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PROCESS DEVELOPMENT STUDIES ON THE BYCONVERSION OF CELLULOSE AND PRODUCTION OF ETHANOL

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Charles R. Wilke and Harvey W. Blanch

June 1979

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PROCESS DEVELOPMENT STUDIES
ON THE BIOCONVERSION OF CELLULOSE
AND PRODUCTION OF ETHANOL

under the auspices of
DEPARTMENT OF ENERGY--SOLAR ENERGY DIVISION
Fuels from Biomass Program

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Berkeley, California
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Harvey W. Blanch, Co-Investigator

Report of Work Progress
June 1, 1979
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I. RAW MATERIAL & PROCESS EVALUATION


Approximately 1.5 pounds of Indiana Corn Stover were sent to Professor Hans Grethlein, at Dartmouth, to be subjected to the pretreatment process developed there. The 40-60 mesh fraction (0.25 to 0.35 mm size) of the 2 mm Wiley milled material was sent as requested. The analysis of this material has been reported. (1) The four samples and controls returned to the Lawrence Berkeley Laboratory were subjected to enzymatic hydrolysis as 2.5 to 3.5 w% suspensions in cellulase enzyme solutions of an activity of 2.42 I.U. per ml. Appropriate quantities of substrate and enzyme solution were used to give approximately 40 I.U. of enzyme per gram of substrate used.

The conditions of the Dartmouth pretreatment are essentially a 5 w/v% suspension of the corn stover in 0.5% acid subjected to a temperature of 194°C for 12 seconds.

The samples were kept frozen at -78°C during shipping and prior to use. This was done to slow down the recrystallization of the material to α-cellulose that occurs after the acid processing which has rendered the microcrystalline cellulose amorphous.

The pretreatment liquors contained an average of 6.8 grams per liter of dissolved sugars with a mean composition of 77% xylose, 6% arabinose and 16% glucose.

The results of enzymatic hydrolysis on the slurried samples (now separated showed that hydrolysis was essentially complete in less than 20 hours. An average of 19 grams per liter of dissolved sugars was
obtained with a composition of approximately 15% xylose, 5% arabinose, 74% glucose and 6% galactose. The carbohydrate conversion to sugar was from 88 to 92% and the glucan to glucose conversion was 97% in all of the samples studied. The solid residues after enzymatic hydrolysis contained from 32 to 37% of the initial substrate.

It would appear that the Dartmouth pretreatment process is one of the more attractive alternative processes studied.

B. High Pressure HCl Conversion of Wood

Re: LBL 9220.

*This section (pages 4-6 inclusive) is deleted because of matter subject to patent not released by DOE.
II. ENZYME FERMENTATION STUDIES

A. Cellulase Production

a. Batch Fermentation

In the present study, evaluations were made of *Trichoderma viride* strain C-30, comparing cellulase productivity with strain QM 9414, upon which most of our previous process development studies have been based.

Fermentation operations were conducted in 5-liter New Brunswick fermentors. The medium as devised by Mandels was used for all experiments except that urea was deleted from the medium unless otherwise indicated. Ball-milled solka floc (BW 200) at 1.0% concentration was used as a substrate. Four-day old, 10% mycelium inoculum was used for all fermentations. The pH was adjusted with 2N NaOH and 2N H₂SO₄.

It was observed by Wilke and Yang (2) that 31°C and a pH of 4.5 for the first 48 hours and then 28°C and maintaining pH above 3.3 for the rest of the fermentation time period was optimum for cellulase production. Similarly Montenecourt and Eveleigh (3) and Mandels (4) found 28°C and pH ≥ 3.3 to be optimum. Wilke and Yang (2) used an 0.02% Tween-80 level, while others used 0.2%. In the present studies 0.02% Tween-80 was used for all runs. Our current studies lead to the conclusion that 25°C is the optimum temperature. As shown in Table 1 there is an increase in cellulase enzyme activities in addition to an increase in soluble protein. The addition of urea (Table 2) to the fermentation medium effects all the activities except β-glucosidase and soluble protein.

There is a three-fold increase in β-glucosidase activity at 25°C as compared to other temperatures. The increased potency in terms of glucose production is probably due to the selectively higher level of β-glucosidase in the culture filtrate. The higher level of β-glucosidase would permit more rapid conversion of cellobiose to glucose.
### Table 1
Effect of Temperature on State Variables

<table>
<thead>
<tr>
<th>State Variables*</th>
<th>Present Process</th>
<th>Previous Method (2)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>31°C (0-2 days)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28°C (rest 0 days)</td>
<td></td>
</tr>
<tr>
<td>FPA (I.U./ml)</td>
<td>3.03</td>
<td>1.72 (8*)</td>
<td>+ All with 1% substrate conc.</td>
</tr>
<tr>
<td>C_1 (I.U./ml)</td>
<td>16.79 x 10^{-3}</td>
<td>5.56 x 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>C_x (I.U./ml)</td>
<td>10.9</td>
<td>6.55</td>
<td>*with 5.6% substrate</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>0.368</td>
<td>0.126</td>
<td>Concentration</td>
</tr>
<tr>
<td>Soluble Protein</td>
<td>4.8</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>(mg. ml^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2
Effect of Urea Addition

<table>
<thead>
<tr>
<th>State Variables</th>
<th>Without</th>
<th>With (0.3g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPA (I.U./ml)</td>
<td>2.3</td>
<td>1.76</td>
</tr>
<tr>
<td>C₁ (I.U./ml)</td>
<td>$25.9 \times 10^{-3}$</td>
<td>$25.05 \times 10^{-3}$</td>
</tr>
<tr>
<td>Cₓ (I.U./ml)</td>
<td>9.07</td>
<td>9.8</td>
</tr>
<tr>
<td>β-glucosidase (I.U./ml)</td>
<td>0.29</td>
<td>0.24</td>
</tr>
<tr>
<td>Soluble Protein (mg.ml⁻¹)</td>
<td>3.4</td>
<td>3.47</td>
</tr>
</tbody>
</table>
This then releases the cellobiose inhibition of the C₁ enzyme, and hence increases the rate of depolymerization of crystalline cellulose.

b. Continuous Cellulase Production (T. viride) 9414)

It was observed in previous work (5, 6, 7, 8) that increasing the cell density or substrate concentration did not proportionally increase enzyme productivity. Extensive studies were carried out in order to optimize individually the 1st and 2nd stage of the two-stage continuous system for cellulase activity by manipulating pH, temperature, Tween-80 level, substrate concentration and dilution rates. The results are shown in Table 3. The experiments were run continuously for about 3 1/2 months.

Runs #1 and #2 show that decreasing Tween 80 level by half increases the filter paper activity by 60% in the first stage of the two-stage fermentation. There is a considerable drop in the filter paper activity in the first stage at pH 5.0 than at other pH levels which severely effects the productivity in the second stage. Although, the filter paper activity of run #1 in the first stage is about 70% less than in run #3, the productivity is higher in the second stage. If run #1 was operated with 0.1% Tween 80 level, it could lead to higher productivity. Similarly if the second stage of run #2 was operated at pH 5, it could also lead to higher productivity. Hence for all practical purposes the first stage can be operated between a pH range of 3.75-4.25 and the second stage at pH 5.0. In terms of inlet substrate concentration, 1.75% gives as good a filter paper activity as 2.5%. If the first stages of run #2 and run #9 are compared, it is seen that there is a decrease in filter paper activity and enzyme productivity from 4.2 and 0.084 to 2.54 and 0.053, respectively. If enzyme activity and production in the second stage are taken into consideration, then it would be profitable to use SW-40 rather than
<table>
<thead>
<tr>
<th>Run #</th>
<th>Inlet Sub Conc. (g 10^-1)</th>
<th>Tween 80 Level (%)</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>Dilution Rate (hr^-1)</th>
<th>Productivity</th>
<th>FPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>0.2</td>
<td>28</td>
<td>28</td>
<td>4.0</td>
<td>5.0</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0.1</td>
<td>28</td>
<td>28</td>
<td>4.0</td>
<td>4.0</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>0.1</td>
<td>28</td>
<td>28</td>
<td>3.75</td>
<td>3.3</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0.1</td>
<td>28</td>
<td>28</td>
<td>4.25</td>
<td>4.0</td>
<td>0.02</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>0.1</td>
<td>28</td>
<td>28</td>
<td>5.0</td>
<td>3.75</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>0.1</td>
<td>28</td>
<td>28</td>
<td>4.0</td>
<td>5.0</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>17.5</td>
<td>0.1</td>
<td>28</td>
<td>28</td>
<td>4.0</td>
<td>5.0</td>
<td>0.02</td>
</tr>
<tr>
<td>8</td>
<td>17.5</td>
<td>0.1</td>
<td>23</td>
<td>28</td>
<td>4.0</td>
<td>5.0</td>
<td>0.054</td>
</tr>
<tr>
<td>9</td>
<td>17.5*</td>
<td>0.1</td>
<td>23</td>
<td>28</td>
<td>4.0</td>
<td>4.00</td>
<td>0.02</td>
</tr>
</tbody>
</table>

For non-recycled system

**Ball milled Solka Floc (200 mesh)  F_1 = first stage ;  F_2 = second stage**
BM200, as the substrate. Table 4 gives the optimum operating conditions for the two-stage continuous cellulase production system using *T. viride* QM 9414.

In the near future, work will be completed on the optimization of the cultural conditions, i.e. pH, temperature, Tween 80 level, aeration, agitation, inlet substrate concentration and dilution rates for the new strains L-5 and Rut-C-30 in a two-stage continuous system. During the remainder of the current year optimization of the blend of C₁, Cₓ and β-glucosidase for faster rate of hydrolysis will be studied. Once correct proportions of these extracellular enzymes are known, the aid of environmental control manipulation will be sought to make the new strains synthesize these enzymes in the right proportions.

**B. Studies on the Composition of Cellulase Enzyme**

Preliminary work has been done on analytical and preparative separations of the components of the cellulase complex derived from the QM 9414 and Rutger's C-30 strains of *Trichoderma viride*. Present work indicates the existence of twelve components. Techniques used include SDS-polyacrylamide gel electrophoresis, gel permeation chromatography and iso-electric focusing.
Table 4
Optimum Operating Conditions

<table>
<thead>
<tr>
<th>Control Variables</th>
<th>1st Stage</th>
<th>2nd Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.75-4.25</td>
<td>5.0</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>28°</td>
<td>28°</td>
</tr>
<tr>
<td>Dilution Rate (hr⁻¹)</td>
<td>0.02</td>
<td>0.027</td>
</tr>
<tr>
<td>Inlet Substrate (%) Conc.</td>
<td>1.75</td>
<td></td>
</tr>
</tbody>
</table>
Future work will include application of techniques of zymography, the results of which will be used in a study of the kinetics of the degradative reactions undergone by cellulosic substrates, and measuring inhibition constants.

C. Xylan Hydrolysis

Carbohydrate in the biomass used (corn stover) consists of 37% cellulose and 27% hemicellulose. Most of the work on the enzymatic hydrolysis has been done on cellulose conversion to glucose by the action of cellulases. This section of the research program involves studying the hydrolysis of hemicellulose to xylose by the action of xylanases.

It was hoped that a process using enzymes alone could be developed. However, the stover was shown to be too contaminated with bacteria to be used without some type of pretreatment. Tests have been run in the presence of merthiolate which retards bacterial growth but also inhibits enzyme activity. Therefore, tests are currently being performed to determine which antibacterial agent would be the best to use in terms of stopping bacterial growth without inhibiting enzyme activity.

The major problem encountered so far is the establishment of analytical procedures to be used in xylanase characterization. Unlike cellulose, which can be obtained in pure form, commercial xylan preparations are not pure. Because of this, a method is needed to treat raw xylan so that a xylan with at least a known composition can be obtained. Tests have been done using various xylan sources and solvents for purification. To date a procedure to obtain purified xylan has not been found.

Tests have shown the xylanase we currently use produces mainly xylobiose (xylose equivalent to cellobiose) from xylan. In order to completely
convert the xylan to xylose a micro-organism must be found that will either complete the entire conversion or one that will convert xylobiose to xylose. A further problem associated with this is that a pure source of xylobiose needs to be found. Since this chemical cannot be obtained from any supply house, it must be isolated in the laboratory. We hope that a chromatograph column with Bio-Gel P-2 packing will give enough separation of a hydrolyzed mixture of xylan so that the xylobiose will be separated.

III. ETHANOL FERMENTATION STUDIES

A. Media Development and Growth Factors in Ethanol Fermentations.

The following conclusions were drawn from previously carried out batch ethanol fermentations using glucose as the substrate.

From Shake Flasks:

1. *Saccharomyces cerevisiae* var. *Anamensis* (ATCC 4126) can synthesize all growth factors needed with sufficient time (Figure 2).

2. Vitamin deficient media produce lower cell mass yields but higher ethanol yields (Figure 3).

3. Biotin synthesis is the rate limiting step in cell growth if no external growth factors are added (Figure 2).

From 1 liter batches:

1. Vitamins and amino acids can increase the specific growth rate. A synthetic mix of vitamins can achieve 85% of the maximum specific growth rate produced with yeast extract (Figure 4).

After the identification of the important growth factors in batch fermentation, continuous cultures were started to determine the optimum levels of all the medium components in ethanol fermentation. A novel procedure extending developments by Mateles and Battat (9) was employed. Each medium component is made the limiting substrate in terms of cell and
Figure 2. Comparison of Growth in Biotin Deficient and Growth Factor Free Medium
Figure 3. Effect of various Growth Factor Combinations on Cell Yield and Ethanol Yield Per Gram of Cells in Shake Flasks.
Figure 4. Effect of Growth Factors on Specific Growth Rates.

- **Yeast Extract**
  - $\mu_{\text{max}} = 0.51 \text{ hr}^{-1}$
  - 0.5 g/l

- **Biotin**
  - $\mu_{\text{max}} = 0.20 \text{ hr}^{-1}$
  - 0.5 mg/l

- **Synthetic Vitamin Mix**
  - $\mu_{\text{max}} = 0.43 \text{ hr}^{-1}$
  - 149 mg/l
ethanol yields. The limiting nutrient is first determined by observing which component when injected as a concentrated shot directly into the fermenter produces a transient increase in cell mass and/or ethanol. When this limiting nutrient is found, its concentration is the feed reservoir, which is feeding at a steady state dilution rate is increased so that it is no longer yield-limiting up to a given level of cell mass and ethanol. When the component is limiting the steady state yield, the stoichiometric requirement of that component per quantity of cell mass and the ethanol yield per quantity of cell mass can be determined. This component can then be eliminated from further testing as the other components in turn are each made yield limiting.

Thus far, it has been found that biotin is a critical vitamin which must supplement the synthetic medium. (Tables 5, 6). Other growth factors may also be important and will be studied further in continuous culture. The growth factors are most economically supplied from a complex medium, such as corn syrup liquor or molasses. These will be investigated next. Use of one of these media in place of yeast extract will reduce ethanol cost considerably. These results and experimental procedure for both batch and continuous fermentations were presented at the recent annual meeting of the American Society for Microbiology (10).

A. Process Development Studies on Production of Ethanol

a. Rapid Fermentation

1. Design Study

(1) Limitations of Previous Work

In our current fermenter design, fermentation is carried out in a continuous, high cell density, atmospheric pressure fermenter. End product inhibition is removed (and productivity thus greatly increased) by cycling fermenter beer to a vacuum flash-pot where an equilibrium ethanol water vapor mixture
Table 5  
Search for Initial Limiting Substrate  
in Continuous Culture

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stepped up Glucose in the Feed (2X)</td>
<td>None</td>
</tr>
<tr>
<td>Pulsed (NH₄)₂SO₄</td>
<td>None</td>
</tr>
<tr>
<td>Pulsed KCL</td>
<td>None</td>
</tr>
<tr>
<td>Stepped Up Yeast Extract in Feed (3.5X)</td>
<td>Cell Mass Increased 3.74X</td>
</tr>
<tr>
<td>Stepped Up Yeast Extract in Feed to 2X Last Level</td>
<td>Cell Mass Increased 1.15X</td>
</tr>
<tr>
<td></td>
<td>Last Level (Glucose Depleted)</td>
</tr>
<tr>
<td>Pulsed Glucose</td>
<td>Cell Mass Increased (Glucose Limiting)</td>
</tr>
</tbody>
</table>
Table 6

Search for Yeast Extract Component Limiting Growth in Continuous Culture

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsed MgSO$_4$</td>
<td>None</td>
</tr>
<tr>
<td>Pulsed NaCl</td>
<td>None</td>
</tr>
<tr>
<td>Pulsed Inositol and p-Aminobenzoic Acid</td>
<td>None</td>
</tr>
<tr>
<td>Pulsed Pyridoxine and Nicotinic Acid</td>
<td>None</td>
</tr>
<tr>
<td>Pulsed Thiamine and Pantothenate</td>
<td>None</td>
</tr>
<tr>
<td>Stepped Up Biotin in Feed 40 X</td>
<td>Transient Overshoot--Cell Mass Increased 138%</td>
</tr>
<tr>
<td>Stepped Up Biotin in Feed to 4 X Previous Level</td>
<td>Steady State Cell Mass Increased 26%</td>
</tr>
<tr>
<td></td>
<td>No Further Change</td>
</tr>
</tbody>
</table>
(22 wt% ethanol) is boiled away.

Preliminary test results, showing feasibility for flash-pot operation at normal fermenter temperature (but infeasibility for elevated temperatures) have been reported (11).

These tests were hampered by equipment limitations, therefore the full rate and conversion data were not obtained.

The overall process design calls for a feed of 50 wt% sugar solution. At this feed composition, the water carried into the fermenter with sugar feed will just balance the water carried over in the flash-pot ethanol-water vapor equilibrium product stream. Operation with a 50 wt% sugar feed has not yet been demonstrated in the laboratory.

A general limitation of past studies has been the inability to achieve high yeast cell densities—cells being lost to a bleed stream, which is required to prevent build-up of potentially toxic cellular metabolites. Maintenance of high cell densities should further improve fermentor productivity and will allow fermentation of more concentrated sugar solutions. Reduced equipment sizing and reduced water load on the final distillation system will result.

(ii) Improved Experimental Design

An improved experimental design has been devised. A modified laboratory flash-pot, which will prevent localized cell overheating and avoid traditional foaming problems has been developed. Yeast cycling is through specially modified finger pumps, which will minimize mechanical damage to the cells (a problem in the earlier flash-pot tests). A continuous centrifuge or improved settler device will be used to separate and recycle cells from the bleed stream.
(iii) Significant Variables to Be Studied

Fermentation rate at high cell density is to be studied. Significant variables will be steady state ethanol concentration, sugar feed concentration and fermenter bleed rate. Temperature and pH optima located by previous experimenters will be maintained. Ethanol removal will be via the vacuum flash-pot with fermentation conducted at atmospheric pressure. Possible adverse effects on the yeast cells due to pressure shock as they are cycled through the flash-pot will be examined.

Nutrient and oxygen requirements are also significant. These are being currently studied and are described in another section of the report.

2. Experimental Design

(i) Equipment Preparation

Experimental apparatus preparation is almost complete. A highly flexible experimental set up has been constructed to allow many possible variations in operating conditions with minimal equipment modification. This apparatus extends considerably the capabilities of our laboratory to conduct fermentations under unusual operating conditions.

Development of a yeast separation process for the continuous laboratory fermenter is made difficult because of the extremely small flow rate and liquid hold-up required of the separation device. Initial testing has been conducted to evaluate the Sharple's "Super Centrifuge" and Westfalia continuous centrifuges. With some modification the Westfalia model may be acceptable. An improved settling device making use of the boycott effect to enhance settling rates is being examined.
b. Ethanol Water Separation

1. Design Study

(i) Limitations of Previous Work

A preliminary design study of the Cysewski/Yang proposed ethanol-water separation process was conducted. Significant limitations were the lack of basic ethanol-water equilibrium data collection and reduction and the failure to consider innovative designs such as complete vacuum distillation.

Data Collection

A study to collect basic engineering data (equilibrium compositions, heats of vaporization, thermal expansion coefficients, etc.) for the ethanol-water system was conducted. This data is now summarized in a report "Equilibrium Data for Optimizing the Distillation Conditions of the Ethanol-Water System," (12) and serves as the basis for further design studies.

Data Reduction

A computer program has been developed to reduce the large amount of ethanol-water equilibrium data at various reduced pressures to a single equation. Initial fits of the data have been achieved. A standard Wilson form was used:

\[ \ln \gamma_1 = -\ln(x_1 + A_{12}x_2) + x_2 \frac{A_{12}}{x_1 + A_{12}x_2} - \frac{A_{21}}{A_{21}x_1 + x_2} \]  
\[ \ln \gamma_2 = -\ln(x_2 + A_{21}x_1) + x_1 \frac{A_{12}}{x_1 + A_{12}x_2} - \frac{A_{21}}{A_{21}x_1 + x_2} \]  
\[ \gamma_1 = \frac{Y_1p}{X_1p_1^s} ; \quad \gamma_2 = \frac{Y_2p}{X_2p_1^s} \]  
\[ A_{12} = B_1 + B_2/T \]
\[ B_3 + B_7/T \]  

With

\[ B_1 = 902.5 \]
\[ B_2 = 176.5 \]
\[ B_3 = 221.8 \]
\[ B_4 = 563.5 \]

A standard deviation of .95% was achieved. This probably represents experimental scatter in the data and not any limitation of the equation. The quality of the fit is crucial since the resulting equation will later be used to determine an optimum distillation pressure (where the high ethanol concentration pinch is minimized). Work to date is reported in detail in "Low Pressure Ethanol-Water Vapor Liquid Equilibrium Curve Fitting." (13)

2. Process Design

(ii) Flash-pot Operation

Based on the new ethanol-water equilibrium data, a design study was conducted (reported in "Current Status of Ethanol Recovery Using Vacuum Distillation") (14). The advantages of vacuum fermentation can be maintained at substantially reduced costs by use of a vapor recompression heated auxiliary vacuum flash pot (see Fig. 5). Flash-pot operating costs are approximately $9.00 per hour (for a 12 million gallon per year plant).

In addition to the reduction in end product inhibition, water flow rates to the ethanol water distillation column are drastically reduced (11.3 thousand pounds per hour as compared to 60.3 thousand pounds per hour for an atmospheric distillation). This excess water would otherwise be heated to the column bottoms temperature (212°F) in the final purification. A savings of 6.8 million BTU per hour (18%) is thus achieved, with a corresponding major savings in column heating costs.
Figure 5. Flash-Pot Separation Scheme.
c. Vacuum Distillation

A complete vacuum distillation affords further advantages (Figure 6). At low pressures the azeotrope is removed, but the equilibrium "pinch" at high ethanol concentrations is so severe as to make total separation by direct distillation unfeasible. At 95.6 weight percent (the atmospheric azeotrope) the pinch is greatly reduced in a vacuum distillation and substantially less reflux is required. Bottoms temperatures are also substantially reduced so that heat loss through the pure water bottoms product is minimized. Energy requirements are thus reduced an additional 22% from 20,400 Btu/gal (for flash-pot and atmospheric column to 16,000 Btu/gal (steam requirements for compression included). The column size must be somewhat increased (approximately a 2-fold increase in diameter) and a small (22 HP) compressor must be added to compress CO₂ gas dissolved in column feed back to atmosphere pressure.

A study to determine optimal operating conditions for a vacuum distillation system and to evaluate the resulting economics is underway.

IV. UTILIZATION OF HEMICELLULOSE SUGARS

A. Xylanase Production

In shake flasks, *Streptomyces xylophagus* nov. sp. grows in pellet form. The size of pellets varies from flask to flask, some being very fine and the largest is about 1 mm in diameter. As pellets grow, the color of the broth changes from milky white to brown. The organism growing in wheat bran medium is thus difficult to observe directly.

The results of batch growth studies are shown in Table 7. The enzyme production rate in shake flask and 14-liter fermenter showed a marked
CONTINUOUS VACUUM DISTILLATION
(12 M GALLON YEAR PLANT)

ENERGY REQUIRED: 16,300 BTU/GALLON PRODUCT

Figure 6. Continuous Vacuum Distillation Scheme.
<table>
<thead>
<tr>
<th>TYPE OF SUBSTRATE</th>
<th>MODE OF OPERATION</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larchwood (L) 1%</td>
<td>Shake Flask (200 ml medium in 500 ml flask)</td>
<td><strong>Washed &amp; Dried Wheat Bran</strong></td>
</tr>
<tr>
<td>Wood Gum (WG) 1%</td>
<td></td>
<td>4.99**</td>
</tr>
<tr>
<td>Wheat Bran (W) 7%</td>
<td></td>
<td>6.89*</td>
</tr>
<tr>
<td></td>
<td>Submerged fermentation</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td>Per vol=142, L/4 vol=102</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Continuous (In 14 litre Fermentor)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0273</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Final Enzyme Activity**
- 8.5* (1.61)*; pH 7.4
- 6.03 (1.75)*; pH 7.9
- 6.83 (1.79)*; pH 8.4
- 7.87 (1.83)*; pH 8.4
- 7.83 (1.56)*; pH 8.4
- 7.92 (1.25)*; pH 8.4
- 7.75 (1.65)*; pH 8.4
- 7.25 (1.65)*; pH 8.5
- 7.25 (1.65)*; pH 8.5
- 7.25 (1.65)*; pH 8.5
- 7.25 (1.65)*; pH 8.5
- 7.25 (1.65)*; pH 8.5

**Bacterial activity** (mg ml⁻¹)
- 9.82
- 8.46
- 7.25
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46
- 8.46

**Soluble protein** (mg ml⁻¹)
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
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- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
- 9.44 (5.16)*
difference because of pH control in the fermenter. Similarly, there was a difference in final enzyme activity in using washed-wheat bran compared with washed-dryed wheat bran. In the 14-liter fermentor where the pH was controlled, wheat bran shows a considerable improvement in enzyme activity as compared to shake flask studies.

In continuous culture dilution rate of 0.027 hr$^{-1}$ gives an enzyme activity of 7.25 mg ml$^{-1}$, while lower or higher dilution rates seems to effect the enzyme activity, soluble protein and cell dry weight. It thus appears that a dilution rate of approximately 0.027 hr$^{-1}$ is optimal.

B. Xylose Fermentation*

Work conducted during this period involved measuring the effect of xylose concentration on the yield of fermentation products using Bacillus macerans. Data are shown in Table 9. The highest per cent conversion occurred at an initial xylose concentration of 2.3%. $U_{\text{max}}$ under these conditions was 0.154 hr$^{-1}$ (doubling time = 4.5 hrs). This compares favorably with the maximum growth rate ($U_{\text{max}} = 0.163$ hr$^{-1}$, $T_d = 4.25$ hrs) seen in the 1% xylose cultures described in our previous report (LBL-8558). The conversion of xylose to ethanol was higher in the 2.3% cultures being 26.6 wt% as compared with 19.6 wt% in the 1% xylose culture.

At a 4.3% xylose concentration, fermentation of the sugar was incomplete, and growth was slower. Product yields were lower, although final concentration of ethanol and acetone in the broth are similar to those seen in the 2.3% fermentation. The data indicate that unmeasured products are being formed.

In a 7.6% xylose fermentation growth was slow, and little of the sugar was fermented. Final ethanol, acetic acid and acetone concentrations all were lower than seen previously.

* Some of the work conducted in the previous period is summarized in Fig. 7 and Table 8. The data presented are for anaerobic growth of Bacillus macerans with 2% xylose.
Table 8
Anaerobic Growth of Bacillus Macerans on 2% Xylose

<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>TIME (HRS)</th>
<th>CONCENTRATION (mg/ml)</th>
<th>$\mu_{\text{max}}$ (mg cell/hr)</th>
<th>MAX CONC. (mg/ml)</th>
<th>$Y$ (mg mg xylose$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CELL MASS</td>
<td>45</td>
<td>0.18</td>
<td>0.15</td>
<td>0.66</td>
<td>0.031</td>
</tr>
<tr>
<td>XYLOSE</td>
<td>45</td>
<td>20</td>
<td>4.29 (minimum)</td>
<td>2.0</td>
<td>--</td>
</tr>
<tr>
<td>EToH</td>
<td>45</td>
<td>0.35</td>
<td>0.39</td>
<td>5.6</td>
<td>0.27</td>
</tr>
<tr>
<td>HAc</td>
<td>45</td>
<td>0.35</td>
<td>0.39</td>
<td>4.8</td>
<td>0.23</td>
</tr>
<tr>
<td>ACETONE</td>
<td>70*</td>
<td>0.24</td>
<td>0.018</td>
<td>0.32</td>
<td>0.015</td>
</tr>
</tbody>
</table>

The maximum cell concentration occurred at 90 hours. The maximum concentration point for all other substances occurred at about 90-100 hrs.

*The acetone concentration did not rise to a measurable level until after 60 hrs. The $\mu$ (acetone) calculated from the data at 70 hours is probably less than the $\mu_{\text{max}}$ (acetone).
Figure 7. *Bacillus mascerans* Growth on Xylose.
Table 9

Fermentation of Xylose to Ethanol, Acetone and Acetic Acid by Bacillus macerans in Batch Culture at pH 6.0
Effect of Increasing Xylose Concentration on the Fermentation

<table>
<thead>
<tr>
<th>Initial [Xylose] %</th>
<th>% Xylose Used</th>
<th>Maximum Concentration</th>
<th>Produce of:</th>
<th>% Conversion of Sugar to:</th>
<th>Minimum time* Required Hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>Acetic Acid</td>
<td>Acetone</td>
<td>Ethanol</td>
</tr>
<tr>
<td>2.3</td>
<td>91</td>
<td>0.558</td>
<td>0.484</td>
<td>0.032</td>
<td>26.6</td>
</tr>
<tr>
<td>4.3</td>
<td>73</td>
<td>0.569</td>
<td>0.394</td>
<td>0.033</td>
<td>18.4</td>
</tr>
<tr>
<td>7.6</td>
<td>23</td>
<td>0.345</td>
<td>0.230</td>
<td>0.027</td>
<td>19.2</td>
</tr>
</tbody>
</table>

* Lag phase subtracted.
In the 1.0% xylose fermentations described previously essentially all (>98%) of the sugar was fermented. At the higher sugar concentrations, fermentation was incomplete. In the 2.3% and 4.3% xylose fermentations similar final ethanol and acetone concentrations were reached. Since other authors who used this organism reported higher yields and concentrations of acetone in their fermentations, a possible explanation for the incomplete sugar utilization is end-product inhibition of the fermentation brought about by ethanol. Alternatively, an unmeasured product (such as lactate or formate) may be responsible.

In the 7.6% xylose fermentation the fact that the previously achieved levels of ethanol were not reached suggests that some other product or possibly the xylose itself, or an impurity, inhibited growth.

To understand the fermentation better, a mass balance will be performed. To this end, analytical procedures for H₂, CO₂, lactic and formic acids are being developed. Modifications are also being made in the fermentation and sampling system to eliminate losses of ethanol and acetone which are known to occur. Future fermentations should, thus, display higher yields of those two volatile products.

Future fermentations will be conducted using a nitrogen sparging system to remove volatile products and test the hypothesis that ethanol levels on the order of 0.5% inhibit growth and fermentation. Removal of volatiles might also allow faster growth as in the case with yeast. We will also carry out fermentations in the presence of varying levels of acetate to see if the fermentation can be directed toward the production of greater amounts of ethanol and acetone. Acetate is a precursor of both these products.
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Published Reports

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Papers in Press


APPENDIX

CELLULOSE BIOCONVERSION AND PILOT PLANT STUDIES
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