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THE BIOLOGICAL PRODUCTION OF LIQUID
FUELS FROM BIOMASS

University of Pennsylvania

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"The Biological Production of Liquid
Fuels from Biomass"

DOE Contract to the
University of Pennsylvania

EXECUTIVE SUMMARY

The purpose of this study is to investigate a scheme for the production of liquid fuels from renewable resources such as poplar wood and lignocellulosic wastes from a refuse hydropulper. The particular scheme being studied involves the conversion of a cellulosic residue, resulting from a solvent delignified lignocellulosic feed, into either high concentration sugar syrups or into ethyl and/or butyl alcohol. The process is aimed at achieving total raw material utilization and maximization of high value by-product recovery. Specific goals of the investigation are the demonstration of the process technical feasibility and economic practicality and its optimization for maximum economic yield and efficiency.

During the period covered by this report the construction of a pilot apparatus for solvent delignifying 150g samples of lignocellulosic feeds has been completed. Also, an analysis method for characterizing the delignified product has been selected and tested. This is a method recommended in the Forage Fiber Handbook. Delignified samples are now being prepared and tested for their extent of delignification and susceptibility to enzyme hydrolysis.

Work is continuing on characterizing the cellulase and cellobiase enzyme systems derived from the YX strain of Thermomonospora with which we have been working. It should be noted that in the past this organism, isolated by workers at the General Electric Company, has been designated as a Thermoactinomyces sp.. Its identification as a member of the genus Thermomonospora has been confirmed by workers at the Waksman Institute of Microbiology, Rutgers University. In their examination of the organism they have noted that the morphology is typical of the bifid type of Thermomonospora and fits the description of Thermomonospora alba (also called Actinobifida alba). Strain improvement work with the organism has resulted in a stable mutant designated N-35 whose culture broth has more than double the extracellular filter paper activity compared with the YX strain as well as double the specific activity. Enzyme from this new strain will be compared with that from the YX strain in our systems for producing high glucose syrups. It should be noted that we have demonstrated with the crude extracellular cellulase and crude β -glucosidase recovered from sonified cell fragments of the YX strain that within 168 hours 20% sugar syrups assaying 90% glucose can be obtained from swollen cellulose.

Enzyme characterization of the YX strain cellulase system has shown it to be extremely heat stable, activated by Ca^{++} and Co^{++} and inhibited by Hg^{++} and Pb^{++} , and strongly end product (cellobiose) inhibited. The β -glucosidase activity of the sonified cell fragments has been shown to be considerably less thermally stable. The immobilized enzyme (on glass beads) has a half life of 6 hours at 55°C . The cellobiase activity is not

significantly product inhibited; activity in a 15% glucose solution is reduced by only 25%. Purified β -glucosidase enzyme has now been prepared and is being studied. A two reactor scheme has been constructed and is in the process of being tested for the production of concentrated sugar syrups. In the first reactor the cellulase system is contained in stirred reactor configuration. Clarified solution from this reactor is sent to a second reactor containing immobilized β -glucosidase in a fixed bed configuration. Most of the solution from this reactor is recycled to the first reactor in order to achieve high conversion yields. In view of the recent success of Dr. Hagerdal, a former member of the group, in converting cellobiose to ethanol by passage through a column containing both immobilized yeast cells and β -glucosidase enzyme, our group is considering the possibility of converting the system to a three reactor recycle system, i.e. the first stage being a cellulase reactor, the second a β -glucosidase and yeast reactor, and the third stage an alcohol extraction system.

With respect to alcohol production systems, the group has constructed and is currently testing 3 different bench scale systems. The first consists of a scheme to simultaneously saccharify, ferment to alcohol using an alcohol tolerant C1. thermocellum strain at 55°C, and then vacuum flash the broth to recover a high ethanol beer. Testing the system with a 1 g/liter solution of ethanol and acetic acid has shown a 9- fold increase of ethanol concentration in the vapor. With respect to the microorganism, a soil Clostridium, strain XD, isolated at Hahnemann was shown to produce 65% of the theoretical amount of ethanol. The second scheme consists of a two stage system in which simultaneous saccharification and fermentation

to butanol is carried out with a strain of Cl. acetobutylicum. The broth is continuously extracted with dibutylphthalate to recover butanol.

A third scheme involves the simultaneous fermentation of glucose syrups by a strain of yeast (S. cerevisiae) and the extraction of the alcohol by polypropylene glycol (P1200).

In addition to these studies the group is continuing its work on the characterization of a xylanase derived from Thermomonospora, strain LL, in order to test the possibility of producing pure xylose from delignified cellulosic wastes. Also, economic evaluation of these various schemes is proceeding hand-in-hand with the process developments.

Sept. 1, 1979

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RESEARCH PERSONNEL SUPPORTED BY PROJECT

September 1, 1978 - August 31, 1979

E. Kendall Pye, Ph.D.
Principal Investigator (50%)

Associate Professor of
Biochemistry & Biophysics

Arthur E. Humphrey, Ph.D.
Co-Principal Investigator (20%)

Dean, Engineering and
Applied Science

Biochemistry & Biophysics

John Ferchak
Harriet Harris
Helena Whitty

Research Specialist (100%)
Research Specialist (50%)
Research Assistant (100%)

Chemical & Biochemical Engineering

Richard I. Mateles
Roy Hernandez-Mena
Mark Holtzapple)
Janice Phillips)
Don Ristroph)
Geoff Slaff)

Visiting Professor (50%)
Research Technician (100%)
Research Assistants/
Graduate Students (80%)

General Electric Company

John R. Forro, Ph.D.
Kathy Detweiler
Edward Nolan

Microbiologist (10%)
Research Technician (50%)
Engineer (50%)

Hahnemann Medical School

James Alexander, Ph.D.
Richard Connors
Anthony Quattrone
Mark Sobel

Professor of Biochemistry (50%)
Technician (100%)
Summer Alumni Fellow (100%)
Summer Fellow (100%)

THE BIOLOGICAL PRODUCTION OF LIQUID FUELS FROM CELLULOSIC BIOMASS:

A PROCESS STUDY FOR TOTAL UTILIZATION OF BIOMASS

University of Pennsylvania/General Electric Company/

Hahnemann Medical College

(U.S. D.O.E. Contract EY-76-S-02-4070)

Introduction

The purpose of this study is to investigate the production of alternative liquid fuels, specifically ethanol and a butanol-lignin slurry, from renewable resources such as wood and cellulosic wastes. Ethanol has long been demonstrated to be an excellent extender of gasoline, both worldwide and in this country, and most recently in Brazil where a national program is underway. Lignin slurries in butanol promise to be excellent pumpable alternative heating and diesel fuels, but their utility has yet to be demonstrated.

It is a fundamental premise of the University of Pennsylvania/G.E. process for the production of multiple liquid fuels from biomass that there be total raw material utilization and that high value by-products recovery be maximized. While the process economics should not ultimately rely on the value of the by-products, it is our belief from preliminary economic studies that by-product credit can be crucial for the early implementation of this process.

With these points in mind, and with the valuable experience of over two years of research behind us, the PENN/GE/Hahnemann group has developed a process scheme designed to convert cellulosic biomass, such as poplar wood chips and solid waste cellulosic residues, to ethanol and lignin-butanol slurries, for use as liquid fuels, together with high value by-products such as animal feed, polymer grade lignin and xylose. This process, shown schematically in the Figure on page 5 is a modification of our earlier scheme and is now the center of research activities of our group. Our goal is to demonstrate its technical feasibility and economic practicality, and to optimize the process for maximum yield and efficiency. The overall project consists of 9 basic tasks (see the Table on pages 6 and 7). The objectives, manpower, results, and future plans of these individual tasks are detailed in the material which follows.

PENN/GE PROCESS FOR TOTAL BIOMASS UTILIZATION

LIST OF PROJECT TASKS

<u>Sub-Project</u>	<u>Investigators</u>	<u>Specific Tasks</u>
Solvent Delignification and Lignin Slurry Fuel Production	M. Holtzapple (UP) A. Humphrey (UP)	1. Investigate solvent delignification as a pretreatment scheme for the production of a readily degradable cellulose and useable by-product streams and measure lignin slurry recovery for use as a fuel.
Hemicellulose Utilization	D. Ristroph (UP) A. Humphrey (UP)	2. Investigate the possibility of xylose recovery from the hemicellulose fraction and the utility of the residual hemicellulose for fuel and solvent production.
Cellulose Saccharification	J. Ferchak (UP) E. K. Pye (UP) H. Harris (UP) H. Whitty (UP)	3. Define the kinetic character of the <u>Thermomonospora</u> cellulase system for optimal enzyme production and cellulose hydrolysis.
Production of Concentrated Glucose Syrups	J. Phillips (UP) H. Harris (UP) A. Humphrey (UP) E. K. Pye (UP)	4. Define an enzymatic system for production of concentrated glucose (>20%) syrups for ultimate use in existing alcohol fermentation processes.
Enhanced <u>Thermo-</u> <u>monospora</u> Mutants	J. Forro (GE) K. Detweiler (GE)	5. Develop <u>Thermomonospora</u> mutants producing cellulase with high unit activity and low product inhibition characteristics.
Use of Ethanol Tolerant <u>Cl.</u> <u>Thermocellum</u> in Combined Saccharification and Vacuum Fermen- tation for Ethanol Production	J. Alexander (HH) G. Slaff (UP) A. Humphrey (UP) R. Connors (HH) A. Quattrone (HH) M. Sobel (HH)	6. Investigate the feasibility of combining, at high temperatures, enzymatic saccharification, <u>Cl. thermocellum</u> fermentation and alcohol recovery by vacuum techniques into a single processing step.
Combined Saccharification and Extractive Fermentation	A. Humphrey (UP) R. Mateles (UP) R. Mena (UP)	7. Investigate the feasibility of combining enzymatic saccharification, <u>Cl. acetobutylicum</u> fermentation and butanol extraction by solvent and/or solid absorption into a single processing step.

Sub-ProjectInvestigatorsSpecific Tasks

Increasing the
Efficiency of
Ethanol Fermen-
tation

J. Ribaud (UP)
R. I. Mateles (UP)
A. Humphrey (UP)

8. Utilize extractive fermentation to increase the efficiency of ethanol production by yeast.

Economic
Analysis

E. Nolan (GE)
A. Humphrey (UP)

9. Perform economic analysis of the integrated process and its intermediate steps for the purpose of identifying cost sensitive steps and reasonable alternatives.

Task 1

Solvent Delignification and Lignin Slurry

Fuel Production

Objective:

To determine the most economic manner for removal of lignin from wood chips by extracting with an aqueous/organic solvent mixture, and to determine operating parameters to obtain highly saccharifiable cellulose.

Manpower:

Mark Holtzapple (75%)

Arthur Humphrey (5%)

Title:

Graduate Research Fellow
Chemical and Biochemical Engineering

Dean, Engineering and Applied Science

Summary of Results

Preliminary studies were conducted in a pressure bomb to determine whether delignification by an aqueous/organic mixture is applicable to the Penn/GE process. The chips were treated for ½-hour with a 50:50 mixture of butanol and water at a pressure of 50 psig (about 150°C). The water contained a buffer of sodium carbonate to prevent hydrolysis of the hemicellulose. Results indicate that about 50% of the lignin was removed with little or no loss of hemicellulose or cellulose (Summary presented in Table 1.1).

The solid chips were saccharified using the enzyme from Thermomonospora YX (Fig. 1.1). Untreated chips showed virtually no hydrolysis in a 6-hour period. Chips which had been exploded at 60 psig and which were extracted with the water/butanol mixture showed increased susceptibility to hydrolysis. Unexploded, water/butanol extracted chips showed the most release of

reducing sugars over the 6-hour period. It is possible to interpret less release of reducing sugars from exploded chips as resulting from a loss of the most readily saccharifiable sugars in the explosion process. This conclusion is preliminary and warrants further investigation.

The results from the preliminary study were encouraging, so it was decided to build a more sophisticated apparatus allowing more complete characterization of the delignification process. A schematic is shown in Fig. 1.2. The reactor 1 is supplied with water and organic solvent from reservoirs 6 by metering pumps 3. Recycle pump 4 allows the liquid to flow through the wood chips which fill the basket 2 which is inside the reactor. The output stream flows into flash chamber 7. Condenser 8 returns vapors to the flash chamber. The pressure, as indicated by gauge 11, is controlled by back-pressure regulator 9. The heating tape 18 is controlled by proportional temperature controller 17. Strainers 12 protect equipment from any particles which may be suspended in the streams. Safety relief valve 10 protects the apparatus and operator from excess pressure. Quick disconnects 14 allow the reactor to be opened so that the basket may be refilled with wood chips.

This piece of apparatus has been fully built and tested. A preliminary run has been completed. The temperature controller provided excellent temperature control. The apparatus provides great flexibility in operation as it may be operated in both a batch and continuous mode of extractant addition. Water and organic streams may be added separately or together. Steam may be used to rapidly bring the system up to temperature. Samples may be drawn throughout the extraction to measure the kinetics of the delignification process.

The pressure is limited to about 160 psig and the temperature is limited to about 500°F. About 150g of chips may be treated at a time.

Before the performance of the extractor can be tested, a reliable means of measuring the lignin content must be found. Three basic methods were found in the literature:

1. Weight loss in making holocellulose
2. Weight remaining after strong acid treatment
3. Weight loss by oxidation with potassium permanganate

The first method involves treating wood fibers with chlorine gas which reacts with lignin leaving the cellulose and hemicellulose (holocellulose) behind. This is prone to error because HCl forms and causes some hemicellulose and cellulose to hydrolyse and be lost. It is also dangerous to work with chlorine.

The second method is prone to error because about 5-10% of the lignin is acid soluble. Also, it assumes complete hydrolysis of the carbohydrates.

The third method seems to be the best. It was taken from Forage Fiber Analysis (Agriculture Handbook No. 379, USDA Agricultural Research Service, Jacket No. 387-598). The fiber is treated for 90 minutes with concentrated potassium permanganate using silver catalyst. The fibers are then washed with demineralizing solutions to leach out the ions.

It is recommended in the reference to treat the sample with boiling 1N H_2SO_4 for 1 hour in a reflux apparatus to remove materials which interfere with the lignin test. It also claims that the removed material in the pre-treatment is an indication of the hemicellulose content.

Since 1N H_2SO_4 and 1 hour seem like rather arbitrary conditions, it was decided to test various acid strengths and treatment times to see the

effect they have on weight loss due to hydrolysis. It was also decided to do the treatment in an autoclave rather than in a reflux apparatus since this would allow many samples to be run at a time and would not require purchasing reflux apparatus.

The poplar wood samples were ground in a Wiley mill and sieved to pass through a 20 mesh screen, but remain on a 40 mesh screen. One gram samples were dried in a 105°C convection oven before the acid pretreatment was done. The results are shown in Fig. 1.3 .

No characteristic shoulders are present which would be expected if weak acid were to hydrolyse only the hemicellulose and then begin hydrolysing cellulose at higher acid concentrations. Probably, at lower acid concentrations hemicellulose hydrolysis dominates and at higher concentrations, cellulose hydrolysis dominates; but this has not been verified experimentally. Even though this test doesn't appear to be hemicellulose specific, it can be useful in defining a "weak acid susceptible fraction" (WASF). This will give a number which will help characterize the effect delignification has on enhancing wood hydrolysis using weak acids. An efficient treatment condition is 25 g/l for 15 minutes because a significant portion of the wood (26%) can be hydrolyzed without much investment in time or acid.

All of the acid pretreated samples were oxidized using the permanganate method described in Forage Fiber Analysis. The resulting weight loss is shown in Table 1.2.

The average weight loss was 15.9% with a standard deviation of 1.8. The standard deviation is only about 10% of the mean, Therefore, it is reasonable to assume that the different pretreatment conditions did not affect the lignin assay results. Further, there don't appear to be any

trends within the data.

The most consistent result for percent lignin is seen with 25 g/l acid concentration (~17%). Also, after 25 g/l acid pretreatment and permanganate oxidation, the fiber was white. Other pretreatment acid concentrations tended to leave fibers off-white or even brown after the permanganate oxidation. White indicates complete lignin removal, while brown means some lignin could be remaining. This consideration further supports the use of 25 g/l as the pretreatment acid concentration.

Table 1.1

Butanol Extraction of Poplar ChipsTreatment

Time = 30 min w/constant agitation

Temp = 150°C (equilibrium temp for 50 psig)

Press = 50 psig

But: H₂O = 1:1 (by volume)

Liquid: Solid = 10:1 (by wgt)

Aqueous pH = 8.2(Na₂CO₃ buffer)Wood Analysis by Madison Technique

	<u>Before Treatment</u>	<u>After Treatment</u>	<u>% Reduction</u>
Dry Weight (Basis), gm	100	85	15
Cellulose, gm	45	48*	0
Hemicellulose, gm	20	20	0
Lignin, gm	24	12	50
Unknown (Ash + Unreacted Above), gm	11	5*	--

* After treatment apparently some unreacted material analyzed as cellulose

FIGURE 1.1

Hydrolysis of Poplar Chips by Extracellular
Cellulase of Thermomonospora

25 dry grams chips/liter

chips wet-milled in blender before hydrolysis

FIGURE 1.2
Apparatus for Solvent Extraction of Biomass

Apparatus Key

1. flanged stainless steel reactor (15" x 3" dia)
2. stainless steel basket
3. metering pump
4. recycle gear pump
5. rotameter
6. graduated cylinder
7. flash chamber
8. condenser
9. back-pressure regulator valve
10. safety pressure relief valve
11. pressure gauge
12. Y-strainer
13. sight glass
14. quick release
15. sample flask
16. platinum resistance temperature probe
17. proportional temperature controller
18. heating tape

FIGURE 1.3

Weight Loss After Acid Pretreatment of Ground Poplar Chips

Table 1.2

Weight Loss with Permanganate Oxidation

<u>acid conc(g/l)</u>	<u>Treatment Time</u>		
	<u>15 min</u>	<u>30 min</u>	<u>60 min</u>
10	20.7	13.7	17.2
25	17.0	17.0	16.1
50	15.5	17.0	14.5
100	16.6	13.8	14.2
200	15.4	13.9	
300	16.7	14.9	17.2

$\bar{x} = 15.9$

Standard deviation = 1.8

Task 2

Hemicellulose Utilization

Objective:

To investigate the possibility of a relatively pure by-product recovery from the hemicellulose fraction of solvent pretreated wood chips and to determine the utility of the residual hemicellulose for fuel and solvent production.

Manpower:

Don Ristroph (80%)

Arthur Humphrey (5%)

Title:

Graduate Research Fellow
Chemical and Biochemical Engineering

Dean, Engineering and
Applied Science

Summary of Results:

The overall objective of this task is to determine if it is feasible to utilize an enzyme system produced by Thermoactinomyces LL to selectively hydrolyze xylan to xylose and thus selectively recover this sugar. In order to achieve this objective it was originally believed that four steps or minor objectives needed to be met. The research was essentially to duplicate similar work performed in this lab with cellulose and cellulase, in which a pure substrate, Avicel, was used to produce enzyme, and begin the saccharification and kinetic studies. The organism used in the cellulase work has been reclassified as a Thermomonospora, therefore it's possible that the Thermoactinomyces LL is also misclassified. The four steps involved in this task were to include:

- 1) obtaining a pure, well defined hemicellulose such as linear xylan
- 2) grow the organism on this material to produce an enzyme with xylanase activity

- 3) harvest this enzyme and determine its optimum conditions for hydrolysis of pure linear xylan
- 4) to study the saccharification of various biomass substrates utilizing this enzyme, to determine if xylose could be recovered from the biomass.

The use of a pure linear xylan was desired so that contaminating sugar polymers would not influence the growth of the organism, the production of xylanases, or the hydrolysis studies involving enzyme and substrate. Unfortunately, such a material has not been obtained. A total of six commercial sources of xylan have been tested for purity. Upon acid hydrolysis followed by HPLC analysis it was concluded that all of the materials contained significant quantities of glucose and several contained additional sugars. The acid hydrolysis was the same method used to hydrolyze wood for sugar studies at the Forest Products Laboratory in Madison. (2.1) The HPLC work was performed using a Waters HPLC complete with one of their carbohydrate columns and using an 85/15 ratio of acetonitrile and water at pH 4.5 as the carrier. Attempts to purify each of these xylan materials by use of enzymes to degrade non-xylan polymers, or by solubilizing the entire material in an autoclave followed by precipitation of xylan by alcohol addition, as studied at Berkeley, failed. (2.2) Ultimately, it became apparent that additional time and effort should not be spent attempting to purify these materials.

In March it was decided to work with an impure xylan from Sigma Chemical Company for the remainder of the research. Upon acid hydrolysis and HPLC analysis this material, Sigma xylan lot #48C-00541, yields approximately

10% glucose, 60% xylose, and some furfural. The remaining material cannot be accounted for. It is known that a significant amount is lignin, and the furfural peak which merges with the solvent peak is also significant. Because of the poor results of acid hydrolysis and HPLC analysis the material is considered as having a maximum of 20% glucose.

The use of impure xylan complicates this research tremendously. It is now necessary to determine if the organism is hydrolyzing and growing on the contaminate or the xylan. The organism readily grows on this impure xylan and supernatant from shake flasks repeatedly yields 1.6-1.8 g/l of reducing sugar from 5.3 g/l xylan in 15 minutes at $T = 55^{\circ}\text{C}$ and $\text{pH} = 7.5$. The amount of reducing sugar is determined by a DNS assay with xylose being used for the standard curve^(2.3). These results were obtained using 1 part of sonified whole fermentation broth containing enzyme in 20 parts total of xylan, buffer and enzyme. Any higher concentration of enzyme results in exponential production of reducing sugar which will reach a plateau before 15 minutes. Using a statzyme assay it was determined that 0.2 g of the 1.8 g of total reducing sugar was glucose.^(2.4) Apparently, less than 0.1 g/l was cellobiose as shown by β -glucosidase treatment of the enzyme hydrolyzed material followed by a statzyme assay. It is believed that the remaining 1.5 g/l is xylose. In the control case no reducing sugar is produced when boiled, killed, enzyme is used to perform the same experiments.

The HPLC was used to attempt to prove that the remaining reducing material was indeed xylose. This work has been frustrated by numerous problems with the machine and the columns. In latter June the system was finally functioning properly. Samples of enzyme hydrolyzed xylan which had

been ultrafiltered by passing approximately 75% of the hydrolysate volume through a 500 molecular weight Amicon UO5 membrane, and had retained 1.1 to 1.2 g/l of reducing sugar by DNS assay were injected into the HPLC. These samples gave only two peaks, the solvent front and 0.2 g/l glucose. This confirmed the Statzyme assays which recorded 0.2 g/l of glucose, but confused the issue of identifying the remaining reducing material. Because of the many problems with the HPLC and its confusing data it is being temporarily disregarded.

The hypothesis that this organism was producing a xylanase and growing on xylan remained unproven at this point. Since conclusive evidence of xylose production could not be obtained with the HPLC, it was decided to first obtain more circumstantial evidence. Three shake flasks, 1 liter flask containing 200 mls of approximately 10 g/l Sigma xylan as the carbon source, were inoculated with the organism previously grown on the same media. Samples of these shake flasks were taken periodically and assayed for carbohydrates, reducing sugar, glucose, and protein (Figures 2.1, 2.2, 2.3, 2.4). According to the phenol-sulfuric acid test for carbohydrates nearly 7.5 g/l of carbohydrate was present before inoculation. Of the 7 g/l carbohydrate present after inoculation 4 g/l were consumed, 55-60% of the original material. See Figure 2.1. The reducing sugar levels as measured by DNS jumped from near zero before inoculation to greater than 3 g/l only 1 minute after inoculation and within 36 hrs leveled off at 0.8-0.9 g/l. See Figure 2.2. This reducing material had to be generated in the flask after inoculation because the inoculum was 48 hrs old and did not contain enough reducing material to substantially affect the levels in the shake flasks. The glucose concentrations were only a small fraction of the reducing material present. See Figure 2.3. The results of the statzyme assays show that the glucose concentration remained

FIGURE 2.1

FIGURE 2.2

Reducing Sugar
equivalence to xylose
g/l

Reducing Sugar Levels in DNS Assay

□ flask 1
△ flask 2
○ flask 3

FIGURE 2.3

Glucose Levels via Statzyme Assay

FIGURE 2.4

between 0.025-0.076 g/l, so low as to be difficult and unreliable for testing by the Statzyme method. The protein content of the whole broth, which unfortunately could not be sonified due to the necessity of using small samples, rose to about 0.34 g/l then leveled out at approximately 0.2 g/l. See Figure 2.4. These results strongly indicate that substantially more than 20% of the impure xylan is being utilized. Since the upper limit of glucose contamination is about 20% this would suggest that xylose from xylan is indeed being used, and xylanase activity to produce this xylose is probably present. Unfortunately, the HPLC analysis does not confirm this. In addition to these tests, some preliminary experiments have shown that the enzyme system displays no activity when used to run CMC or filter paper assays, indicating that no cellulases are present. These assays are identical to the CMC and filter paper assays used in this lab for the cellulase studies (2.5).

Xylan usually exists as a linear backbone of 1,4 β linked xylose with branches of either xylose or other pentoses or hexoses periodically appearing. Because of the rapid appearance of reducing sugar, as measured by DNS, in both these shake flask runs and in the saccharification of xylan with enzyme, it would appear that this xylan may have branches of glucose at which junction hydrolysis of the branch unit and breaking of the xylan backbone may occur to release glucose and small units of xylan accounting for the large levels of reducing material. If the xylanase enzyme of this organism is similar to the cellulase enzyme system being used by this lab then the xylanase enzyme system could be strongly end product inhibited so that only very low levels of xylose or xylobiose are generated. Perhaps only enough xylose or xylobiose is produced for maintenance.

Enzyme from this organism produces reducing material on the order of 30g/100g of xylan in a very short period of time. That is to say that the reducing material has the same reducing capacity as 30g of xylose. The xylan is substantially contaminated with lignin, therefore, this ratio cannot be said to be very precise. More than 50% of this xylan is consumed during a fermentation. The xylan is much less than 50% non-xylose material. Yet enzyme hydrolyzed xylan displays no xylose or xylobiose on an HPLC. The circumstantial evidence indicates xylanase activity on a large scale but the direct evidence, the presence of xylan hydrolysate products, has failed to be demonstrated.

References

- 2.1) Marilyn Effland (personal communications)
- 2.2) Harvey Blanch (personal communications)
- 2.3) Miller, Gail L., Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, Anal. Chem. Vol. 31, No. 3, p. 426-428, 1959.
- 2.4) Worthington Statzyme Glucose Assay produced by Worthington Diagnostics, Division of Millipore Corp., Freehold, New Jersey 07728.
- 2.5) Janice Phillips (personal communications)

Task 3

Investigation of the Cellulase System of Thermomonospora sp and the Characteristics of the Cellulose Saccharification Process

Objectives:

To optimize the production of cellulase by Thermomonospora sp., to investigate the nature and functional characteristics of the crude cellulase obtained from Thermomonospora sp., and to characterize the saccharification process. The purpose is to reduce the cost of saccharification and to find a way to produce high glucose syrups from cellulose for use in standard fermentations.

Manpower:

Title:

John Ferchak (80%)

Research Specialist II

Helena Whitty (100%)

Research Assistant

E. Kendall Pye (25%)

Associate Professor of Biochemistry
& Biophysics

Summary Results:

I. Immobilization of β -Glucosidase/Cellobiase Activities

The crude filtrate obtained after sonication of the Thermomonospora sp culture cell-solids contains the entire β -glucosidase/cellobiase activities of the culture. These activities were successfully immobilized by binding to a controlled-pore glass carrier (CPG-10, 50/80 Mesh, 475.5 Å pore diameter; Electro-Nucleonics, Inc., N.J.). Preliminary tests indicate that a major fraction of the available activity was bound to the carrier (> 30%). The β -glucosidase activity (20 λ enzyme suspension, 1.5 ml of 20 mM p-nitrophenyl- β -D-glucoside, held at 55°C for 30 min.) of this preparation was considerably greater than the cellobiase activity (100 λ enzyme

suspension, 1.5 ml 20 mM cellobiose, held at 55°C for 30 min., and analyzed for glucose by the Glucostat method). 13 μ moles PNP/ml enzyme-hr were released, compared to only 2.2 μ moles glucose/ml enzyme-hr, which is consistent with prior results.

One of the primary goals of immobilization is enhanced thermal stability of the relatively heat labile β -glucosidase/cellobiase activities (half-life at 55°C of activity in whole cells is ~6 hours). Our initial tests indicate that some increase in thermal activity was achieved with the above method of immobilization. However, it should be emphasized that optimization of the process must be completed before any final conclusions can be drawn, and determinations of the new pH optima must be made.

II. Ethanol Inhibition

The effect of ethanol on the filtrate (cellulase enzymes) was studied using HPLC. The cellobiose levels produced by the filtrate during an 8 hour saccharification of 5% Solka-floc, in the presence of varying concentrations of ethanol, were measured. In Fig. 3.1, it is seen that the presence of 8% ethanol had a slight effect on the filtrate, reducing cellulolytic activity to 80% of full value. No glucose accumulated since cell solids were absent.

The effect of ethanol on whole broth, including the cell-solids (cellobiase activity) concentrated four-fold by decanting the supernatant after settling, was similarly studied in an 8 hour saccharification of 5% Solka-floc. Fig. 3.1 shows that ethanol has a significant effect on glucose production, which was reduced to 30% of the control in the presence of 8% ethanol. In the control (0% ethanol), the concentrated cellobiase activity kept the cellobiose concentration at 0.6 mg/ml; but as the ethanol

Figure 3.1. Effect of ethanol on the saccharification of Solka-Floc cellulose, using either filtrate or whole broth from Thermomonospora culture. Soluble sugar assayed by HPLC using a reverse phase "carbohydrate" column from Waters, Inc., Mass.

concentration was increased, the strongly inhibited cellobiase activity allowed the cellobiose concentration to rise. The total soluble sugar level, calculated as the sum of the cellobiose and glucose concentrations, showed only a small decline, indicating that the rate limiting step in the saccharification of cellulose was the production of cellobiose.

III. Effect of Metal Ions and Sulfur Compounds on Avicelase Activity and the Thermal Stability of the Avicelase

Of the compounds tested, Co^{++} was found to have both an appreciable activating and stabilizing effect in some buffer systems. Ca^{++} had a stabilizing effect. Hg^{++} and Pb^{++} were the most inhibitory metal ions tested. The procedure and results are summarized in Table 3.1. The results for Co^{++} led us to study its effects in greater detail.

Using the same procedure as above, but with varying time periods, it was found that Co^{++} in the concentration range of 0.1 - 0.5 mM enhanced the stability and activity of the Avicelase activity after incubation with filtrate at 65°C (Fig. 3.2).

To determine whether Co^{++} had a similar effect when present during a saccharification, 0.3 mM Co^{++} was added to a 60°C saccharification of phosphoric-acid swollen cellulose using filtrate from a 10% fermentor culture; an identical experiment was performed with a 65°C saccharification of Avicel using filtrate from a shake-flask culture. (A different protein profile is found in the culture broth depending on the method of culture.) Figure 3.3 shows that only a slight enhancement of activity was found under these conditions.

It is possible that the presence of cellulose stabilizes the cellulase system sufficiently such that the effect of Co^{++} is negligible. Further studies at higher temperatures are planned.

Table 3.1. Effect of metal ions on *Thermomonospora* sp cellulase activity and stability.

PROCEDURE - Incubate enzyme filtrate containing additive at 65°C for 4 hours. Measure Avicelase activity (1 ml enzyme + 2.5 ml of 1.4% Avicel PH 105 suspension at 60°C for 45 min. → DNS → reducing sugar).

RELATIVE ACTIVITY:
(BUFFER AND ADDITIVE CONC.)

ADDITIVE	TIME (HRS.)	PHOSPHATE; 1 mM	MALEATE; 2 mM	MOPS; 2 mM
NONE	0	100	100	100
	4	100	100	100
CaCl ₂ ·2H ₂ O	0	98	100	100
	4	115	95	101
Co(NO ₃) ₂ ·6H ₂ O	0	122	98	127
	4	134+	83	73
Pb(Ac) ₂ ·3H ₂ O	0	58+	83+	5+
	4	52+	85+	1+
FeSO ₄ ·7H ₂ O	0	101	90	97+
	4	73+	54+	82+
FeCl ₃ ·6H ₂ O	0	74+	88	84+
	4	74+	62+	43+
CuSO ₄ ·5H ₂ O	0	100		
	4	47		
MgCl ₂ ·6H ₂ O	0	83	88	
	4	82	73	
ZnSO ₄ ·7H ₂ O	0	81	49	
	4	94+	36	
CrCl ₃ ·6H ₂ O	0	91+	87	
	4	73+	62	
LiCl	0	95		
	4	107		
HgCl ₂	0	40		
	4	0		
MnCl ₂ ·4H ₂ O	0	95+	90	
	4	70+	42	

† Indicates formation of precipitate.

Table 3.1 (cont)

ADDITIVE	TIME (HRS.)	RELATIVE ACTIVITY: (BUFFER AND ADDITIVE CONC.)		
		PHOSPHATE; 1 mM	MALEATE; 2 mM	MOPS; 2 mM
L-Cysteine	0	98		
	4	91		
NiCl ₂	0		89	
	4		63	
Dithiothreitol	0		93	
	4		55

Figure 3.2. Thermal stability of *Thermomonospora* sp filtrate at 65°C.

Effect of added Co⁺⁺. Assay conditions (Avicelase activity).

Figure 3.3. Saccharification of cellulosic substances in the presence and absence of Co^{++} ion. 65°C, pH 6.5. *Thermomonospora sp* culture filtrate.

A. 1.4% dry wt. acid swollen cellulose

B. 2% Avicel

IV. Effect of Sugars/Analogues on β -Glucosidase Activity

A series of sugars and analogues were tested for effect on Thermomonospora sp (cell-solids) PNPG'ase activity. D-Gluconic Acid, Lactone was shown to be the most inhibitory substance tested. The results are given in Figure 3.4.

The purpose of this study was to search for effective inhibitors of β -glucosidase which are sugars or sugar analogues and which might be employed in techniques to select mutants containing high β -glucosidase activity. High β -glucosidase producers would be extremely useful for production of concentrated glucose syrups.

V. Cellobiose Inhibition of Cellulase Activity

A gravimetric study of the effect of cellobiose on the cellulase activity of Thermomonospora filtrate, acting on 2% (dry wt) acid-swollen cellulose confirmed the previously reported results of strong inhibition by cellobiose of saccharification of pretreated cellulose (Figure 3.5). These results demonstrate the advantages in saccharification kinetics of using either combined saccharification and fermentation, or in converting the cellulose primarily to glucose.

VI. Inhibition of Cellulase Activity by Glucose

To study the effect of initial glucose concentration on the extracellular (cellulase) enzymes, a saccharification series was prepared using 5% Solka-floc cellulose and filtrate alone (no cellobiose activity present), with the initial glucose concentration varying up to 20%. Surprisingly, it was found that glucose had a considerable effect on the production of cellobiose; an initial glucose concentration of 5% reduced the activity to about 50%, and 20% glucose reduced it to about 30% of the

Figure 3.4 Effect of sugars and sugar analogs on PNPGase activity.

* Neutralized to pH 6.5 in phosphate buffer prior to assay.

Figure 3.5 Cellobiose inhibition of Thermomonospora sp cellulase (culture filtrate) acting on a 2% suspension of acid-swollen cellulose.

control without glucose (Figure 3.6).

VII. Inhibition of PNPGase Activity by Glucose

The effect of glucose on the β -glucosidase activity was studied using cell solids that were washed and resuspended in 4 to 9 volumes of pH 6.5 phosphate buffer. The β -glucosidase activity was measured using the p-nitrophenyl- β -D-glucosidase assay, in the presence of glucose concentrations up to 20%. Each sample in the series was compared to the control containing no glucose. The series was repeated several times, accumulating 7 to 11 determinations for each concentration. The results are shown in Figure 3.7. It appears that the presence of glucose has an activating effect at concentrations up to 2%, with inhibition occurring at higher concentrations. However, the error bars, showing the standard deviation, do not allow a firm conclusion to be drawn on this point. In any case, inhibition is not seen until the glucose concentration is 3%. Afterwards, the inhibitory effect is clear, and activity is reduced to about 35% of the original activity when a 20% glucose concentration is present.

VIII. Inhibition of Cellobiase Activity by Glucose

The effect of varying initial concentrations of glucose on the ability of Thermomomospora sp cell-solids (cellobiase activity) to catalyze the hydrolysis of cellobiose was determined.

1% cellobiose solutions containing 0 - 15% glucose were incubated with washed cell-solids in buffer at 55°C for ~4 hours. The extent of hydrolysis was ~50%; i.e., at 0% initial glucose, the final cellobiose concentration was ~0.5% and the final glucose concentration was ~0.5%. HPLC was used to measure the change in cellobiose concentrations.

The results shown in Figure 3.8 indicate that very little product

Figure 3.6 Saccharification of 5% Solka-Floc by Thermomonospora sp filtrate in the presence of varying initial concentrations of glucose. Incubation: 55°C, 8 hrs. HPLC analysis of soluble sugar (cellobiose).

Figure 3.7 Inhibition of PNPase activity from Thermomonospora sp by glucose.

Figure 3.8 Hydrolysis of cellobiose by Thermomonospora sp cell-solids (cellobiase activity) in the presence of varying initial concentrations of glucose. Cellobiase activity was determined by HPLC quantitative analysis of the change in cellobiose concentration.

inhibition occurred, which is different from the results obtained with p-nitrophenyl - β -D-glucosidase. Figure 3.9 shows the results of HPLC analysis using two concentrations of initial glucose (0% and 15%). It shows the decrease in cellobiose concentration that occurred in each case. A small peak (shown at RT300 in B, t=4hrs) occurs when the initial glucose is present and increases with increasing initial glucose concentration. It may represent a transglycosylation or a synthesis product, such as gentiobiose.

IX. Hydrolysis of Cellobiose Using Culture Cell-Solids

The weak inhibitory effect of glucose on the ability of culture cell-solids of Thermomonospora sp containing the cellobiase activity to catalyze the hydrolysis of cellobiose suggested the following experiment.

Cellobiose and centrifuged cell-solids were added to a buffer solution to a concentration of ~10% each. The suspension was held at 50°C for ~24 hrs, centrifuged, and fresh cellobiose and cell-solids added to the decanted solution. Repeating this process five times resulted in a sugar solution of approximately 20% glucose (by Glucostat and HPLC assay), 5% residual cellobiose (HPLC assay), and 5% unidentified sugar (by difference, using the phenol-H₂SO₄ assay for total sugar). Results are shown in Figures 3.10 and 3.11.

X. Glucose Syrup Production

An initial experiment was made to determine whether a high concentration glucose syrup could indeed be made from a cellulosic substrate using Thermomonospora cellulase and β -glucosidase. Conditions were optimized in the following way. Phosphoric acid - swollen cellulose ("Walseth cellulose"), prepared from Avicel PH105, was lyophilized to remove all moisture.

A

0% INITIAL GLUCOSE

t= 0 hrs

NAME	CONC(%)	RT(SEC.)	AREA	KF
CELLOBIOS	.983	324	63995	65120

t= 4 hrs

GLUCOSE	.549	194	34386	62600
CELLOBIOS	.429	320	27949	65120

B

15% INITIAL GLUCOSE

t=0 hrs

GLUCOSE	14.71	215	921165	62600
CELLOBIOS	1.1	335	71632	65120

t= 4 hrs

GLUCOSE	14.69	214	919919	62600
CELLOBIOS	.548	334	35667	65120

Figure 3.9. HPLC analysis of cellobiose hydrolysis by Thermomonospora sp cellobiase activity.

Figure 3.10 Hydrolysis of cellobiose 50°C, pH 6.5. ~10% W/V cellobiose and ~10% centrifuged cell-solids from Thermomonospora sp culture (β -glucosidase activity) were added after 24 hr period, after removal of spent cell-solids. The calculated final total sugar concentration was ~31%, as compared to the assayed value of ~35.5%. Total sugar was assayed by the phenol-sulfuric acid method, and glucose by the glucose oxidase assay and HPLC. Cellobiose was estimated by HPLC.

Std. (.01 ml): 2.0% each Glucose & Cellobiose

SUGAR ANAL 79 JAN 19 10:57:11
 CHANNEL 1 RUN 3 FILE 2 METHOD 5
 INDEX 3 SAMPLE 1
 NAME CONC RT AREA KF % conc.
 1 82 127934
 2 145 11344
 GLUCOSE 1.001 174 57938 L 57880 x 2 = 2.0
 CELLOBIOS 1.075 281 64102 59650 x 2 = 2.1
 TOTALS 2.076 261318

#5 (.005 ml): Final sample from cellobiose hydrolysis

SUGAR ANAL 79 JAN 19 12:59:56
 CHANNEL 1 RUN 11 FILE 2 METHOD 5
 INDEX 11 SAMPLE 1
 NAME CONC RT AREA KF % conc.
 1 83 16494 L
 2 127 2950 L
 GLUCOSE 5.231 181 302791 S 57880 x 4 = 20.9
 4 254 39026 L
 CELLOBIOS 1.001 293 59740 L 59650 x 4 = 4.0
 6 351 19626 L
 TOTALS 6.232 440627

Figure 3.11 HPLC analysis of the final sample from cellobiose hydrolysis, and comparison with standard (Waters, Inc. HPLC equipment, Spectra-Physics 4000 Integrator). The final sample shows a greatly reduced water peak (RT 83 sec), a predominating glucose peak (RT 181 sec), and two large peaks (RT 254 sec, RT 351 sec), in addition to what is identified as the cellobiose peak (RT 293 sec). Presumably, transglycosylation products have been formed. The peak at RT 351 may be gentiobiose.

Thermomonospora filtrate containing extracellular cellulase activity, and crude extract from cell solids, containing β -glucosidase activity, were also lyophilized. Using the lyophilized preparations, a suspension was prepared with 10% cellulose, 1.8% cellulase, and 0.9% crude β -glucosidase extract. The mixture was incubated at 50°C for 24 hrs, centrifuged and fresh lyophilized preparations were then added to the supernatant in the same proportions. Figure 3.12 illustrates the results over a 7 day time span. It can be seen that a solution containing 20% soluble sugar was attained, of which ~18% was glucose. These results indicate that a process for the production of glucose syrups, attainable on a laboratory scale, may be developed for commercial application. Such a process could provide low cost fermentable sugar from biomass for use in a standard yeast alcohol plant. Therefore further investigation of the highly desirable goal is warranted.

XI. Saccharification of Cotton

The activity of Thermomonospora sp cellulase against two-roll-milled cotton was appreciable, and similar to the activity reported for Trichoderma (Mandels, et al, Fuels From Biomass, 2nd Ann. Symp.). Two-roll-milled cotton from Natick was passed through a kitchen strainer sieve and then exposed to Thermomonospora sp enzyme filtrate for 11 days at 55°C. Absorbent cotton was used as a control. Gravimetric and chemical analysis was performed and the results are shown in Table 3.2.

XII. Relationship of β -Glucosidase and Cellobiase Activities

Shake flask cultures of Thermomonospora sp grown either on Avicel or cellobiose were prepared for analysis (after growth through five serial transfers) by centrifugation, washing in buffer, and resuspension of culture

GLUCOSE SYRUP PRODUCTION FROM CELLULOSE

Figure 3.12 Enzymatic hydrolysis of swollen cellulose to yield soluble sugars. Lyophilized Thermomonospora sp cellulase and β -glucosidase activities were added at 24 hr. intervals to fresh cellulose. Arrows indicate one addition of supplemental β -glucosidase activity. Total sugar was assayed by phenol-sulfuric acid assay and glucose by glucostat assays.

Table 3.2 Saccharification of absorbent and two-roll-milled cotton by Thermomonospora sp. culture filtrate.

<u>Substrate</u>	<u>% Hydrolyzed</u>
0.5% Abs. cotton	7
1.0% Abs. cotton	5
0.5% Milled cotton	43
1.0% Milled cotton	40

cell-solids. The Avicel-grown and cellobiose-grown culture solids were concentrated 2-fold and 5-fold, respectively, as compared to the original whole broth.

- A. Activities against PNPG (β -glucosidase) and cellobiose were examined. The following results were found:

<u>Assay</u>	<u>Ratio of Activities</u>	
	<u>Growth Substrate</u>	
	<u>Avicel</u>	<u>Cellobiose</u>
PNPGase	8-13 ^(a)	1 (a) 15-30 μ moles PNP/ml enz./hr
Cellobiase	1	4-7 ^(b) (b) 2-3 μ moles Glucose/ml enz./hr

It is apparent that different enzymatic activities are induced, depending on the substrate. The Avicel-grown cell solids contain high PNPGase activity and low cellobiase activity, while the opposite is found for the cellobiose-grown cell solids. These results indicate the possibility of independent β -glucosidase and cellobiase activities.

- B. The respective activities were tested using the filtrate from an actual saccharification of swollen cellulose by Thermomonospora sp extracellular cellulase. This solution was ~ 7 mg/ml soluble sugar as determined by the phenol- H_2SO_4 assay, and ~ 0.14 mg/ml glucose by Glucostat assay. The predominant soluble sugar is called Avicel-derived 'cellobiose'. It shows the same retention time as commercial cellobiose (Sigma Chemical Co.) used as a standard during HPLC analysis (Waters Associates, Inc. carbohydrate column) and is considered to be the predominant sugar found from cellulose by numerous cellulolytic systems. Therefore, one might expect a similar ratio of activity as reported in A.

(above) for the cellobiase assay. However, the following results were found:

<u>Culture Substrate</u>	<u>Cell Solids Activity</u> moles glucose/ml-hr	
	<u>1 hr assay</u>	<u>3 hr assay</u>
Avicel-grown	4.6 $\pm$ 1.5	1.7
Cellobiose-grown	3.8	2.9

Comparable activities are found in the 1 hr assay, while the Avicel-grown cell solids activity declines with time, as shown by the 3 hr assay.

Further studies will attempt to clarify these seemingly contradictory results.

XIII. Protein Identification by HPLC

Recent developments in HPLC column technology may allow the identification and purification of protein mixtures by this new and powerful technique.

We have had samples of culture filtrates from Thermomonospora sp analyzed through the courtesy of a representative from Waters Associates, Inc., using a new HPLC molecular sieving protein-separation column being test-marketed by that company. Figure 3.13, A & B, shows the results of a single (non-optimized) injection of filtrates from shake-flask and 10% fermentor grown cultures. The profiles are clearly different, and show numerous peaks, consistent with our previous observation of protein differences using isoelectric focusing techniques.

Optimization and repeated injection of samples using microprocessor controlled HPLC equipment holds the promise of a rapid and accurate separations

Figure 3.13 HPLC analysis of culture filtrates from Thermomonospora sp containing cellulolytic enzymes, Waters, Associates, Inc. HPLC equipment and protein column; 0.01 ml sample injection. Simultaneous measurement of refractive index and UV₂₈₀ absorbance.

- A. Sample from 10% fermentor culture.
- B. Sample from shake flask culture.

technique for protein purification and characterization.

We are presently acquiring the necessary equipment to proceed with development of this method.

Task 4

Production of Concentrated Glucose Syrups

Objective:

To define a cellulase system for production of concentrated glucose (>20%) syrups for use in existing fermentation processes for the production of liquid fuels and chemical feedstocks.

Manpower:

Janice Phillips (80%)

Arthur Humphrey (2%)

Title:

Research Fellow,
Chemical and Biochemical
Engineering

Dean, School of Engineering
and Applied Science

Summary of Results

The purpose of this task is to demonstrate the feasibility of producing concentrated glucose syrups (>20% sugar) by enzymatic hydrolysis of cellulose utilizing the cellulase system produced by the cellulolytic thermophile Thermomonospora (formerly Thermoactinomyces).

Preliminary experiments indicated that the crude cellulase preparation, isolated as supernatant from Thermomonospora fermentation of cellulose, has little β -glucosidase activity. Consequently, in saccharifications carried out with this crude enzyme, cellobiose accumulates as the major product. This effectively inhibits the rate of further hydrolysis. This inhibition could be removed by a variety of steps, primary among which is supplementation of the enzyme with a source of β -glucosidase to convert the cellobiose to glucose. Ideally, β -glucosidase from Thermomonospora, if judged suitable

for use in the hydrolysis, would be best applied in this circumstance, eliminating the requirement for an additional enzyme production fermentation in the overall process scheme.

β -Glucosidase from Thermomonospora is cell-associated and, consequently, naturally separated from the major endo-/exo-glucanase activity. Therefore, separation and, if necessary, purification of this enzyme may be relatively easy. The crude cellulase broth could be supplemented with β -glucosidase, as purified soluble or immobilized enzyme, or as immobilized cells or crude extract, to increase the rates and extent of hydrolysis.

The work over the past year has been aimed, therefore, at isolating and purifying the β -glucosidase from Thermomonospora, characterizing its substrate specificity and kinetic properties and outlining a reactor scheme for the hydrolysis of cellulose to glucose employing Thermomonospora β -glucosidase.

The cellulase enzymes are produced by fermentation of Thermomonospora on 1% Avicel at 55°C and initial medium pH of 7.2 in the media listed in Table 4.1. Crude endo-/exo-glucanases are harvested as the supernatant after centrifugation of the whole broth; this supernatant is filtered through glass wool/glass fiber filter paper and stored at 4°C, after addition of 0.03% azide, for use in subsequent hydrolyses. The solids after centrifugation, the source of β -glucosidase, are washed four times with 0.05 M pH6.5 $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer containing 0.1 M mercaptoethanol, with intermediate centrifugation to settle solids, before final resuspension to 1/5 the original culture volume in 0.05 M pH6.5 $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer containing 1 mM dithiothreitol. The crude preparation of β -glucosidase is obtained by sonification of these washed solids.

Table 4.1.

Thermomonospora Media

Quantities are per liter of media:

100 ml 0.67M pH 7.8 $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$

20.0 ml Sigma salt solution consisting of

2.5 gms/l EDTA (trisodium salt)

10.0 gms/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.4 gms/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$

1.0 gm/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

1.0 gm/l $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$

1.0 gm/l CaCl_2

1.5 gms NaCl

3.1 gms $(\text{NH}_4)_2\text{SO}_4$

100 mg yeast extract

1 mg biotin

1 mg thiamine HCl

Kinetic data for the action of this crude β -glucosidase against p-nitrophenyl- β -D-glucoside, the aryl analog of cellobiose, and against cellobiose have been obtained. Values of the various kinetic parameters for hydrolysis of these substrates are given in Table 4.2. Reaction rate constants and half-lives as a function of temperature are given in Table 4.3.

Values of K_m indicate that the crude β -glucosidase has a greater affinity for the aryl analog, pNp β G, than for cellobiose. K_m values for pNp β G are comparable to those for β -glucosidase isolated from other natural and microbial sources.

The half-lives of this enzyme at operational temperatures of interest (50, 55 and 60°C) are quite short. These higher temperatures represent the optimum range of thermal stability for the endo-/exo-glucanases. If β -glucosidase were to be utilized in the same reactor with these cellulases, its thermal stability would have to be increased. Otherwise, continuous addition of the enzyme would be required.

K_I values for glucose, the end product inhibitor of β -glucosidase action, have been obtained for both substrates. Analysis of the inhibition data for the cellobiose/glucose system has not been completed so no value is listed.

A scheme for purification of this enzyme, consisting of the following steps, has been outlined:

1. saturation to 50% ammonium sulfate of the crude β -glucosidase fraction;
2. elution of supernatant from step 1 on an Ultrogel molecular sieve column;
3. elution of the pooled β -glucosidase fractions from step 2 on a Sepharose ion exchange chromatography column.

Table 4.2.

Kinetic Parameters for the Hydrolysis of
 pNp β G and Cellobiose with Crude
 β -Glucosidase from Thermomonospora

	pNp β G	cellobiose
K_m (mM)	0.50-0.80	6-12
V_{max} (μ moles/ml enzyme/minute)	2.50-3.00	4-5
K_I (mM)	25-30	
pH range (buffer)	5.9-6.7 (KH_2PO_4 - K_2HPO_4)	5.9-6.6 (KH_2PO_4 - K_2HPO_4)
Temperature optimum ($^{\circ}C$)	60	60
Energy of activation (kcal/mole)		12
Energy of thermal inactivation (kcal/mole)	42	53

Table 4.3.

Reaction Rate Constants and Half-Lives
for Crude β -Glucosidase from Thermomonospora

Temperature ($^{\circ}\text{C}$)	Substrate pNp β G		Cellobiose	
	k	$t_{1/2}$	k	$t_{1/2}$
	(hr^{-1})	(hours)	(hr^{-1})	(hours)
37			0.0035	201
45			0.0408	17
50	0.053	13.1	0.1382	5
55	0.260	2.7	0.3323	2.1
60	0.435	1.6	1.7157	0.4

The fractions from these steps have been applied to an analytical scale isoelectric focusing gel for determination of relative purity. Preliminary evaluation of the patterns resulting on this gel indicate that two pure enzyme fractions are obtained from the above scheme.

Initial design of a laboratory scale reactor for the cellulose hydrolysis has been completed. The preliminary scheme, outlined in Figure 4.1, consists of a two reactor system. The first reactor will be operated for the conversion of cellulose to cellobiose, the second for the conversion of cellobiose to glucose. Fresh cellulase and cellulose will be fed to the first reactor which consists of a well-mixed reaction zone and two unmixed zones for solids settling. Sugar solution from the first reactor will be pumped to a second reactor containing β -glucosidase. The resulting sugar syrup, consisting mainly of glucose, will be recycled to the first reactor or removed as product. The temperature of each enzyme reactor may be independently controlled. Reaction temperatures, residence times and enzyme concentrations can be varied to optimize sugar production. Stage 1, with a volume of approximately 1.5 liters, has been constructed. Initial data on the cellulase reaction is being obtained to determine proper operational conditions. Construction of Stage 2, the β -glucosidase reactor, will commence when a suitable source of β -glucosidase is obtained.

FIGURE 4.1

Laboratory Scale Apparatus for Cellulose Hydrolysis

Relative Volumes Not To Scale

Task 5

Enhanced Thermomonospora Mutants

Objective:

Develop Thermomonospora mutants producing cellulase with high unit activity and low product inhibition characteristics.

Manpower:

Title:

K. F. Detweiler (40%) Research Microbiologist

Dr. J. R. Forro (5%) Senior Microbiologist
General Electric Co.

Summary of Results

The microorganism that is being studied in this project and which was previously designated as belonging to the genus Thermoactinomyces has now been more properly identified as a member of the genus Thermomonospora. This has been confirmed by Drs. Hubert and Mary Lechevalier of the Waksman Institute of Microbiology, Rutgers University. Studies have shown this strain to produce single conidia mainly on aerial mycelia, with the conidia being borne on dichotomously branched sporophores. This morphology is typical of that of the Thermomonospora of the bifid type, and fits the description of Thermomonospora alba (also called Actinobifida alba).

Further separation from the genus Thermoactinomyces was made on the basis of the heat resistance of the spores with those of the thermoactinomycetes forming true endospores which are resistant to high temperatures. The GE strain forms heat-sensitive conidia (aleuriospores), confirming assignment to the genus Thermomonospora. Further, cell wall analyses are also consistent with this assignment - high percentage of meso-diaminopimelic and no madurose, xylose or arabinose.

Because attempts to definitely speciate this organism are not contemplated at this time, we have chosen to designate the parent working strain as Thermomonospora YX.

Research into the mutational enhancement of cellulase activity in Thermomonospora YX has resulted in the production of a variety of mutant strains which were found to initially possess greater unit activity (filter paper activity) than the parent YX strain. Upon further cultivation and study, it was found, however, that the cellulase activity in the majority of these "mutants" quickly decreased to the original parental level. Strain N-35 (derived from YX by treatment with N-methyl-N'-nitro-N-nitrosoguanidine) is, however, apparently stable and has consistently been found to produce approximately twice the cellulase activity of the YX strain.

As shown in Table 5.1, extracellular cellulase activity in strain YX is produced mainly during the first 24 hours of culture and although additional extracellular protein is produced during the next 48 hours, little increase is noted in cellulase activity. The specific activity of the cellulase therefore actually decreases slightly.

In contrast, while strain N-35 has been found to produce lesser quantities of extracellular protein than strain YX, both the activity and the specific activity of its extracellular cellulase complex actually increase with time. It appears therefore that strain N-35 is not only producing more cellulase, but also reduced quantities of non-cellulase proteins (i.e., proteases):

The increase in cellulase activity noted in this strain is thus basically different from that reported in hyperproducing mutants of Trichoderma in which increased activity has been correlated with a generalized increase in total extracellular protein.

Table 5.1

Comparison of Cellulase Activity in Thermomonospora YX and
Mutant Strain N-35

Culture	Culture Time	Filter Paper Activity (mg Reducing sugar/ml)	Extracellular Protein (mg/ml)	Specific Activity (mg RS/ mg Protein)
YX	24 hrs	1.05	0.43	2.4
	48 hrs	1.27	0.59	2.1
	72 hrs	1.31	0.69	1.9
N-35	24 hrs	0.75	0.25	3.0
	48 hrs	1.78	0.38	4.7
	72 hrs	2.44	0.44	5.6

Task 6

Combined Saccharification and Vacuum Fermentation with an Ethanol Tolerant Mutant of Clostridium thermocellum

Objective:

The objectives of this task are four-fold: 1) Obtain highly ethanol-tolerant organisms and high yields of ethanol; 2) Facilitate saccharification of hemicellulose and cellulose by optimization of combined action of Thermomonospora cellulase and growth of Clostridium thermocellum; 3) Ferment hemicellulose with production of ethanol by xylan utilizing organism; 4) Study the effectiveness of combined saccharification-vacuum fermentation as a method of increasing the ethanol yield and to determine the kinetics of a Clostridium thermocellum fermentation.

Manpower:

James Alexander (50%)

Geoff Slaff (50%)

Arthur Humphrey (2%)

Title:

Prof. of Biochemistry
Hahnemann Medical College

Research Fellow
Chemical and Biochemical Engineering
University of Pennsylvania

Dean, Engineering and Applied Science
University of Pennsylvania

Summary of Results:

Chemically-defined medium for Cl. thermocellum. A chemically defined medium which supports growth of strains 651, 27405, and TET is shown in Table 6.1 (Medium A). Medium A does not support growth of S4 (obtained from Dr. D. Wang) because this strain requires tryptophane. Medium B (Table 6.1) supports growth of S4 as well as strains 651, 27405, and TET.

S4 has a generation time of about 170 min. in medium B, whereas, 651 has a generation time of about 90 min. in medium A. The relative amount of growth

obtained was 26 g cells per mole of cellobiose for S4 in medium B and 50 g cells per mole of cellobiose for 651 in medium A. The pH optimum is 7.0-7.5 for both S4 and 651.

Isolation of ethanol resistant mutants. Spontaneous mutants of 651 have been isolated which will grow in the presence of 3 to 4% (W/V) ethanol. Isolation of additional mutants is in progress.

HG-2 (a xylan-utilizing organism). It appears that C1. thermocellum is unable to utilize xylan because of its lack of ability to ferment D-xylose. Therefore, to ferment hemicellulose as well as cellulose from biomass it will be necessary to use a xylan-utilizing organism as well as C1. thermocellum. A xylan-utilizing organism, HG-2, has been obtained from Dr. D. Wang for this purpose. HG-2 utilizes xylan, D-xylose, and cellobiose but is non-cellulolytic. The optimum pH for growth is about 6.0. HG-2 produced from 1.8 to 3.0 moles of ethanol per mole of cellobiose and it is relatively resistant to growth inhibition by ethanol. HG-2 grows slowly on defined media, however, the growth rate can be enhanced by yeast extract.

Isolation of a xylan-utilizing organism. Since very little is known about anaerobic thermophilic xylan-utilizing organisms several organisms were isolated from a soil sample. One produced butyric-acid, a characteristic of Clostridium thermosaccharolyticum, therefore, it will not be studied further. Another isolate, namely XD, resembles the organism described by Lee and Blackburn (1975) in that it utilizes D-xylose, is stimulated by glutamine, and has an optimum pH of about 6.0. Both organisms grow very rapidly. However, XD differs from the organism of Lee and Blackburn in that XD is non-cellulolytic. Two other isolates remain to be characterized. Because of the rapid growth rate and simple growth requirements, XD may prove

to be superior to HG-2 (and derivatives). A comparison of ethanol and lactate formation by XD and HG-2 is shown in Table 6.2.

Demonstration of combined saccharification - fermentation. Demonstration of the combined saccharification - fermentation has been complicated by the presence of a heat labile substance produced by Thermomonospora which inhibits growth of Cl.thermocellum and other thermophilic organisms. As shown in Table 6.3, the amount of inhibitor produced by strain LT was much less than the amount of inhibitor produced by strain YX. The inhibitor was removed by absorption on activated charcoal (Table 3). Although a slight amount of cellulase was absorbed, it has been found that the amount of charcoal can be reduced by at least 70% which may result in more selective absorption of the inhibitor. Another activated charcoal, Norit A, effectively removed the inhibitor and no absorption of enzyme was found. With the successful elimination of the growth inhibitor, saccharification-fermentation experiments are currently being carried out. In Figure 6.1 saccharification-fermentation is shown with Thermomonospora cellulase and the non-cellulolytic strains, HG-2 and XD. Cellulase degradation and ethanol production occurred only in the presence of both enzyme and bacteria. With HG-2 and enzyme, 1.5 moles of ethanol were produced for each mole of glucose solubilized, which represents a yield of 75% of theory. These data clearly show a synergism between the enzyme and the bacteria.

Vacuum Fermentation System. Figure 6.2 shows a schematic of the vacuum fermentation system that has been built in the laboratories. The fermentor is a specially designed vessel and built for Penn by Bellco (See Fig. 6.2). It has a working volume of 500 mls, and a surrounding jacket for the circulation of water for temperature control. Using a constant feed rate a constant

level is maintained by a hydraulic level control. The overflow is pumped to an expansion chamber which is kept at 60°C and 173 mm Hg, where the vapor becomes enriched in alcohol. The vapor passes through a -4°C condensor and there is solidified in an acetone-dry ice bath.

Testing the system by using a known solution of ethanol and acetic acid (0.1-1.0 gm/l), has shown a nine-fold increase of ethanol concentrations in the vapor product over that in the liquid product.

Table 6.1 Chemically Defined Media for C. thermocellum

	<u>Medium A</u>	<u>Medium B</u>
	<u>g</u>	<u>g</u>
$(\text{NH}_4)_2\text{SO}_4$	0.3	0.3
K_2HPO_4	0.05	0.05
KH_2PO_4	0.05	0.05
NaCl	0.12	0.12
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.01	0.01
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.002	0.002
Na_2CO_3 (gas phase CO_2)	0.3	0.3
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.001	0.0005
Zn acetate $\cdot 2\text{H}_2\text{O}$	0.0001	0.00005
$\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$	0.0001	
Co acetate	0.0002	
Cysteine	0.05	0.05
Cellobiose	0.5	0.68
Resazurin	0.0001	0.0001
EDTA		0.0025
	<u>ug/ml</u>	<u>ug/ml</u>
Niacin	1	1
Riboflavin	1	1
Pyridoxine	1	1
Thiamin	1	
Pantothenate	1	
Biotin	0.01	0.01
B_{12}	0.01	0.01
Folate	0.01	
p-Aminobenzoate		0.1
L-Tryptophane		50

Table 6.2 Production of Ethanol and Lactate by HG-2 and XD

	<u>Ethanol^a</u> (mM)	<u>%Theoretical^b</u>	<u>Lactate^a</u> (mM)	<u>%Theoretical^b</u>
HG-2	47	58	3.6	4.5
XD	52	65	0.92	1.2

^aEthanol was measured with alcohol dehydrogenase. Lactate was measured with L-lactate dehydrogenase. Since both organisms appear to produce D-lactate as well as L-lactate, the amount of L-lactate measured was multiplied by 2.

^bTheoretical is assumed to be 4 moles of either ethanol or lactate per mole of cellobiose.

Table 6.3 Growth Inhibitor in Thermomonospora Cellulase Preparations

<u>Thermomonospora cellulase (ml)</u>	<u>Strain</u>	<u>Growth^a O.D. 650</u>
None		1.70
0.1	YX	0.01
0.2	YX	0.004
0.5	YX	0.01
0.5 (heat-inactivated)	YX	1.08
0.2 (absorbed) ^b	YX	1.80
0.5 (absorbed) ^b	YX	1.50
0.2	LT	0.59

^aGrowth of Cl. thermocellum strain S4 in 5 ml Medium B in 24 hr.

^bCellulase absorbed by addition at 1g charcoal (activated Amoco PX-23) per 100 ml culture supernatant.

FIGURE 6.1

Saccharification-fermentation with Thermomonospora cellulase and strains HG-2 and XD. The amount of enzyme (culture supernatant) added was 0.1 ml per ml of culture medium. The curve labeled "Enz" contained enzyme and no bacteria.

FIGURE 6.2

Vacuum System

Task 7

Combined Saccharification and Extractive Fermentation

Objective:

To develop a combined saccharification and extractive fermentation within a two or three stage continuous culture system and to obtain kinetic data for the acetone/butanol production.

Manpower:

Roy Hernandez-Mena (100%)

Richard I. Mateles (5%)

Title:

Research Technician

Visiting Professor, Chemical and Biochemical Engineering

Summary of Results

In order to achieve our above objective, we have found it necessary to direct our research toward (1) developing and optimizing a growth medium, (2) optimizing the fermentation to increase butanol production, (3) testing the effect of the extractant (dibutylphthalate) on growth of the micro-organism (Clostridium acetobutylicum), (4) doing preliminary two-stage continuous culture studies to see its effect on butanol production, and (5) developing a system by which continuous extraction can be done. Below are some of the results of these studies as well as a system we are testing for use with our continuous solvent extraction fermentation.

Our overall process requires that the saccharification products of the enzyme hydrolysis of cellulose be used as the carbohydrate source for the fermentation to produce butanol. Since this fermentation had previously been done on cereal grains and molasses, we felt it necessary to find a medium that would allow the organism to grow on glucose and minimal salts.

Because we had been using a medium (Table 7.1) of this type very successfully on another strain of Clostridium, we chose it as a starting point. We found that this medium would not allow growth of the microorganism unless some type of amino-nitrogen source were present. We therefore modified it using soy peptone. Additional studies to determine growth limiting components of the medium were done and it was found that all materials in the medium were in sufficient quantities except for the soy peptone. By adding 1% soy peptone to this medium we were able to increase the cell mass concentration from 1 gm/liter to 2-2.5 gm/liter.

Although good growth was achieved in the medium, solvent yields were very poor. We therefore moved to manipulating conditions in the fermentation. By using techniques of continuous culture we manipulated the pH of the medium and were able to increase solvent yields up to 30% (Table 7.2). This work, although not complete, has given us an indication that pH control will be necessary to produce high solvent yields.

Preliminary studies have also been done to check effects of two-stage continuous culture on production of butanol. This data indicates (Table 7.3) that a 40% increase in solvent concentrations up to now are achievable. These results coupled with the results of effects of extractant on growth of the microorganism (Table 7.4) seem to indicate that the coupling of these two systems may indeed increase solvent yields. The goal of the work, currently being done and that will be continued for the rest of this year, will involve showing that continuous solvent extraction will increase solvent yields. We are currently developing a system (Figure 7.1) that will hopefully allow us to prove this. At this point we are making modifications in the system which will allow for release of gases and longer settling times in the phase separator.

We are also testing different mixing chambers as well as testing different types of phase separators to avoid the possibility of extractant entering the main fermentation vessel.

Table 7.1

Medium Components

gm/liter		mg/ml	
3.0	$(\text{NH}_4)_2\text{SO}_4$	2.0	pyridoxine
1.2	NaCl	1.0	panthetheine
0.75	KH_2PO_4	1.0	niacin
0.75	K_2HPO_4	0.5	thiamine
0.1	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.01	folate
0.02	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.01	Biotin
0.001	Resazurin		
0.5	cysteine		
0.01	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$		
0.002	$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$		
0.001	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$		
0.001	$\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$		
50.0	glucose		
10.0	soy peptone		

Table 7.2

Effect of pH on Solvent Production

<u>pH</u>	<u>acetone</u>	<u>ethanol</u>	<u>butanol</u>	<u>acetic</u>	<u>butyric</u>
4.2	1.1	0.6	3.2	1.4	2.1
4.4	1.5	0.5	4.7	1.0	2.1
5.2	2.1	0.5	7.4	1.7	3.6

Table 7.3

Continuous Culture Studies with First and Simulated Second Stage

	<u>pH</u>	<u>Dry cell mass gms/liter</u>	<u>Acetone</u>	<u>Ethanol</u>	<u>gms/liter</u> <u>Butanol</u>	<u>Acetic</u> <u>Acid</u>	<u>Butyric</u> <u>Acid</u>
first stage:	4.0	1.24	1.1	0.2	3.4	1.0	2.3
second stage:*	4.0	2.23	1.6	0.3	5.8	1.3	2.3

*Second stage 15a sample taken from effluent of first stage and allowed to incubate for 24 hrs. at 37°C.

Table 7.4

Effect of Dibutylphthalate on Growth of Clostridium Acetobutylicum

Dry Cell Mass Grams/Liter

Control: Medium-No Dibutylphthalate

0.63

Medium + Dibutylphthalate

0.94

FIGURE 7.1

General Scheme of Extraction System (batch operation)

Task 8

Increasing the Efficiency of Ethanol Fermentation

Objective:

To study the utility of extractive fermentation for increasing the production of ethanol by yeast anaerobically grown on an analog of enzymatically hydrolyzed cellulose.

Manpower:

Jeanne Ribaud (65%)

Richard Mateles (10%)

Title:

Research Fellow
Chemical and Biochemical
Engineering

Visiting Professor
Chemical and Biochemical
Engineering

Summary of Results

To achieve a feasible extractive fermentation it is necessary to first find an extractant that is non-toxic to the organism and possesses a relatively good distribution coefficient for dilute solutions of ethanol. Distribution coefficient measurements were made by mixing the dilute solution of ethanol in fermentation media (4.5% by volume) and the proposed organic extractant, waiting for equilibrium to be achieved and measuring the amount of ethanol in each of the two phases. Radioactive ethanol was used and the concentration in each phase was measured using scintillation spectrometry. Results achieved thus far are given in Table 8.1; the aqueous solution compositions are in Table 8.2.

Those chemicals possessing the greater distribution coefficients were then further tested for their toxicity to the yeast. The polypropylene glycol,

P-1200, was also tested because it was believed to be non-toxic and the only one previously known to meet this criterion. A summary of the toxicity studies results along with a brief review of the procedure for each are given in Tables 8.3 - 8.6. It is evident that the P-1200 is the only extractant of practical value from this viewpoint.

Two tests have been made to examine the effect of the P-1200 on ethanol production. Only final harvest time samples were analyzed and these results are presented in Tables 8.7 and 8.8.

Three fermentations have been run in a fed-batch mode with enough sugar added for ethanol production but keeping the sugar concentration at a level that was non-inhibitory to the yeast. Fifty to 100 g. glucose/l of broth in the fermenter were added when the glucose concentration fell below 10 g/l. These fermentations were inoculated with relatively large concentrations of fresh yeast cream to achieve ethanol production, rather than growth, from the glucose. The results of these experiments, along with others yet to be run, will be used as a control with which to compare the results obtained using extractive fermentation.

Table 8.1

Results of distribution coefficient measurements

<u>family</u>	<u>chemical</u>	<u>aqueous solution</u>	<u>k.</u>
alcohol	1 pentanol	A	1.13
		B	3.12
		C	1.14
	2,4 dimethyl-3-pentanol	A	0.84
		B	2.26
		C	0.83
	hexanol	A	0.97
	2-methyl-2-hexanol	A	0.96
	5-methyl-2-hexanol	A	0.82
	1-heptanol	A	0.87
	dl-3-heptanol	A	0.89
	4-heptanol	A	0.80
	octanol	A	0.75
	nonanol	A	0.69
	decanol	A	0.60
ketone	4-methyl-2-pentanone	A	0.54
	2-hexanone	A	0.58
	2-heptanone	A	0.54
	5-methyl-2-hexanone	A	0.45
	4-heptanone	A	0.41
	3-octanone	A	0.34
	methyl heptyl ketone	A	0.34
	diisobutyl ketone	A	0.29
	5-nonanone	A	0.30
alkanes	benzene	A	0.20
	iso octane	A	0.16
	tri methyl pentane	A	0.15
	hexane	A	0.13
esters	dibutyl phthalate	A	0.28
	diisobutyl phthalate	A	0.29
	dioctyl phthalate	A	0.18
ethers	butyl ether	A	0.23
glycols	polypropylene glycol P-1200	A	0.50
		B	0.98
		C	0.60
	polypropylene glycol P-2000	A	0.45
	polypropylene glycol P-4000	A	0.37

Table 8.1 (continued)

<u>family</u>	<u>chemical</u>	<u>aqueous solution</u>	<u>k.</u>
oils	olive oil	A	0.16
	soy bean oil	A	0.17
	sesame seed oil	A	0.18
	peanut oil	A	0.22
	corn oil	A	0.18
	cod liver oil	A	0.17
	vegetable oil	A	0.17

Table 8.2

Aqueous solutions used in distribution coefficient studies

Aqueous solution	A	B	C*
ml/l ethanol	45.0	45.0	79.4
gm/l KH_2PO_4	10.0	10.0	--
gm/l yeast extract	5.0	5.0	--
gm/l NaCl	0.0	200.0	--
gm/l glucose	50.0	100.0	30.4

* made using final fermentation broth supernate

Table 8.3

Toxicity Study 1

Test 1 - carried out in test tubes containing 5.0 ml of media and 2.5 ml of extractant inoculated with 1 ml of 3.63 gm cells/1 inoculum to give an initial concentration of 0.07 gm/l. Harvested at 92 hours.

<u>extractant</u>	conc cells, gm/l		
	<u>initial</u>	<u>final</u>	<u>growth</u>
hexanol	0.07	0.05	0
1-pentanol		0.08	0.01
3-heptanol		0.09	0.02
P-1200		1.59	1.52
2,4 dimethyl-3-pentanol		0.05	0
1-heptanol		0.08	0.01
5-methyl-2-hexanol		0.08	0.01
octanol		0.06	0
2-octanol		0.09	0.02
control		5.72	5.65

Table 8.4

Toxicity Study 2

Test 2 - carried out in test tubes containing 5.0 ml of media mixed with 3.0 ml extractant. Tubes then centrifuged and the organic phase plus 1/5 of the aqueous phase removed. Inoculated with 1.0 ml of 3.45 gm cells/l inoculum to give an initial concentration of 0.07 gm/l. Harvested at 63 hours.

<u>extractant</u>	<u>conc cells, gm/l</u>		<u>growth</u>
	<u>initial</u>	<u>final</u>	
hexanol	0.07	~0	0
1-pentanol		~0	0
3-heptanol		~0	0
P-1200		1.24	1.17
2,4 dimethyl-3-pentanol		~0	0
1-heptane		~0	0
5-methyl-2-hexanol		~0	0
octanol		~0	0
2-octanol		~0	0
control		1.40	1.33

Table 8.5

Toxicity Study 3

Test 3 - carried out in 250 ml shake flasks containing 50 ml of media and 0.5 ml extractant. Half of the samples were put on the shaker and half were put on the shelf in the shaker and left stationary. Inoculated with 1 ml of 8.15 gm cells/l inoculum to give an initial concentration of 0.16 gm/l. Harvested at 52 hours.

<u>extractant</u>	conc cells, gm/l		<u>growth</u>
	<u>initial</u>	<u>final</u>	
P-1200 agitated	0.16	2.05	1.89
P-1200 stationary		2.71	2.55
control agitated		2.45	2.29
control stationary		1.84	1.68

Table 8.6
Toxicity Study 4

Test 4 - carried out in 250 ml shake flasks containing 50.0 ml media and 0.5 ml extractant. Half were agitated and half left stationary. Inoculated with 1 ml of 14.207 gm cells/l inoculum to give an initial concentration of 0.284 gm/l. Harvested at 69 hours.

<u>extractant</u>	<u>conc cells, gm/l</u>		<u>growth</u>
	<u>initial</u>	<u>final</u>	
hexanol agitated	0.28	0.33	0.05
stationary		0.42	0.14
1-pentanol agitated		0.26	0
stationary		0.28	0
3-heptanol agitated		0.37	0.09
stationary		0.36	0.08
P-1200 agitated		1.97	1.69
stationary		8.09	7.81
2,4 dimethyl-3-pentanol agitated		0.28	0
stationary		0.26	0
1-heptanol agitated		0.38	0.10
stationary		0.40	0.12
5-methyl-2-hexanol agitated		0.29	0.01
stationary		0.33	0.05
octanol agitated		0.38	0.10
stationary		0.41	0.13
2-octanol agitated		0.30	0.02
stationary		0.35	0.07
control agitated		1.88	1.60
stationary		4.54	4.26

Table 8.7

Ethanol Productivity Test 1

Test 1 - carried out in 250 ml shake flasks containing 50 ml of media with 25 g/l glucose. Those with extractant contained 0.75 ml P-1200. Glucose added at inoculation to bring the concentration to 150 g/l and at 6 hours 5 g glucose were added to each flask. Harvested 138 hours.

<u>sample</u>	<u>g/l cells</u>	<u>g/l glucose</u>	<u>g/l ethanol</u>
P-1200 agitated	8.84	152.60	0.69
P-1200 stationary	3.53	52.98	62.31
control agitated	9.16	122.74	1.25
control stationary	2.65	42.53	68.62

Table 8.8

Ethanol Productivity Test 2

Test 2 - carried out in shake flasks containing 50 ml of media with 150 g/l glucose. Those with extractant contained 0.5 ml of P-1200. Harvested at 41.5 hours.

<u>sample</u>	<u>g/l glucose</u>	<u>g/l ethanol</u>
P-1200	60.62	14.35
control	73.88	10.68

Task 9

Economic Analysis

Objective:

The two objectives of this part of the research are as follows:

1. Evaluate the economics of the proposed integrated process for producing n-butanol and ethanol from cellulose.
2. Where warranted, recommend new process designs which will result in a least cost pathway for producing these two fuels.

Manpower:

Title:

E. J. Nolan (50%)

Senior Chemical Engineer
General Electric Co.

Arthur Humphrey (2%)

Dean, Engineering and
Applied Science
University of Pennsylvania

Summary of Results:

A preliminary estimate of the cost to produce ethanol by enzyme hydrolysis from poplar wood has been made for the University of Pennsylvania/GE process. This estimate was prepared by using cost information obtained from two principal sources:

- (1) The enzyme hydrolysis economics reported by Gulf for those parts of the Gulf enzyme hydrolysis process which are similar to the University of Pennsylvania/GE process; this includes the hydrolysis, fermentation and distillation steps.

(2) The cost information published by Katzen for the wood

handling and treatment steps prior to enzyme hydrolysis.

The resulting information is shown on Table 9.1 as a function of yield variation. Potential by-product credit is broken out in each case.

Moreover, we have assumed that the wood feedstock contains 56% cellulose, 28% hemicellulose and 16% lignin; the yield numbers are, therefore, given on a dry basis. Two different raw material feedstock cases are also considered: poplar sawdust and poplar wood chips. Poplar sawdust is assumed available at a delivered cost of \$11.25 per oven dry ton (O.D.T.) while the cost of wood chips is assumed to be delivered at \$20/ODT.

The 13.3 lb. ethanol/100 lbs. dry feed yield listed in the first column is based on: 56% cellulose; 50% yield to glucose; 50% conversion of glucose to alcohol, 95% overall yield. Different individual yield steps are specified in the second and third columns as noted. The yield in the last column includes utilization of the hemicellulose, so that a yield of 95% of the theoretical results. It is seen that, as a function of yield, the manufacturing cost varies from \$1.07/gal. (at 31.7% of theoretical) to \$0.74/gal. (at 95% theoretical) for the sawdust feedstock, while the manufacturing cost using wood chips varies from \$1.29/gal. to \$0.81/gal. The manufacturing costs for the chip feedstock are plotted as ordinates on Figure 9.1 with yield as abscissa. Potential credits for fermentation and distillation by-products are also shown. These could conceivably reduce the manufacturing cost from \$.80 to \$0.65 per gallon respectively as the yield is improved from 31.7% to 95% of the theoretical for the sawdust feedstock; in the case of wood chips the corresponding cost can decrease from \$1.08 to \$0.72 per gallon on the same yield basis. For comparison

purposes the new plant manufacturing cost of producing ethanol is shown on the ordinate. This cost is \$1.22/gal including by-product credits and a feedstock cost at \$2.50/bushel. The cost reduction challenge is reflected on Figure 9.1 where a "learning" curve is superimposed. The learning curve suggests where future research should focus. Yield improvement is of paramount importance; by-product credit is always important but has more incremental effect on manufacturing cost when yields are low, as would be expected.

Heat Pump Application

An initial investigation was made into the use of a heat pump as a potential for reducing the energy requirement during distillation. In this particular method the column reboiler is replaced by the refrigerant condenser, and the column condenser is replaced by the refrigerant evaporator.

The case of an ethanol distillation at atmospheric pressure was considered in which a 20% (wt.) ethanol fuel is concentrated to 92% (wt.). It is found that under these conditions, about 16 pounds of steam per gallon of ethanol is necessary to effect the separation. Thus, when producing 25 million gallons of 95% (vol.) ethanol per year, the reboiler duty is approximately 50×10^6 BTU/hr, and the corresponding heat pump requirement is about 4,200 tons of refrigeration.

Depending on the refrigerant selected, the heat pump compressor above can range up to 5,600 b.h.p.

If fuel and power are purchased at costs of \$3.50/1,000 lb. of steam and 6¢/kwh respectively, the use of the heat pump in the aforementioned case is not at all attractive; at a steam cost of \$5.50/1000 lb. the situation is more favorable. The corresponding energy cost is:

(a) steam \$3.50/1000 lb 5.5¢/gallon of ethanol

\$4.50/1000 lb 7.1¢/gallon

(b) heat pump power 7.9¢/gallon

Steam Consumption

In the process of computing the theoretical quantity of steam necessary to distill the ethanol from ethanol water mixtures, it became evident that a review of the ethanol-water vapor-liquid equilibria is in order. A number of ethanol water vapor liquid data sources were kindly furnished by Drs. H. Blanch and Brian Maiorella of the University of California, Berkeley. Moreover, we have uncovered some other references (1) (2) to experimental ethanol-water vapor-liquid equilibria which, when coupled with the Berkeley references, sheds some new light on the commonly used data of Carey and Lewis (3) as quoted in Perry (4). Some of this data when plotted as Figure 9.2 depicts the resulting ethanol-water vapor-liquid equilibrium diagram at 1 atmosphere. This curve represents the work of eight different investigations including that of Carey and Lewis. The measurements of Carey and Lewis covered only the range of 1.9 mole % to 74.7 mole % in the liquid (corresponding to .17 mole % and 78.1 mole % vapor respectively); whereas the Henry law region extends from .0 to 2 mole % giving a relation $Y = 9.75 X$ ($0 < X < 2$) mole percent. Here Y is the vapor composition in equilibrium with the liquid X (both Y and X are in mole units). In addition Carey and Lewis did not measure the composition of the constant boiling mixture. This latter value was specified by them as the average of data obtained by Young and Fortey in 1902 (5) and Wade and Merriman in 1911 (6). The following values in Table 9.2 are given by these investigations, standard deviations were not specified.

Table 9.2

760 mm. Ethanol-Water "Constant Boiling" Mixture

<u>Investigator</u>	<u>Ethanol Mole % in liquid</u>	<u>Ethanol Mole % in Vapor</u>
Young and Fortey	84.00	84.70
Young and Fortey	86.00	86.40
Young and Fortey	88.00	88.20
Wade and Merriam	89.43	89.43

The "best curve" drawn through the data plotted in figure (9.2) gives the azeotrope at about 89.4 mole % (95.6 wt. %) ethanol in substantial agreement with the Wade and Merriam work.

The impact of the variation in the vapor liquid data arose when computing minimum reflux ratios. In cases where the product and feed compositions are such that the operating line becomes tangent at the pinch region, it is found that variations of up to 15% in the $\left(\frac{L}{D}\right)_{\min}$ occur between the commonly accepted Carey-Lewis vapor liquid curve and the vapor liquid curve shown in figure (9.2). Errors in the computed theoretical steam consumptions of about 10% result when the feed composition is approximately 16% wt. or higher (feed at the boiling point).

Table 9.3 contains steam consumption estimates for concentrating 5 and 10 wt. % ethanol water mixtures to 80% (wt.) ethanol (170.6 proof) as a function of feed temperature. Two extreme conditions of reflux were considered 10% in excess of the minimum and 10% less than the maximum (total) reflux ratio. All other reflux ratios fall between (obviously) minimum and total reflux and do not represent operating conditions inasmuch as they correspond

to an infinite number of plates and an infinite column diameter respectively.

It may be seen from Table 9.3 that when an ethanol-water mixture of 5 wt % is distilled to 80 wt % ethanol, about 60% more steam is needed if the feed mixture is initially at 30°C than if the feed is at its boiling point; and about 40% more if the feed is 10% ethanol. It should be noted however that for cases in which the feed temperatures are lower than the boiling point, the composition on the feed plate will be richer in ethanol than the feed stream itself as a consequence of the "q" line (7). Therefore, whenever ethanol feed compositions are below these boiling points, the heat required to raise the feed to the feed plate temperature is less than the heat necessary to raise the corresponding ethanol feed composition to its boiling point. One should bear this in mind when comparing the theoretical heating requirement for ethanol feed compositions both at and below their respective boiling points. Moreover, sufficient heat is released during the condensation of the overhead product to preheat the feed to at least 60°C.

Minimum Reflux Ratios

Minimum reflux ratios have been computed using the recommended ethanol-water vapor liquid data shown in Figure 9.3 for feed at the boiling point ranging from 3.0 to 39 wt %. The reflux ratios shown on Figure 9.3 are for three different ethanol product compositions: 92 wt % (189.4 proof); 86 wt % (180.4 proof); and 80 wt % (170.6 proof). As expected, the minimum reflux varies over a wide range of values. For example, in the case of a 92% wt ethanol product, the result of the "tangent pinch" inflection point in the vapor-liquid equilibrium curve is clearly shown at compositions of about 16 wt % and above; a constant value of $\left(\frac{L}{D}\right)_{\min}$ results which is equal to 1.45. For product compositions less than 92% (wt), the "tangent pinch" effect does not arise when the feed is at its boiling point. Minimum reflux ratios

varying from 6.7 to about 0.5 correspond to feed concentrations of 3 to 39 % (wt) respectively when the product composition is 86 wt %. When the product composition is 80 wt %, these minimum reflux ratios vary from about 5.6 to 0.25 over the same range of feed composition as above (i.e. 3 to 39 wt %). It is to be expected however that when feed compositions below 16% are encountered, at temperatures less than their boiling point prior to distillation, the constant value of $\left(\frac{L}{D} \right)_{\min}$ (which arises because of the "tangent pinch") will also occur at compositions much less than 16% because of q line construction. Establishment of a number of additional cases is currently under investigation.

References

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Table 9.1 - Cost Estimate for the Manufacture of Ethanol
by the Enzyme Hydrolysis of Poplar Wood

Yearly Production 25 Million Gallons of 95% Ethanol

Yield lbs Ethanol/100 lb Feed		13.3		26.6		26.6 + 13.3	
Yield Calculations*		(.56)(.50)(.50)(.95)		(.56)(.50)(.95)(1.0)		(.56)(.50)(.95)(1.0) + (.28)(.50)(.95)(1.01)	
Cases		\$/Gal. Ia	\$/Gal. Ib	\$/Gal. IIa	\$/Gal. IIb	\$/Gal. IIIa	\$/Gal. IIIB
Base Case: (No byproduct) (credits)	Capital related	.555		.456		.413	
	Raw material**	.283 (Sawdust)		.142		.095	
	Power, Maint., Labor	.232		.232		.232	
	Sawdust	1.07	1.07	0.83	0.83	0.74	0.74
	Wood Chips	1.29	1.29	0.94	0.94	0.81	0.81
Credits							
1.	Animal Feed @ \$120/ton	\$(-0.15) (31,485) tons/yr	\$(-0.15)	(-.076) (15,743) tons/yr	(-.076)	(-.05) (10,495) tons/yr	(-.05)
2.	Spent Solids @ \$20/ton	(- .06) (74,458) tons/yr	--	(- .03) (36,729) tons/yr	--	(- .02) (24,464) tons/yr	
3.	50% lignin recovery						
	a. 20¢ as polymeric lb material	--	(-.094)		(-.047)		(-.03)
	b. \$20/ton as fuel	--	(-.03)		(-.015)		(-.01)
Net Production Cost							
	Sawdust	0.86	0.796	0.724	0.69	0.67	0.65
	Chips	1.08	1.02	0.83	0.80	0.74	0.72

* Feed composition - 56% cellulose, 28% hemicellulose, 16% lignin

** Avg. Cost \$11.25 per ton sawdust dry basis

' Assumed/Avg. wood chips cost \$20/ton dry basis

Table 9.3

Steam Consumption for Ethanol-Water Distillation
 80 wt % Ethanol in distillate - 99.9 wt % of
 feed ethanol is recovered in the distillate

Ethanol Concentration in Feed Mixture		STEAM CONSUMPTION* (#10 psig Steam/gallon anhydrous ETOH)						
Weight %	Volume %	Feed Temperature:	30°C	40°C	50°C	60°C	70°C	B.P.
5	6.25	110% (L/D) min	27.5	25.8	23.9	22.4	21.2	18.3
5	6.25	90% of total reflux	60.8	58.3	55.7	53.2	51.0	44.0
10	12.40	110% (L/D) min	16.5	15.7	14.7	13.9	12.9	11.5
10	12.40	90% of total reflux	51.5	50.2	48.9	47.6	46.7	43.5

*Sufficient heat released during condensation of overhead product to preheat feed to 60°C.

FIGURE 9.1
PENN/GE PROCESS

Anticipated Manufacturing Cost of Ethanol by Enzymatic Hydrolysis

Yearly Production: 25 Million Gallons (95%)

Wood Chips: \$20 Oven Dry Ton

56% Cellulose, 28% Hemicellulose, 16% Lignin

SUMMARY (Selling Price =
Manufacturing Costs + 14¢/gal. R.O.I.)
Today = \$1.43/gal.
Tomorrow = \$0.82/gal.

FIGURE 9.2