammonia explosion,⁽⁷²⁾ ammonia vapor,⁽⁷³⁾ ammonia plus ketones,⁽⁷⁴⁾ aqueous ethanol,⁽⁷⁵⁾ and aqueous butanol⁽⁷⁶⁾ have been described in terms of properties of the resulting pulp. Since many of these pulping agents also enhance the susceptibility of wood fibers to enzymatic hydrolysis, there appears to be a strong rationale for a detailed investigation of their suitability as pretreatment agents in combination with steam. Unlike pulping where the objective is to produce lignin and hemicellulose-free cellulose fibers of optimum length, bio-conversion requires only a high recovery of the original wood charge and sufficient lignin dissolution to allow quantitative microbial conversion to alcohol.

Summary

On the basis of energy input efficiency, processing time, and ease of scaling, chemical supplemented wood steaming appears

to be the most attractive pretreatment process that can be developed on a short-term basis. In the next section, a revised research plan is presented based upon this rationale.

IX. REVISED RESEARCH PLAN

Goals and Objectives

The goals of the revised research program remain consonant with our original philosophy of developing a bioconversion process for converting plant biomass to ethanol. The experimental difficulties encountered in culturing C. pruinatum (M-10) and sporocytophaga strain US necessitates the adoption of an alternative approach in order to demonstrate technical feasibility within the time frame of this contract. In place of the biological delignification pretreatment step, we have substituted a chemically supplemented steam pretreatment step so as to partially delignify wood and to enhance its accessibility to microbial utilization. Part of the steam energy input can be recovered in the form of defiberized fine wood particles by rapid decompression. The adoption of this approach permits the use of coarse green (50% moisture) wood chips as the raw material feed, conserves steam and avoids many material handling problems associated with lower consistency wood slurries.

Depending upon the optimum pretreatment conditions selected, the pretreated wood will either be fermented directly to ethanol or be separated into a cellulose and lignin, and a hemicellulose fraction and fermented to ethanol separately by Clostridium thermocellum and a thermophilic bacillus, respectively.

The yield of ethanol from both cultures requires improvement in order to enhance the overall conversion yields from wood. The genetic improvement approach discussed in Part I will be applied to both cultures.

The principal elements of the revised process for direct fermentation of chemically supplemented steam exploded hardwood to ethanol are schematically presented in Figure 36.

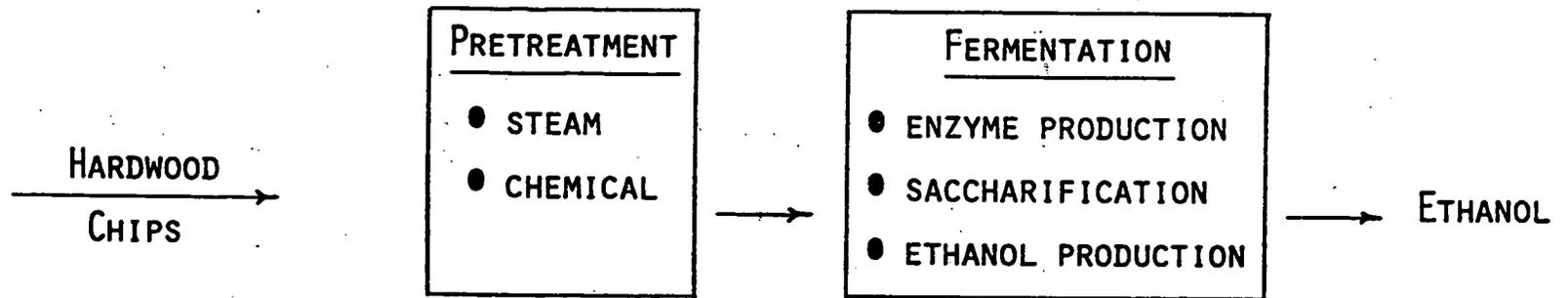
The scope of the work and the experimental methodology required to accomplish the aforementioned goals are conveniently discussed in terms of the major research objectives.

A. Chemically Supplemented Wood Steaming

The objective of this research is to develop a chemical delignification pretreatment process for increasing the accessibility of hardwood to microbial utilization. Experimental work is required to determine the minimum lignin-carbohydrate disruption and lignin solubilization (if required) needed to permit the quantitative conversion of wood carbohydrate to ethanol. The optimum pretreatment reagent and conditions (time and pressure) will be selected after an initial screening of the more promising candidates reagents.

FIGURE 36

DIRECT ETHANOL PRODUCTION FROM CELLULOSIC FIBER



To rule out the possibility that just steam exploding of wood chips would provide the required increase in accessibility, the soluble sugar yields from acid and enzymatic hydrolysis of steam exploded wood were examined and compared to milled wood. Steam samples were provided by Dr. Robert Bender of the Stake Technology, Ltd. (56) The results are given in Table XXI.

Steam explosion of poplar yields a significant quantity of soluble sugars and a more accessible substrate for hydrolysis. The soluble sugars are probably xylans and can be readily removed by water or base. The steam exploded fiber was found to be hydrolyzed in the first hour of incubation by T. viride cellulase faster than the untreated or Wiley milled fibers and at least as fast as the two-roll milled fibers. However, no substantial increase in soluble sugar production was observed thereafter. The removal of soluble sugars did not result in increased saccharification which eliminates the possibility that the initial presence of soluble sugars inhibited further saccharification. These results confirm the need for chemical addition, in order to achieve adequate lignin-carbohydrate disruption and lignin solubilization.

The construction of a suitable experimental apparatus for evaluating the optimum conditions for enhancing the microbial utilization of wood has been initiated and will be completed within two weeks. The primary components of the pretreatment reactor are schematically presented in Figure 37. The steam

TABLE XXI

Comparative Hydrolysis of Pretreated Wood

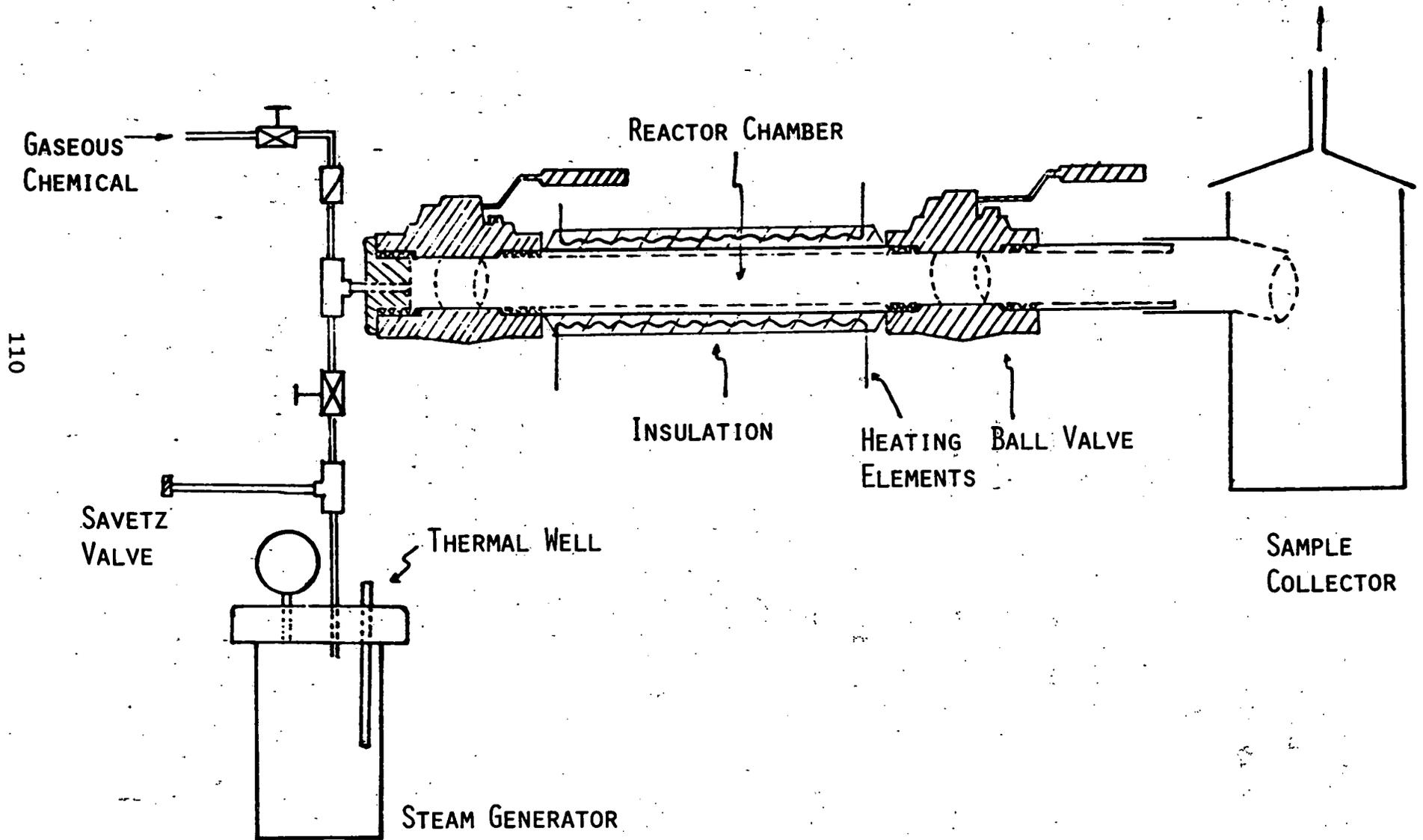
<u>Wood</u>	<u>Acid Hydrolysis*</u>	<u>Enzyme Hydrolysis</u>		
	<u>Total mg SS** produced/100 mg fiber</u>	<u>Total mg SS** produced/100 mg fiber</u>		
	<u>1 hr. 96°C</u>	<u>T₀</u>	<u>T₁-T₀</u>	<u>T₂₄-T₀</u>
Poplar Sawdust	9.0	0.55	0.4	0.7
Poplar Steam Exploded	7.0	7.8	5.0	5.4
Poplar 2 Roll Milled	18.6	1.0	5.05	9.1
Poplar Wiley Milled	9.0	3.25	0.65	1.1

Beech Sawdust	9.0	1.35	0.3	0.2
Beech 2 Roll Milled	16.8	1.6	4.95	8.7
Poplar Steam Exploded Distilled Water Wash	7.8	0.4	5.0	-
Poplar Steam Exploded 1N NaOH Wash	3.6	0.5	3.8	-

*6N HCl; **soluble sugars, T₀ = zero time; T₁ = 1 hour; T₂₄ = 24 hours.

FIGURE 37

HIGH TEMPERATURE AND PRESSURE FIBER PRETREATMENT REACTOR



generator is a high pressure bomb that can withstand 1800 psi. The capacity of the bomb is 1 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 500 psi at 250°C. The ball valves have a response time in the millisecond range to ensure uniform fiber discharge conditions. The use of ball valves allows for rapid front loading and discharge of wood chips and fibers respectively. Under these experimental conditions, the minimum residence time is limited primarily by the loading time into the preheated pretreatment chamber. The addition of chemicals before or after steaming is accomplished via a high pressure gas port on the input side of the pretreatment chamber. The pretreatment chamber is a one inch 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 accessibility of chemically pretreated fibers to microbial conversion is conveniently evaluated in shake flasks by incubation with C. thermocellum. Comparative evaluations of the more promising pretreated fibers using T. viride cellulase will provide a means of relating these studies to other research projects in the DOE Fuels from Biomass Program.

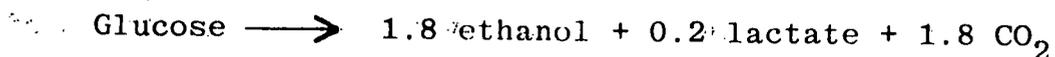
B. Genetic Improvement of Ethanol Yields

As indicated earlier, the development of thermophilic strains with enhanced ethanol and reduced acetic acid production is critical in obtaining a favorable overall yield of ethanol from wood. In principal, there appears to be no fundamental biological reason why a mutation program should not lead to strains with enhanced ethanol yields from soluble sugars.

The ability to produce ethanol from glucose is widely distributed among different microorganisms; however, the yields vary considerably from almost two moles of ethanol per mole of glucose fermented, characteristic of yeast, to very much smaller amounts formed by many bacteria.⁽⁷⁷⁾ Of the four known metabolic pathways leading to ethanol, three involve pyruvic acid as an obligatory intermediate. Pyruvate may be formed from glucose, either by the Embden-Meyerhof or the Entner-Doudoroff glycolysis pathways. Conversion of pyruvate to ethanol occurs either via reduction of acetaldehyde or acetyl-coenzyme A.

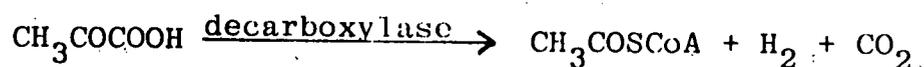
The microbial metabolism of glucose via pyruvate and acetaldehyde leads to essentially quantitative conversion of glucose to ethanol and carbon dioxide. Yeast is best known for utilizing this pathway; however, bacteria are known which possess a yeast-like pathway and ferment glucose almost quantitatively to ethanol.⁽⁷⁷⁾ Gibbs and DeMoss⁽⁷⁸⁾ have described the anaerobic

dissimilation of glucose or fructose by Zymomonas lindneri, formerly named Pseudomonas lindneri or Thermobacterium mobile.

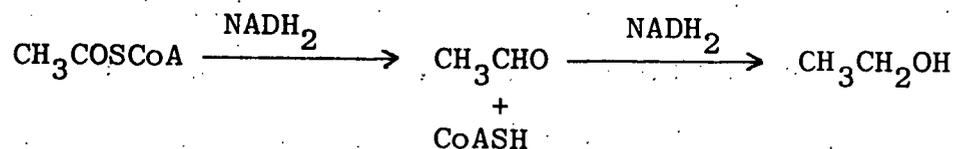


This bacterium is one of the principal organisms employed in the commercial mixed fermentation of Mexican pulque. (79) A second species anaerobia in the genus Zymomonas has also been described and found to quantitatively ferment glucose to ethanol but has different nutritional requirements. (80)

Clostrida cleave pyruvate to acetyl CoA by the following "thioclastic" reaction: (77)



The reduction of acetylcoenzyme A is thought to proceed via acetaldehyde to ethanol and to consume 4H in the process.



For quantitative conversion of glucose to ethanol, the production of hydrogen must be suppressed to provide additional reducing power for ethanol production.

Zymomonas ventriculi is presently the only organism reported to possess both decarboxylase and thioclastic enzymes for pyruvate. (81)

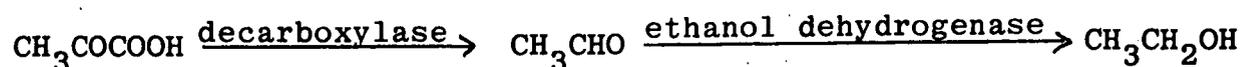
Heterolactic organisms such as Leuconostoc mesenteroides

ferment glucose to lactate and ethanol via xylulose 5-phosphate which is subsequently cleaved to give acetyl phosphate and glyceraldehyde 3-phosphate. The latter is converted to pyruvate and then reduced to lactate. Acetyl phosphate is reduced to ethanol with the reducing power derived from the conversion of glucose to xylulose 5-phosphate.

Yeast ferment glucose to pyruvate via Embden-Meyerhof glycolysis:



and use two enzymes - pyruvate decarboxylase and ethanol dehydrogenase, to convert pyruvate to ethanol.



The four pathways for ethanol production are schematically presented in Figures 38, 39, 40, and 41, after Wilkerson and Rose. (79)

The yeast fermentation of glucose to ethanol is a mature technology. The only apparent method of improving upon the productivity of this fermentation is to employ a continuous cell recycle vacufermentation⁽⁸²⁾ or to conduct the fermentation at higher temperatures to increase the rate of fermentation and to eliminate the cooling requirements associated with conventional yeast fermentations.

Thermophilic ethanol producing yeast are not known and are not likely to be accessible via mutation. Alternatively, the

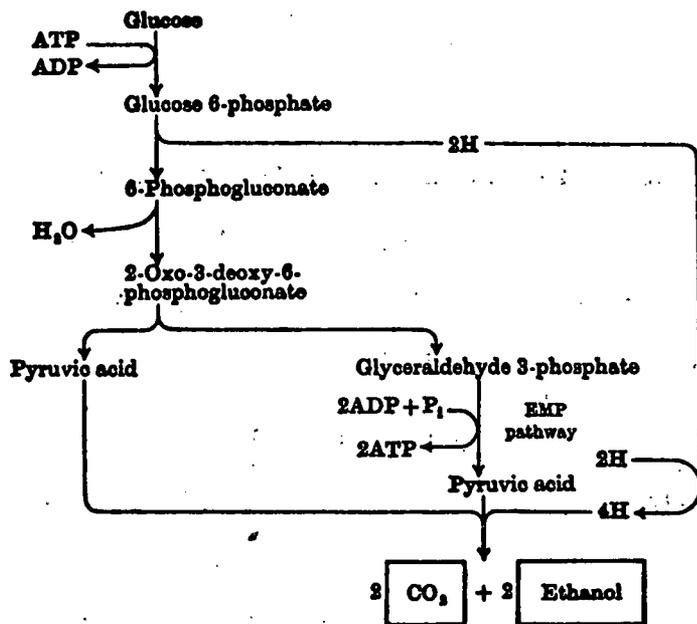


FIGURE 38. The Ethanolic Fermentation of Glucose by *Pseudomonas lindneri* Using the Entner-Doudoroff Pathway. (79)

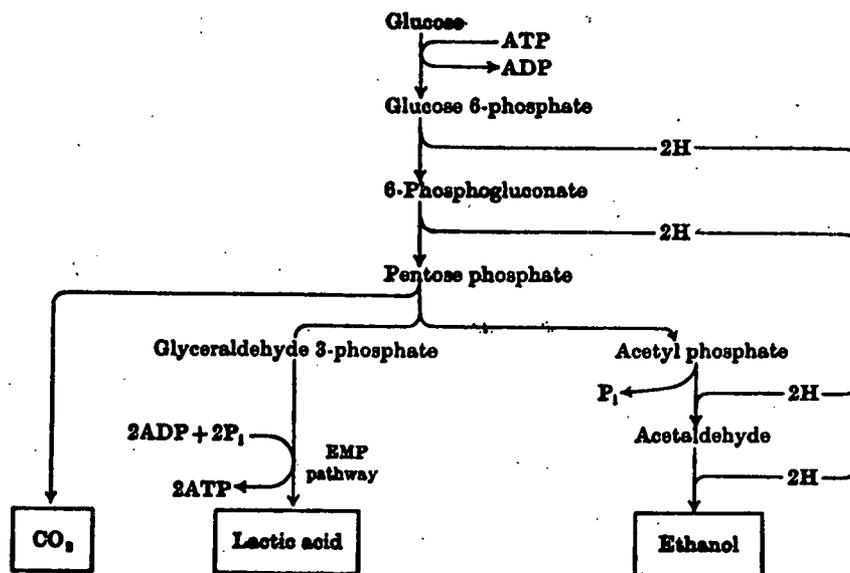


FIGURE 39. The Heterolactic Fermentation of Glucose by *Leuconostoc mesenteroides* Using the Pentose Phosphate Pathway. (79)

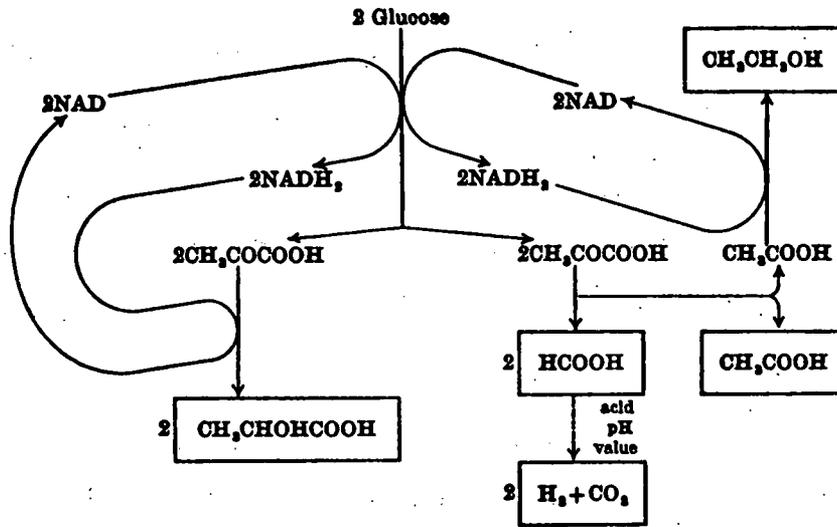


FIGURE 40. The "mixed acid" Fermentation of Glucose by Escherichia coli. (79)

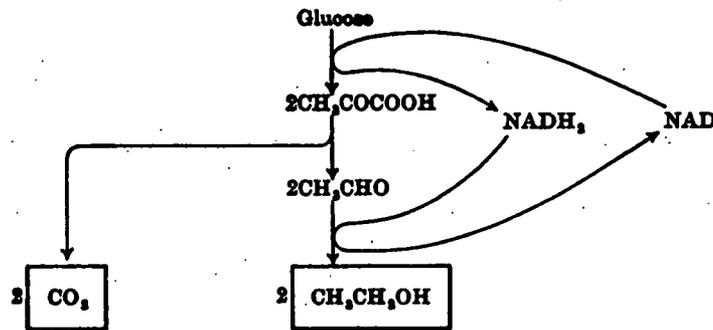


FIGURE 41. The Ethanolic Fermentation of Glucose by Yeasts. (79)

best prospects for improving upon conventional ethanol fermentation technology appears to be in mutating thermophilic ethanol producing strains for higher yields of ethanol.

The objective of this phase of our research is to develop mutants in which acetic and formic acid production is blocked and the available reducing power is used to channel pyruvate to ethanol. The generalized scheme for accomplishing this objective is presented in Figure 42. The mutational protocol discussed in Part I will be employed to develop suitable mutant strains of C. thermocellum and thermophilic bacillus strain ZB-B2.

C. Bench-Top Fermentation Studies

The objective of this segment of the research is to demonstrate the technical feasibility of an integrated process for the direct production of ethanol from chemically pretreated wood fibers.

Chemically pretreated hardwood fibers will be evaluated in 5 liter bench-top fermentor experiments. The kinetics of substrate utilization and ethanol production will be evaluated under batch fermentation conditions. Separate fermentations will be conducted on the hemicellulose fraction to determine the yield and conversion rate to ethanol.

The size of the fermentors and, therefore, the costs, depends to a large measure on the concentration of substrate that

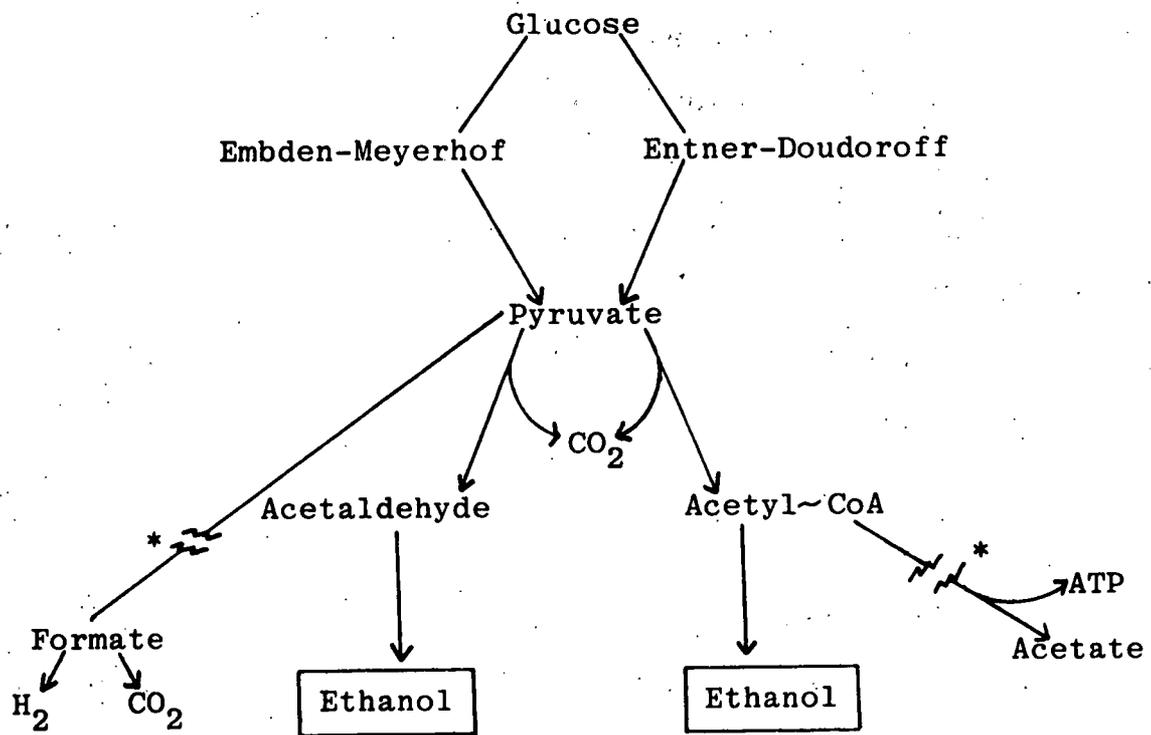


FIGURE 42. Glucose Metabolism to Ethanol

*Blocked pathways

can be fermented. The effect of substrate concentration on the rate of ethanol production will, therefore, be examined.

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X. ECONOMIC CONSIDERATIONS

A. Economics of Ethanol Production

The most thoroughly studied process for producing ethanol from biomass is the enzymatic conversion of agricultural waste to soluble sugars and subsequent fermentation to ethanol by yeast. Recent preliminary economic evaluations^(83,84) of the process, developed by Professor Wilke of Berkeley, provide some indication of the principal cost elements. The distribution of costs associated with ethanol production (exclusive of raw material costs) from wheat straw and newsprint via this process are given in Table XXII. Saccharification costs dominate because the fermentor capacity required to produce sufficient T. viride cellulase enzyme is between 30 - 40 times that needed to ferment the resulting sugars (Table XXIII). Consequently, the volumetric production efficiency which is often used to evaluate the economics of a fermentation process is significantly lower than conventional molasses fermentation by yeast and reflected in the conversion cost estimates (Table XIV and Figure 43). In this regard, the direct fermentation of biomass to ethanol which avoids the separate saccharification step is seen to be potentially capable of higher volumetric efficiency (Figure 36).

TABLE XXII

DISTRIBUTION OF ETHANOL COST FROM CELLULOSIC FIBER

(Except Raw Material Cost)

<u>Substrate</u>	<u>Pretreatment</u>	<u>Saccharification</u>	<u>Ethanol Fermentation</u>
newsprint ⁽⁸³⁾	6.8 %	64.4 %	26.8 %
wheat straw ⁽⁸⁴⁾	12.0 %	78.0 %	10.0 %

TABLE XXIII
ETHANOL PRODUCTION BY FERMENTATION

<u>Substrate</u>	<u>Ethanol Output</u> <u>10⁶ gallon/yr</u>	<u>Required Fermentor Size (10⁶ gal.)</u>	
		<u>Enzyme Production</u>	<u>Ethanol Fermentation</u>
newsprint ⁽⁸⁵⁾	8.5	5.25	0.125
wheat straw ⁽⁸⁴⁾	25.0	9.54	0.336
molasses ⁽⁸⁶⁾	25.0	----	0.300

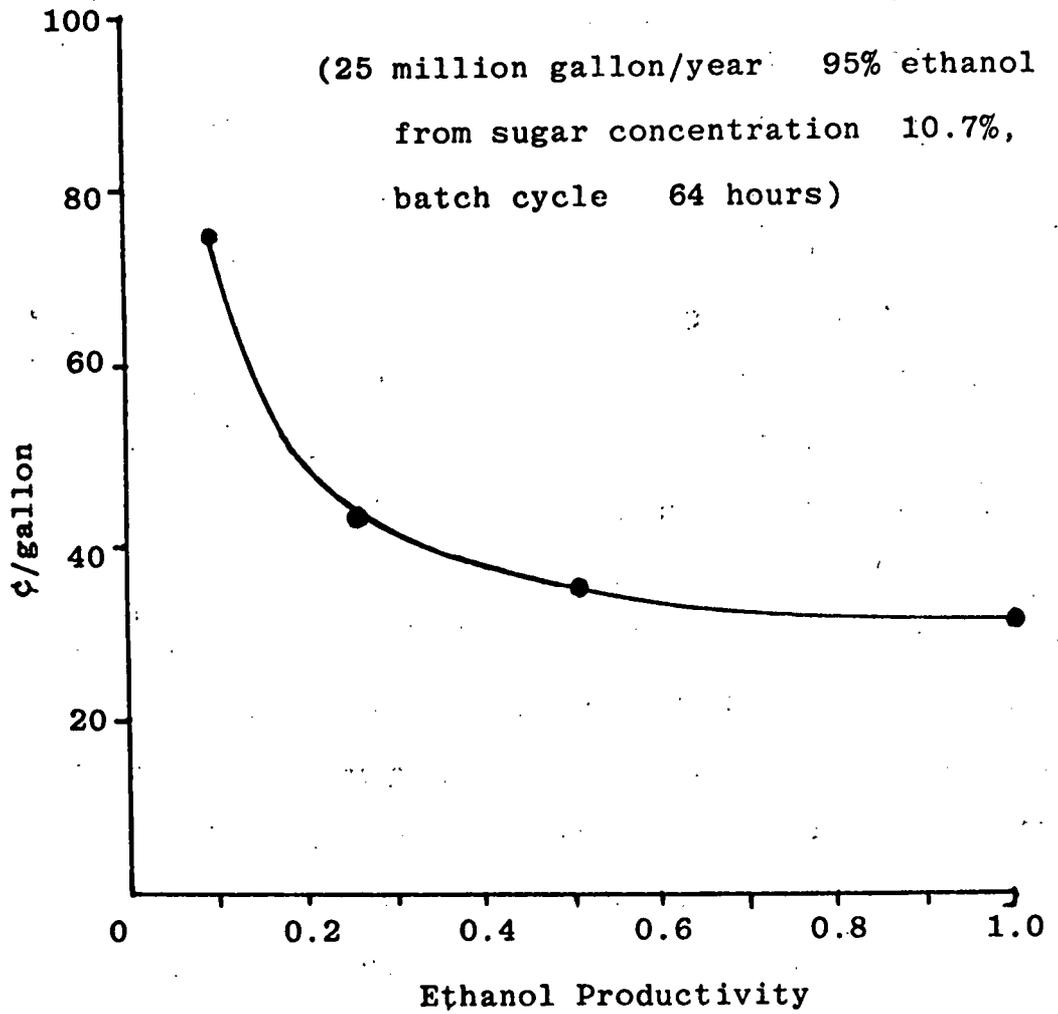
TABLE XXIV

ETHANOL PRODUCTION BY FERMENTATION⁽⁸⁷⁾

<u>SUBSTRATE</u>	<u>NEWSPRINT⁽⁸⁵⁾</u>	<u>WHEAT STRAW⁽⁸⁴⁾</u>	<u>MOLASSES⁽⁸⁶⁾</u>
<u>ETHANOL OUTPUT</u>			
10 ⁶ GALLON/YEAR	8.5	25	25
<u>FERMENTOR SIZE 10⁶ GALLON</u>			
<u>ENZYME PRODUCTION</u>	5.25	9.54	
<u>ETHANOL FERMENTATION</u>	0.125	0.336	0.30
<u>VOLUMETRIC PRODUCTION</u>			
<u>EFFICIENCY G/L-HR.</u>	0.2	0.026	10
<u>CONVERSION COST</u>			
¢/GALLON	132.2	395.4	30.5

FIGURE 43

ETHANOL PRODUCTION COSTS AS FUNCTION OF PRODUCTIVITY⁽⁸⁶⁾



B. Preliminary Economic Considerations of Direct Ethanol Production

It is not yet possible to evaluate the economic viability of our revised process for direct ethanol production from pretreated hardwood chips. However, based upon the similarity of the proposed process to ethanol fermentation from molasses and the reported costs,⁽⁸⁶⁾ one can estimate with suitable assumptions what ethanol might cost in such a process.

The cost estimate is based, for convenience, on a 25 million gallon per year 95% ethanol plant from hardwood chips. The first stage is a chemically supplemented wood steaming pretreatment followed by a second stage direct fermentation to ethanol. The principal assumptions for this preliminary cost estimates are:

1. Raw material is received as a coarse hardwood chip, and no further pretreatment is needed before chemical steaming.
2. The high temperature chemical pretreatment involves the reaction of moist wood chips (water/wood \approx 3) with gaseous SO_2 (30 psi at room temperature, weight ratio of wood/ SO_2 \approx 22) under pressure for 2 hours at a temperature of 120°C . These conditions approximate those employed by Millet⁽³³⁾ to obtain a substrate which could be quantitatively converted into sugars. Following pretreatment, the fibers are neutralized with alkali. Approximately one third of the SO_2 is assumed to react with lignin to form sulfonate.

3. The productivity of the ethanol fermentation is calculated on the basis of a 21% fiber concentration in the fermentor, 50% of which is fermentable. The yield of ethanol is assumed to be 50% of the fermentable sugar during a 128 hour batch fermentation cycle. The productivity of the ethanol fermentation is assumed to be one half of that presented in the SRI report. (84)

The principal costs estimates based on these assumptions are presented in Table XXV.

TABLE XXV
Cost Estimates^a

	<u>Pretreatment</u>	<u>Ethanol Production</u>	<u>Raw Material</u>
Capital Investment	\$7.1 x 10 ⁶	\$22.7 x 10 ^{6c}	
Capital Related Cost (¢/gallon)	3.4	14.72	
Direct Cost (¢/gallon)	19.1 ^b	19.8	72 ^d
Manufacturing Cost (¢/gallon)	22.5	33.8	72

^aExcludes general service facility cost, start-up cost, working capital, and land cost.

^bUnit price for SO₂ 8¢/lb; NaOH 11¢/lb (chemical marketing report); steam \$2.50/M lb.

^cBased on SRI Report (1978) Figure

^dUnit cost of wood chip \$30/ton.

C. Areas for Further Process and Cost Improvements

In the aforementioned cost estimate, a conventional stainless steel autoclave was employed as the pretreatment reactor. In practice, a large diameter screw-fed stainless steel pipe should prove adequate for commercial operation and provide a significant cost savings. The SO_2 concentration and treatment time employed by Millet were not optimum, and there is reason to expect that by going to high temperatures that the size of the pretreatment reactor can be reduced significantly. The assumed yield of ethanol was based only on the conversion of the cellulose fraction of the pretreated wood. The conversion of the hemicellulose fraction ($\sim 20\%$ of raw material) to ethanol would enhance the overall conversion yield to products. Finally, the employment of a continuous fermentation process with cell recycle should provide a means of reducing the required fermentation capacity and associated costs.

The preliminary cost estimate of ethanol production via direct fermentation of pretreated fibers suggests that there is considerable merit to this approach. Additionally, several areas where further costs and productivity improvements can be achieved were identified. Accordingly, we have initiated experimental work on this process and will follow the experimental study plan discussed in the next section.

XI. EXPERIMENTAL STUDY PLAN AND PERFORMANCE SCHEDULE

The objectives of this research is to demonstrate on a laboratory scale the technical feasibility of an integrated process for the bioconversion of chemically pretreated hardwood chips to ethanol by direct fermentation.

A. Chemically Supplemented Wood Steaming

The objective of the pretreatment studies is to determine the minimum required lignin-carbohydrate disruption and the optimum pretreatment reagent and conditions for producing a substrate suitable for direct microbial conversion to ethanol. This study will consist of the following tasks:

1. Pressure-time pretreatment conditions. The digestibility of hardwood chips treated at different steam pressures for variable times will be examined. Conditions yielding the highest digestibilities will be employed to study the effects of selected chemical additives.
2. Screening of chemical additives. The effects of added delignification agents; ammonia, ethanol, sodium hydroxide/air, and sulfur dioxide on the microbial utilization of steam-exploded wood chips will be examined. The reagent and conditions showing the highest increase in hardwood digestibility will be employed to prepare sufficient pretreated substrate for subsequent bench-top fermentor studies. The digestibility of the

water or alkali extracted hemicellulose fraction will be examined separately and compared with growth on exploded fibers.

3. Evaluation of pretreated wood. The accessibility of chemically pretreated fibers to microbial utilization will be initially evaluated in shake flasks by incubation with C. thermocellum. Comparative evaluations of the more promising pretreated fibers will also be performed by T. viride cellulase enzymes, as a means of relating these studies to other research projects in the DOE Fuels from Biomass Program.

B. Genetic Improvement of Ethanol Yields

The objective of the mutation studies is to develop mutant strains of C. thermocellum and thermophilic bacillus ZB-B2 in which the acetic acid and formic acid production is blocked and available reducing power used to channel pyruvate to ethanol. The mutation protocol and mutant screening procedure discussed in Part I will be employed to select suitable mutants.

C. Bench-Top Fermentations

The objective of this phase of the research is to demonstrate the technical feasibility of fermenting pretreated hardwood fibers directly to ethanol.

1. C. thermocellum. Base line studies of the growth of C. thermocellum strain LQ8 growing on amorphous cellulose and soluble sugars will be examined while the pretreatment studies

are in progress. The growth of C. thermocellum on the most promising pretreated fibers will then be studied in batch fermentation. The most promising high yield ethanol mutant will also be studied in batch fermentation on pretreated fibers.

2. Thermophilic bacillus ZB-B2. The most promising mutant ZB-B2 strain will be examined for growth and ethanol production from the hemicellulose fraction of the pretreated fibers.

3. Modified vacuform. In the event that mutant strains with high ethanol yields show low ethanol tolerance, a modified vacuum fermentation will be studied using soluble sugars as a substrate.

D. PERFORMANCE SCHEDULE

Research Activity	Months After Authority to Proceed											
	1	2	3	4	5	6	7	8	9	10	11	12
A. <u>Chemically Supplemented Wood Steaming</u>												
Select Optimum Time, Pressure Conditions	→	▲										
Screen Chemical Additives	→	→	▲									
Evaluate Pretreated Wood												
● <u>T. viride</u> saccharification			→	→	▲							
● <u>C. thermocellum</u> digestibility		→	→	▲								
B. <u>Genetic Improvement of Ethanol Yields</u>												
<u>C. thermocellum</u>	→	→	→	→	→	→	→	→	→	→	▲	
Thermophilic Bacillus ZB-B2	→	→	→	→	→	→	→	→	→	→	→	▲
C. <u>Bench-Top Fermentation</u>												
<u>C. thermocellum</u>												
● amorphous cellulose, soluble sugars, and pretreated wood		→	→	→	→	→	→	→	▲			
● mutant growth on cellulose and soluble sugars								→	→	→	▲	
Thermophilic Bacillus ZB-B2								→	→	→	→	▲
● mutant growth on soluble sugars								→	→	→	→	▲
<u>Modified vacuform</u> of high yield ethanol strain on soluble sugars										→	→	▲

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