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ENZYMATIC HYDROLYSIS OF CORN STOVER PROCESS DEVELOPMENT AND EVALUATION

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DEVELOPMENT AND EVALUATION

Javier Perez*, Charles R. Wilke and Harvey W. Blanch
(*M.S. thesis)

December 1981

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ENZYMATIC HYDROLYSIS OF CORN STOVER
PROCESS DEVELOPMENT AND EVALUATION

Javier Perez*
Charles R. Wilke and Harvey W. Blanch

*M.S. Thesis
December 1981

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and
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Berkeley, California 94720

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ABSTRACT

The hydrolysis of acid treated corn stover with cellulase from *T. reesei* Rut-C-30 was evaluated. Experiments were conducted with substrate concentrations of 5-25% by weight, enzyme activities of 0.5-7 IU/ml and residence times of 24-48 hours. Maximum conversion was 55% for specific cellulase activity of 25-30 IU/g. Optimum cellobiase activity for minimum cellobiose production was found to be approximately 1.8 cellobiase units to 1 FPA unit. Hydrolysis experiments with steam exploded corn stover led to a maximum conversion of 80%, significantly higher than the results obtained for acid treated substrate. Steam exploded corn stover was demonstrated as a suitable carbon source for growth of *T. reesei* in submerged cultures.

Computer aided process evaluations were conducted for several processing alternatives. Among the alternatives considered were steam explosion or dilute acid pretreatment, equilibrium solid-liquid contacting or ultrafiltration for enzyme recovery, and batch, continuous or continuous with cell recycle enzyme production. Using dilute acid pretreated materials, glucose may be produced for 12-22 cents per pound for corn stover cost ranging from 0-30 $/ton. This constitutes a 33% reduction over previous processing schemes employing cellulase from *T. reesei* QM9414. Enzyme production is the single most expensive processing step, accounting for 40% of processing costs. Preliminary evalu-
tion of processing with steam exploded corn stover leads to a glucose cost of 10-15 cents/pound for corn stover costing 0-30 $/ton; with further improvement possible. At the current level of development, ethanol may not be manufactured profitably from corn stover; furthermore, the dominance of raw material cost tends to dampen the effect of process improvements on the cost of manufacturing ethanol.
# TABLE OF CONTENTS

1. Introduction .................................................. 1
2. Background ....................................................... 4

2.1. Enzymatic Hydrolysis: Substrate and Enzyme Properties and Interaction ........ 4

2.1.1. Corn Stover Composition ................................... 4
2.1.2. Structure and Morphology of Cellulose ..................... 4
2.1.3. Cellulase Enzymes ........................................ 6
2.1.4. Enzymatic Hydrolysis Models ............................. 12

2.2. Process Components .......................................... 15

2.2.1. Pretreatments ............................................. 15
2.2.2. Enzyme Production ........................................ 18
2.2.3. Enzyme Recovery .......................................... 23
2.2.4. Enzymatic Hydrolysis ..................................... 26

2.3 Enzymatic Hydrolysis Processes ................................ 29

2.3.1. Natick Stirred Tank Process ............................. 29
2.3.2. Gulf-Arkansas Simultaneous Fermentation and Saccharification Process .......... 31
2.3.3. MIT Mixed Culture Process ................................ 33
2.3.4. Penn/GE Multiproduct Process ............................ 35

3. Previous Work ................................................... 43

3.1. Development of the Berkeley Process ........................ 43

3.1.1. Process Recovering Cellulase in Solution and Adsorbed onto Solids ........... 43

3.1.2. Process with Increased Cellulase Productivity Using Cell Recycle ............ 45

3.1.3. Corn Stover Hydrolysis Process ........................... 47

3.1.4. Corn Stover Process Optimization ........................ 48
3.2. Process Evaluation

4. Experimental Procedures

4.1. Corn Stover Composition Determination

4.2. Substrate Pretreatment

4.2.1. Dilute Acid Pretreatment

4.2.2. Explosive Steam Decompression

4.2.3. Acid-Base Pretreatment

4.3. Enzyme Production

4.3.1. Cellulase Production for Use in Hydrolysis Experiments

4.3.2. Shake Flask Studies with Steam Exploded Corn Stover as Substrate

4.3.3. Fermentation with Corn Stover as Carbon Source

4.4. Hydrolysis Experiments

4.4.1. Hydrolysis of Acid Treated Corn Stover

4.4.2. Hydrolysis with Steam Exploded Corn Stover

4.5. Miscellaneous

4.5.1. Hydrolysis Spent Solids Washing

4.5.2. Relative Filtration Rates of Hydrolysis Suspensions

5. Experimental Results and Discussion

5.1. Substrate Composition and Pretreatment

5.1.1. Corn Stover Composition

5.1.2. Dilute Acid Pretreatment

5.1.3. Steam Explosion Pretreatment

5.2. Cellulase Production with \textit{T. reesei} (Rut-C-30)

5.2.1. Batch Cultures Employing Solka Floc as Carbon Source
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.2. Cellulase Production with Steam Exploded Corn Stover as Substrate</td>
<td>76</td>
</tr>
<tr>
<td>5.2.3. Batch Fermentation of \textit{T. reesei} with Corn Stover</td>
<td>76</td>
</tr>
<tr>
<td>5.3. Enzymatic Hydrolysis of Acid Treated Corn Stover</td>
<td>78</td>
</tr>
<tr>
<td>5.3.1. Glucose Production</td>
<td>78</td>
</tr>
<tr>
<td>5.3.2. Xylose Production</td>
<td>87</td>
</tr>
<tr>
<td>5.3.3. Celllobiose Production</td>
<td>87</td>
</tr>
<tr>
<td>5.3.4. Enzyme Recovery</td>
<td>91</td>
</tr>
<tr>
<td>5.3.5. Performance Comparison of Cellulase from QM9414 and Rut-C-30</td>
<td>95</td>
</tr>
<tr>
<td>5.4. Hydrolysis of Steam Exploded Corn Stover</td>
<td>95</td>
</tr>
<tr>
<td>5.4.1. Glucose Production</td>
<td>95</td>
</tr>
<tr>
<td>5.4.2. Enzyme Adsorption</td>
<td>99</td>
</tr>
<tr>
<td>5.4.3. Xylose Production</td>
<td>99</td>
</tr>
<tr>
<td>5.5. Miscellaneous Process Development Studies</td>
<td>102</td>
</tr>
<tr>
<td>5.5.1. Hydrolysis Solids Washing</td>
<td>102</td>
</tr>
<tr>
<td>5.5.2. Relative Filtration Rates</td>
<td>102</td>
</tr>
<tr>
<td>5.6. Kinetic Model For Acid Treated Corn Stover Hydrolysis</td>
<td>105</td>
</tr>
<tr>
<td>6. Process Design and Evaluation</td>
<td>112</td>
</tr>
<tr>
<td>6.1. Basic Design and Alternatives</td>
<td>112</td>
</tr>
<tr>
<td>6.1.1. General Basis</td>
<td>112</td>
</tr>
<tr>
<td>6.1.2. Base Case Process</td>
<td>113</td>
</tr>
<tr>
<td>6.1.3. Processing Alternatives</td>
<td>113</td>
</tr>
<tr>
<td>6.2. Equipment Sizing Criteria</td>
<td>115</td>
</tr>
<tr>
<td>6.2.1. Size Reduction</td>
<td>115</td>
</tr>
<tr>
<td>6.2.2. Mixing</td>
<td>116</td>
</tr>
<tr>
<td>6.2.3. Heat Transfer</td>
<td>118</td>
</tr>
</tbody>
</table>
6.2.4. Vessels
6.2.5. Materials Transfer
6.2.6. Separation
6.2.7. Miscellaneous
6.3. Process Components, Description and Assumptions
6.3.1. Milling
6.3.2. Pretreatment
6.3.3. Hydrolysis
6.3.4. Enzyme Recovery
6.3.5. Enzyme Production
6.3.6. Evaporation
6.3.7. Storage
6.4. Process Evaluation
6.4.1. Capital Cost
6.4.2. Labor
6.4.3. Raw Materials
6.4.4. Utilities
7. Computer Simulation and Evaluation
7.1. Computer Algorithm Capabilities
7.1.1. General Organization
7.1.2. Required Information and Operating Options
7.1.3. Information Distribution and Output
7.1.4. Limitations
7.2. Subroutine Outlines
7.2.1. Main Program Subroutine
7.3. Material Balance
7.3.1. Process Selection Routines
7.3.2. Process Operation Subroutines
7.3.3. Support Subroutines
8. Economic Evaluation, Results and Discussion
8.1. Glucose Production Cost, Base Case
8.1.1. Production Cost Excluding Corn Stover Cost
8.1.2. Production Cost Including Corn Stover Cost
8.1.3. Contribution of Processing Operations to the Cost of Manufacturing Glucose
8.1.4. Effect of Enzyme Activity on Cost
8.1.5. Effect of Substrate Concentration
8.1.6. Effect of Residence Time
8.1.7. Economics of Scale, Direct Fixed Capital
8.1.8. Cost of Ethanol Production
8.1.9. Comparison with QM9414
8.2. Process Alternatives
8.2.1. Enzyme Production
8.2.2. Enzyme Recovery
8.2.3. Steam Explosion
9. Conclusions and Recommendations
9.1. Conclusions
9.1.1. Experimental
9.1.2. Process Evaluation
9.2. Recommendations
9.2.1. Experimental
9.2.2. Process Evaluation
APPENDIX A
APPENDIX B
1. Introduction

Part of current research efforts aimed at developing alternate sources of energy are being devoted to the development of processes which produce ethanol from cellulose. As a raw material, cellulose is attractive because it is renewable and abundant; an estimated 250 billion metric tons are available for processing each year (1). As a product, ethanol is of interest as a liquid fuel and as a raw material employed in the manufacture of bulk chemicals.

Currently most ethanol intended for industrial use is manufactured from ethylene, a petroleum product. Ethanol produced by fermentation is reserved for beverage use and cannot compete with petroleum based ethanol for industrial or fuel applications. The primary reason for the high cost of fermentation ethanol is the high cost of glucose, which is used in the food and beverage industries. In 1979 molasses accounted for over two thirds of the manufacturing price of ethanol (2).

Production of ethanol from cellulosic materials takes place in two discreet steps. First, cellulose is hydrolyzed to produce glucose. Subsequently, glucose is fermented by yeasts to produce ethyl alcohol. Thus, if ethanol is to be manufactured from cellulose at competitive price, an economical means of carrying out the hydrolysis step is essential.

Cellulose may be hydrolyzed to produce glucose by
employing acid or cellulase enzymes. Acid hydrolysis requires high temperature and low pH to be effective. These severe conditions make it necessary to employ expensive corrosion resistant equipment and limit the conversion efficiency due to product degradation (3). Enzymatic hydrolysis, while not requiring severe operating conditions, is hampered by limited accessibility of the substrate to the enzyme and slow reaction rates due to inhibition by products and reaction intermediates (4). In addition, the manufacture of enzymes is costly, making recovery of utilized enzyme necessary to achieve profitability and thus leading to more complicated processing.

The objective of this work was to evaluate an enzymatic hydrolysis process being investigated at the University of California, Berkeley in light of recent developments of high yielding mutant organisms. The raw material employed was corn stover, chosen because it is an abundant agricultural waste product. Hydrolysis experiments were carried out to evaluate the performance of cellulase enzymes produced by a new mutant of Trichoderma reesei. The effect of a steam explosive decompression pretreatment on hydrolysis efficiency was investigated. Preliminary studies were performed to find less expensive raw materials for cellulase production, and to evaluate ultrafiltration as a means of recovering enzymes. A computer algorithm was developed to enable an economic evaluation of these and future process alternatives and to identify areas needing further study.
References: Chapter 1


2. Background

2.1. Enzymatic Hydrolysis: substrate and enzyme properties and interaction

2.1.1. Corn Stover Composition

Corn stover, like other agricultural residues, is composed of carbohydrates, lignin, inorganic compounds, proteins, and soluble organic compounds. Table 2-1 presents a summary of typical compositions of agricultural residues found in California (1). The main constituents are carbohydrates, accounting for 50-60 percent of the total weight.

Among the carbohydrates commonly present are glucans, mannans, galactans, xylans, and arabinans. The most abundant component is cellulose, a polymer of glucose, accounting for 70-80 percent of the available polysaccharides. The second most abundant constituent is hemicellulose, a heteropolymer of the saccharides listed. Lignin, an aliphatic-aromatic polymer of oxygen substituted phenyl-propane units, serves to bind cellulose fibers together by intertwining with cellulose and covalently bonding with hemicellulose, and forms a protective sheath around cellulose fiber bundles. This sheath is partly responsible for hindering the digestion of cellulose by enzymes (2).

2.1.2. Structure and Morphology of Cellulose

Cellulose is a linear polymer composed of anhydroglu-
TABLE 2-1 Composition of California Agricultural Residues

<table>
<thead>
<tr>
<th>MATERIAL (A)</th>
<th>CARBOHYDRATES</th>
<th>ASSAY (C)</th>
<th>AZEOT.</th>
<th>ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLUC (B)</td>
<td>MANN</td>
<td>GALAC</td>
<td>XYL</td>
</tr>
<tr>
<td>BARLEY STRAW</td>
<td>37.5</td>
<td>1.26</td>
<td>1.71</td>
<td>15.0</td>
</tr>
<tr>
<td>CORN STOVER</td>
<td>35.1</td>
<td>0.25</td>
<td>0.75</td>
<td>13.0</td>
</tr>
<tr>
<td>COTTON GIN TRASH</td>
<td>18.0</td>
<td>1.9</td>
<td>0.1</td>
<td>4.0</td>
</tr>
<tr>
<td>RICE HULLS</td>
<td>32.5</td>
<td>2.7</td>
<td>0.1</td>
<td>12.3</td>
</tr>
<tr>
<td>RICE STRAW</td>
<td>36.9</td>
<td>1.6</td>
<td>0.4</td>
<td>13.0</td>
</tr>
<tr>
<td>SORGHUM STRAW</td>
<td>32.5</td>
<td>0.8</td>
<td>0.2</td>
<td>15.0</td>
</tr>
<tr>
<td>WHEAT STRAW</td>
<td>32.9</td>
<td>0.72</td>
<td>2.16</td>
<td>16.9</td>
</tr>
</tbody>
</table>

(A) 2mM Wiley Milled, 40-60 mesh fraction (0.25 to 0.35mM), and 100% dry.

(B) Wgt.% Glucan, Mannan, Galactan, Xylan, Arabinan, Lignin, Azeotropic Benzene Alcohol, Extractives, Acid Insoluble Material.

(C) Average of 3-5 Det'm.

From: Wilke, C.R., et al. (1)
cose units joined by β-1,4-glucosidic bonds. Native cellulose has a degree of polymerization of 3500-10000 (3). Cellulose molecules are linked together by hydrogen bonding into an ordered, parallel arrangement, or fibril. Fibrils are bound together by polyoses to form microfibrils. Current microfibril models describe regions where fibrils are oriented in an ordered, parallel fashion surrounded by less ordered regions containing cellulose, hemicellulose, and lignin (4). Along the length of the microfibril, cellulose molecules experience regions of disorder commonly referred to as amorphous regions. Chang (5) reviewed existing models of fibril structure and concluded that models describing folding chains of cellulose polymers are consistent with existing knowledge. Figure 2-1 presents a summary of common models of cellulose structure (4,5).

Among structural features of cellulosic materials that influence its susceptibility to enzymatic attack are extent of swelling by water, size and surface area of interstitial voids, degree of crystallinity, and the presence of other substances. Despite considerable research activity, the relative importance of these factors has not been established.

2.1.3. Cellulase Enzymes

Although a large number of fungi displaying cellulytic activity have been investigated (6) current research is focused mainly on strains of the fungus *Trichoderma*
Figure 2.1 Common Models of Cellulose Structure.
After Cowling, E.B. (4) and Chang, W.J. (5).
Background

T. reesei. These fungi are of interest because they produce all cellulase components necessary for hydrolysis of cellulose, the cellulase produced is resistant to chemical inhibitors and is stable at temperatures up to 50 degrees centigrade for up to 48 hours (7).

Figure 2–2 presents a genealogy of T. reesei strains (6). The wild type QM6a was isolated by Reese in 1950 (8). Considerable work has been performed on strain QM9414, but this strain suffers the disadvantage of being deficient in the enzyme necessary to hydrolyse cellobiose. This work employed strain Rut-C-30, developed at Rutgers University by Montenecourt and Eveleigh (9). The Rut-C-30 strain has attracted attention because it is hyperproducing, resistant to catabolite repression, and it displays high B-glucosidase and xylanase activities.

The exact mode of action of cellulases is still under investigation. Current knowledge leads to grouping cellulases by function into three groups: B-1,4-glucanglucanohydrolase is a randomly acting endoglucanase which generates new chain ends from the interior of the polymer; B-1,4-glucancellobiohydrolase attacks chain ends to produce cellobiose; B-glucosidase produces glucose from cellobiose. Table 2–2 presents a summary of molecular weights and isoelectric points of each of the enzyme groups (6). Figure 2–3 presents a summary of a current model for cellulase action on cellulose (7). Work with isolated
Figure 2-2. GENEALOGY OF HIGH YIELDING CELLULASE MUTANTS

Trichoderma reesei (viride)
QM 6a (wild type)

High Energy electron
QM 9123

UV
Rut-M-7
(intermediate)

Nitrosoguanidine
QM 9414

Rut-L-5

Rut-NG-14
Hyperproducing
UV
Catabolite repression
Resistant

-------------------
From Wilke, C.R., et al. (6)
<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular Weight</th>
<th>Isoelectric Points</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exoglucanases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(EC 3.2.1.91)</td>
<td>49,000</td>
<td></td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>61,000</td>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td></td>
<td>46,000</td>
<td>3.79</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td>48,000</td>
<td></td>
<td>(20)</td>
</tr>
<tr>
<td>Endoglucanases</td>
<td>76,000</td>
<td></td>
<td>(9)</td>
</tr>
<tr>
<td>(EC 3.2.1.4)</td>
<td></td>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>30,000; 43,000</td>
<td></td>
<td>(18)</td>
</tr>
<tr>
<td></td>
<td>12,500; 50,000</td>
<td>4.60, 3.39</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>37,200; 52,000</td>
<td></td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>49,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-Glucosidase</td>
<td>47,000</td>
<td>5.74</td>
<td>(17)</td>
</tr>
<tr>
<td>(EC 3.2.1.21)</td>
<td>76,000; 76,000</td>
<td></td>
<td>(13)</td>
</tr>
</tbody>
</table>

From Wilke, C.R., et al. (6)
Figure 2-3 MODE OF ACTION OF CELLULASE ON CELLULOSE

\[ \text{B-1,4-glucan\-glucanohydrolase} \]
\[ \text{cellulose} \rightarrow \text{cellulose (susceptible)} \]

\[ \text{B-1,4-glucan\-celllobiohydrolase} \]
\[ \text{celllobiose + glucose} \]

\[ \text{B-glucosidase} \]
\[ \text{glucose} \]

---

enzyme components has shown that cellulases act synergistically (10), Table 2-3 presents an example of this behavior.

2.1.4. Enzymatic Hydrolysis Models

A full description of the enzymatic hydrolysis reaction requires consideration of cellulose structural characteristics, the mode of action of the cellulase species present, and the nature of the interaction between enzymes and cellulose.

Factors commonly considered when modeling cellulose structural characteristics are substrate accessibility, surface area, and crystallinity. Amemura and Teori (52) employed the concept of effective substrate concentration to account for the amorphous/crystalline nature of cellulose. Similarly, Howell and Majal (11) postulated that a certain percentage of the substrate is entirely inaccessible to the enzyme. Kim (12) and Humphrey (13) employed a two substrate model in which the crystalline fraction was converted to amorphous cellulose prior to being involved in product formation. Accessibility limitations have been frequently modeled by assuming the substrate is a shrinking sphere (14).

Accurate modeling of hydrolysis kinetics must account for various enzyme components and their synergistic action, for rate inhibiting effects by products and intermediates, and for deactivation of enzymes by physical means such as
### TABLE 2-3  Synergistic Effect of Cellulase Components

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>culture filtrate</td>
<td>100%</td>
</tr>
<tr>
<td>A. B-1,4-endoglucanases</td>
<td>1% or less</td>
</tr>
<tr>
<td>B. B-1,4-exoglucanases</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>C. B-glucosidase</td>
<td>0%</td>
</tr>
<tr>
<td>D. A + C</td>
<td>5%</td>
</tr>
<tr>
<td>E. B + C</td>
<td>4%</td>
</tr>
<tr>
<td>F. A + B + C</td>
<td>103%</td>
</tr>
</tbody>
</table>

(During hydrolysis of cotton, components added at same level present in original filtrate)

---

After: Wood, T.M. and McCrae, S.I. (10)
temperature and shear. Suga (15), Okazaki and Moo-Young (16), and Lee (17) have proposed models which include two, three, and four enzyme components respectively. These models also account for the relationship between degree of substrate polymerization and reaction rates.

Mandels and Reese (18), and Ghose and Das (19) have reported strong competitive inhibitory behavior by cellobiose, and a less pronounced competitive inhibition by glucose. Huang (20) has incorporated a competitive inhibition mechanism in his model. In addition to competitive inhibition models, several non-competitive inhibition models have been proposed (14, 16).

In modeling the interaction between substrate and enzymes two factors which may be influential are diffusional limitations stemming from the heterogeneous nature of the system, and the adsorption behavior of various enzyme components onto the substrate. Fickian diffusion models have been employed (15, 16), but a more common approach has been to neglect mass transfer resistances. The approach to modeling has been varied, with Langmuir isotherms (16, 20), Freundlich isotherms (14), and linear models proposed (12).

In general kinetic models describing cellulose hydrolysis have evolved from simple Michaelis-Menten models to incorporate the increasing number of factors now understood to influence hydrolysis kinetics. However, even with the appearance of increasingly sophisticated models, a
generalized model fully describing this process is not available.

2.2. Process Components

2.2.1. Pretreatments

Substrate pretreatment techniques have been developed in an attempt to enhance hydrolysis yields. The primary aim of pretreatment is to enhance the porosity of cellulose, decrease its crystallinity and disrupt the lignin sheath. Pretreatments may be grouped by method as physical and chemical.

Physical pretreatments employ some type of size reduction equipment to increase the area available for reaction and disrupt the lignin sheath surrounding cellulose microfibrils. Mandels, Hontz, and Nystrom (21) compared mulchers, shredders, roll, hammer, vibro energy, fluid energy, colloidal, and ball mills. They concluded that ball milling had the greatest effect on increasing the susceptibility of various cellulosic materials to enzymatic attack. Mandels et al. (22) have also successfully employed a two roll compression mill which apparently consumes less energy than a ball mill. Favorable results were also obtained by Kelsey and Shaffizadeh (23) on a process combining wet milling simultaneously with enzymatic hydrolysis.

Table 2–4 presents a summary of some pretreatments investigated in the recent past (6). Although most of the
<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Procedure</th>
<th>Purpose</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water swelling</td>
<td>Solids soaked in boiling water</td>
<td>Capillary swelling</td>
<td>Does not permanently change capillary structure</td>
<td>21</td>
</tr>
<tr>
<td>Alkali swelling</td>
<td>1% NAOH, boil 3 hours</td>
<td>Capillary swelling</td>
<td>Conversions enhanced</td>
<td>24</td>
</tr>
<tr>
<td>Ammonia</td>
<td>Contact solids with ammonia under pressure</td>
<td>Delignification and swelling</td>
<td>Corn stover conversion increased 26 to 61%</td>
<td>24</td>
</tr>
<tr>
<td>Dilute Acid</td>
<td>0.1-1% H₂SO₄, 3-50 hr, 100°C,</td>
<td>Pentosan Extraction</td>
<td>50-80% of available pentosans extracted</td>
<td>1</td>
</tr>
<tr>
<td>Extraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilute Acid</td>
<td>Short residence time, high temperature</td>
<td>Crystalinity disruption</td>
<td>97% conversion corn stover</td>
<td>36</td>
</tr>
<tr>
<td>(Dartmouth)</td>
<td></td>
<td>Pentosan extraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitric acid</td>
<td>5 parts nitric oxide, 100 parts solids, 20°C, 24 hours</td>
<td>Pentosan and lignin extraction</td>
<td>High conversions</td>
<td>37</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>Procedure</td>
<td>Purpose</td>
<td>Results</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------</td>
<td>------------------------------</td>
<td>-----------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Steam decompression</td>
<td>500-1000 PSI steam</td>
<td>Delignification,</td>
<td>Conversions over 60% with</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>5-300 seconds vented</td>
<td>increase</td>
<td>wood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rapidly</td>
<td>surface area,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pentosan formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High temperature ethanol</td>
<td>50% ethanol</td>
<td>Delignification</td>
<td>Minor increase in enzymatic</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>180 °C, 1 hour</td>
<td></td>
<td>hydrolysis conversion</td>
<td></td>
</tr>
<tr>
<td>WAN treatment</td>
<td>Water swelling, alcohol wash, nonpolar hydrocarbon wash</td>
<td>Permanent swelling</td>
<td>Increased conversions</td>
<td>39</td>
</tr>
</tbody>
</table>
processes described are effective, most are also not currently economically feasible. Alkali swelling has been reported to consume about 40 percent of the sodium hydroxide required for operation (24). In general, the processes listed which employ costly chemicals, such as the nitric acid and the Cadox process are difficult to justify economically.

The dilute acid extraction employed by the University of California, Berkeley is sufficiently inexpensive to be considered if acid recycle is employed (25). However, current research on pentose utilization is not sufficiently developed to ensure a use for the dilute xylose stream produced.

Currently attracting much interest is a steam explosive decompression developed by the Iotech Corporation. Studies have shown that the process is successful in enhancing the digestibility of soft woods (26). This work included a preliminary evaluation of the digestibility of corn stover subjected to this method of pretreatment.

2.2.2. Enzyme Production

Process development work aimed at producing cellulases on an industrial scale have focused mainly on aerobic fermentation of strains of T. reesei.

Batch studies have been employed to select nutrients and operating conditions leading to optimal cellulase
production for several strains including strains QM9414 and C-30. Mandels and co-workers (27) developed a successful nutrient media that includes ball or roll milled crystalline cellulose (Solka Floc) as both carbon source and inducer and proteose peptone as a nitrogen source. A slightly modified version of this media was employed by Tangnu et al. (28) for work on strain Rut-C-30. Although this media is effective it employs expensive organic nitrogen and carbon sources not feasible for industrial scale applications. Work has been reported (22, 29) aimed at replacing these with less expensive materials such as agricultural or municipal waste cellulose for carbon sources and corn steep liquor or recycled mycelia for nitrogen sources, but further work is needed.

_T. reesei_ fermentations require pH control to sustain cell growth and cellulase production. Acids are secreted by the organism during carbohydrate consumption and serve a regulating function, inactivating the enzymes at pH below 3 and preventing cell growth at pH below 2 (7). Different strains have slightly different optimal pH points in the range of pH 4-5. Optimal temperature has been reported at 25-28°C (7, 28). Agitation is required to provide adequate mixing of insoluble cellulose, suspend the mycelia and maintain good oxygen transport. Oxygen requirements call for maintaining oxygen tension at 15-20% of saturation (28, 30).

Table 2-5 presents typical batch enzyme production data for Rut-C-30 and QM9414 strains. Batch cycle times of 144
### TABLE 2-5 Batch Enzyme Production Results

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate Conc.</th>
<th>B-g Activity (IU/ml)</th>
<th>FPA Activity (IU/ml)</th>
<th>Productivity (IU/l/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QM9414</td>
<td>5% Solka Floc</td>
<td>1</td>
<td>6.06</td>
<td>42</td>
</tr>
<tr>
<td>QM9414</td>
<td>2.5% &quot;</td>
<td>1.2</td>
<td>4.3</td>
<td>30</td>
</tr>
<tr>
<td>C-30</td>
<td>5% &quot;</td>
<td>26</td>
<td>14</td>
<td>97</td>
</tr>
<tr>
<td>C-30</td>
<td>2.5% &quot;</td>
<td>10</td>
<td>5.2</td>
<td>36</td>
</tr>
</tbody>
</table>

1. After Wilke, C.R., et al. (6)
Background

Levels of both cellobiase and overall activity (FPA) are higher for the C-30 strain. The Rut-C-30 strain also produces a higher ratio of β-glucosidase activity to overall activity against filter paper (FPA). This ratio for C-30 is in excess of the 1.5 ratio reported (7) to be optimal for minimum cellobiase production during hydrolysis. Figure 2-4 presents a typical profile of T. reesei fermentation (7).

Production of β-glucosidase for addition to cellulase mixtures was investigated by Yamanaka (31) utilizing Brotryodiplodia theobromae and by Howell (32) using Aspergillus phoenicis. Although the advantage of having sufficient cellobiase activity during hydrolysis and the feasibility of the scheme was confirmed, economic evaluations did not justify large scale production of β-glucosidase by these methods. Furthermore, the current availability of strains producing high cellobiase activity makes this approach less attractive.

Work has been reported on continuous and semicontinuous enzyme production schemes, but productivities reported were lower than those obtained with batch systems. In early work, Nystrom and Kornuta developed a semicontinuous process with enzyme productivity of approximately 10 IU/l/hr (33). Von Stockar, Yang and Wilke (34) developed a two stage continuous process employing cell recycle to boost productivities to 19 IU/l/hr using strain QM9414. By
Figure 2.4. Typical Fermentation Profile with Trichoderma reesei, QM-9414. (a) pH, and (b) enzyme cell concentration and soluble protein. ○, Dry WT. □, Cellulose. ●, Soluble Protein. △, Mycelial Protein (g/l): ▲, FPU (IU/ml).

1After Mandels, M. and Ryu, D.V.P. (7).
increasing substrate concentrations to 2.5%. Tangnu et al. (28) achieved a productivity of 50 IU/L-hr with QM9414 at dilution rates of 0.04 hr$^{-1}$ for the growth stage and 0.02 hr$^{-1}$ for the induction stage respectively. Work on Rut-C-30 is still in progress, however preliminary work (6) achieved productivities in the range of 50 IU/L-hr. In earlier work Mitra and Wilke (35) reported an optimum dilution rate of 0.02 hr$^{-1}$ for the cellulase producing stage with strain QM9414. This value has not been substantially improved in more recent work (6).

2.2.3. Enzyme Recovery

A predominant contributor to the high cost of producing glucose from waste cellulose is the cost of producing the required cellulose (24). Attempts at cost reduction have therefore led to investigation of enzyme recovery schemes. Most work has been focused on adsorbing the enzymes onto fresh cellulose, precipitating the enzymes by salting out or filtering the cellulase with membranes.

It has been demonstrated that cellulose is adsorbed readily onto cellulose during the early stages of hydrolysis (40). This behavior enables the recovery of enzymes from hydrolysis product streams by contacting fresh cellulose with the hydrolysate. By using countercurrent staging recoveries in excess of equilibrium are obtained.

This process, although inexpensive (24), is complicated
by selectivity differences for adsorption of various enzyme components onto cellulose. Castanon (40) showed that endoglucanases were adsorbed more readily than exoglucanases. Yamanaka (31) observed little adsorption of B-glucosidase. Although potentially still useful, this technique may alter the ratio of component cellulases used in hydrolysis and may result in reduced savings if a limiting cellulase component is not recovered. Considerably more work on the interaction of cellulases during hydrolysis is needed to resolve this problem.

Ige (41) investigated the precipitation of cellulases by adding acetone to enzyme solution. Figure 2-5 is a profile of enzyme recovery as percent of initial activity versus acetone concentration. Recovery for both endo- and exo-glucanases was in excess of 95% when 2-3 volumes of acetone to enzyme solution were used. Yamanaka (31) reported over 90% recovery of B-glucosidase activity with the same amount of acetone. This method overcomes the problem of recovery selectivity but recovery of acetone by distillation is necessary and costly.

Molecular ultrafiltration is a feasible method of concentrating proteins. Wang (42) reported recovery efficiencies ranging from 70-90% of original activities in experiments with several enzymes. The reason for loss of activity was due to inactivation of protein deposited on the membrane. Other problems involved in protein separation with
C activity (●) and C activity (■) recovered from a 2-fold concentration enzyme solution with initial activities of 7.84 C units and 1.88 C units. From Ige, R.I. and Wilke, C.R.

Figure 2.5 Enzyme Recovers as % of Original Cellulase Activity Vs. Acetone Concentration.
UF membranes are low fluxes, 5-15 gal/ft$^2$/day, fast rate of fouling, and short membrane life (43).

Ultrafiltration systems which maintain a high flux and reduce or eliminate backflushing downtime are commercially available (44). The sustained high fluxes are obtained by pumping the liquid at high velocity and shear parallel to the membrane surface, and thus constantly scraping it clean. Although shear denatures some proteins this apparatus warrants investigation. Another potentially feasible method of achieving high flux in membranes is to pulse the liquid to be filtered at high frequency (45).

Riaz (46) investigated compounds which may desorb enzymes bound to cellulose. He reported recovering 45% of bound enzyme upon washing cellulose with 6 molar urea. Adding urea to hydrolysis reactions to prevent cellulases from binding has been studied and found to be effective but costly (47).

2.2.4. Enzymatic Hydrolysis

Enzymatic hydrolysis has been investigated using stirred tank and fluidized bed reactors and packed beds. Methods of continuous product removal to reduce glucose inhibition have included combined saccharification and fermentation, and membrane filtration.

Stirred tank hydrolysis processes have included semibatch and fully continuous methods of operation. Semibatch
techniques take advantage of viscosity drops during the first few hours of hydrolysis to achieve solid concentrations of 30%. With this technique, product streams of 10% glucose have been reported (22).

Cellulases immobilized onto beads have been used in a fluid bed reactor to hydrolyze dilute recirculating cellulose suspensions. Although quantitative conversion of cellulose was obtained and cellular half-life was long, large residence times were required (48).

Packed bed hydrolysis has involved both stationary enzyme and stationary cellulose beds. Both of these techniques were found to be inferior to fluidized bed counterparts. Packed enzyme beds plugged and packed cellulose beds led to high pressure drops (6).

A combined saccharification-fermentation scheme developed by Gulf Chemicals Company has been reported to yield higher conversions than conventional stirred tank hydrolysis (Figure 2-6). Hydrolysis was conducted with 8% pasteurized cellulose feed, whole cellulase culture broth and yeast cake (49).

Mixed culture processes combining cellulase production, hydrolysis and ethanol fermentation have been reported (50). A culture employing Clostridium thermocellum to hydrolyze cellulose and produce ethanol and Clostridium thermosaccharolyticum to catabolize xylose has reported good conver-
Gulf/Arkansas Comparison of Simultaneous Saccharification/Fermentation (SSF) and Saccharification

From Wilke, C.R., et al. (6) XBL803-4882
Figure 2-6.
sion of Solka Floc and corn stover pretreated with sodium hydroxide (6). Stirred tanks and fluidized cellulose beds have been employed as mixed culture bioreactors.

2.3. Enzymatic Hydrolysis Processes

Roughly half a dozen enzymatic hydrolysis processes are currently under investigation. Their degree of development ranges from conceptual, backed by some bench scale experimentation to pilot plant demonstration for a few. The following is a summary of key aspects of the more advanced processes. Discussion of the process being developed at the University of California, Berkeley is deferred until Chapter 3.

2.3.1. Natick Stirred Tank Process

Figure 2-7 shows a schematic diagram of the stirred tank hydrolysis process being developed by the U.S. Army Natick Research and Development Command (Natick). Urban waste is fed to a two roll compression mill prior to entering the hydrolysis vessel. Hydrolysis takes place in 24 hours, using a solids concentration of 30% and an enzyme specific activity of 14 IU per gram of cellulose to achieve a conversion of 45%. Enzyme is made batchwise at a productivity of 125 IU/L-hr. The 10% glucose solution produced is fed directly to an ethanol fermentation bioreactor (22).

This process has been operated in a pilot plant equipped with a 250 liter stirred tank hydrolysis vessel.
Cellulose
Substrate
4.95 $\times 10^8$ kg/yr

Cellulose
Milling
3.7 $\times 10^8$ kg/yr

Hydrolyzable
Cellulose
Hydrolysis
(45% yield)

Glucose
(10% Solution)
1.67 $\times 10^8$ kg/yr

Ethanol
Fermentation
(40% yield)

Ethanol (95%)
2.5 $\times 10^6$ gal/yr
6.7 $\times 10^7$ kg/yr

Enzyme
Production
(125 IU/l/hr)
Cellulase
5 $\times 10^{12}$ IU/yr

Raw Material

Natick Process

From Wilke, C.R., et al. (6)
Figure 2-7.
The Natick process is attractive because it has achieved high cellulase productivity and because it produces a glucose stream concentrated enough to be fermented without evaporation.

2.3.2. Gulf-Arkansas Simultaneous Fermentation and Saccharification Process

Figure 2-8 shows a schematic of a process developed by Gulf Chemicals Company (51). Raw material consists of a mixture of municipal and pulp mill waste. After milling, part of the municipal waste is sterilized and used as carbon source for enzyme production. Milled pulp waste is mixed with the remaining municipal waste, is pasteurized, and is fed to a set of reactors where simultaneous saccharification and fermentation takes place. Hydrolysis-fermentation takes place in 24 hours, using 8% cellulose, fresh and recycled cellulases, and yeast.

Cellulase is produced continuously utilizing three reactors in series with a combined residence time of 48 hours. Unreacted solids are used to generate steam or electricity.

Preliminary design and evaluation of the Gulf/Arkansas process was carried out by Raphael Katzen and Associates (51) who concluded that the process may compete with processes manufacturing ethanol from grain. However, Katzen's study assumed a 90% cellulose utilization; thus far
Gulf/University of Arkansas Process

After Wilke, C.R., et al.

Figure 2-8.
only a 60% conversion has been demonstrated (6). Even though Katzen's estimate is optimistic, the Gulf/Arkansas process has undergone pilot plant testing and remains a potentially desirable method of producing alcohol from waste cellulose.

2.3.3. MIT Mixed Culture Process

Figure 2-9 shows a schematic of the Batelle Columbus design of a hydrolysis process being developed by the Massachusetts Institute of Technology (MIT). This process carries out cellulase production, hydrolysis and ethanol fermentation simultaneously in mixed culture of C. thermocellum and C. thermosaccharolyticum.

The mixed culture bioreactors are fed milled corn stover, and sterilized medium utilizing recycled process water. Ethanol beer produced is separated in a rotary filter; solids from the filter are burned to generate steam. The ethanol beer is combined from ethanol recovered from the carbon dioxide stream leaving the fermentors prior to entering a distillation train.

The Batelle design of the MIT process assumes that corn stover at a concentration of 245 g/L produces a stream containing 45 g/L ethanol (6). Currently, MIT's best fed-batch data indicates only 10 g/L ethanol produced from 100 g/L corn stover. In spite of this liberal assumption, the MIT process is potentially feasible and offers the advantage of
Battelle Design of M.I.T. Process

From Wilke, C.R., et al. (6)

Figure 2-9.
reduced capital requirements.

2.3.4. Penn/GE Multiproduct Process

Figure 2-10 is a block flow diagram of a process being developed by the University of Pennsylvania and the General Electric Company (6). Although still conceptual and lacking data on some of the processing steps, the Penn/GE process is attractive because it intends to manufacture a variety of products. By manufacturing Lignin-butanol fuel, polymer grade lignin, butanol, acetone, ethanol, animal feed and xylose total biomass utilization and lower ethanol cost may be achieved.
University of Pennsylvania/General Electric (PENN/GE)

From Wilke, C.R., et al. (6).

Figure 2-10.
References: Chapter 2


44. Technical Literature, Western Dynetics, Inc.

45. Spall, K. University of California, Berkeley. Personal communication.


3. Previous Work

Much of the information and assumptions made by this work to conduct a process evaluation of new experimental results is based on the work of previous investigators at this University during 1975-1980. Section 3.1 presents the chronological development of the Berkeley process, and points out design innovations. Section 3.2 presents a summary of economic evaluation results obtained for the Berkeley process and the processes discussed in Chapter 2.

3.1. Development of the Berkeley Process

3.1.1. Process Recovering Cellulase in Solution and Adsorbed onto Solids

Figure 3.1 presents a flow sheet of a process developed by Mitra and Wilke (1) to produce 429 tons/day of reducing sugars from 885 tons/day of newsprint. Substrate pretreatment consists of shredding followed by hammermilling. Hydrolysis takes place at 50°C and pH 4.8 for 40 hours. Using 10% solids suspension and cellulase activity of 2.7 filter paper units, 82% conversion of available cellulose is obtained. Enzymes are recovered from the product stream by contacting with incoming solids in five countercurrent mixer-filter stages. Cellulase bound to unreacted solids are recovered by washing with water in 6 countercurrent mixer-filter stages, total enzyme recovery reported was 85%.

Make-up enzyme consisting of 15% of the amount used in
Figure 3.1 Flow sheet, Process of Wilke and Mitra. From (1).
Chapter 3

Hydrolysis is produced in a two stage continuous fermentation scheme. Utilizing T. reesei QM9414 a cellulase solution having an activity of 1.3 F.P.A. is obtained. Dilution rates of 0.181 and 0.0181 reciprocal hours for the cell growth and induction stages are used.

High cellulase concentration in the hydrolysis step are achieved by precipitating the make-up enzyme with acetone and feeding solid protein to the reactor. Acetone is recovered by distillation. The ratio of acetone to cellulase solution used is 3:1 v/v found by Ige (2) to be optimal for activity recovery.

This process incorporates a number of features used in subsequent designs, including two stage continuous cellulase production with separate growth and induction stages, combustion of unreacted solids to generate steam and electricity, and recovery of cellulases by adsorption onto fresh solids. A key assumption made was that hammermilling would produce -200 mesh solids; in conducting hydrolysis experiments, Mitra employed -200 mesh newsprint obtained by ballmilling.

3.1.2. Process with Increased Cellulase Productivity Using Cell Recycle

Figure 3-2 presents a processing scheme developed by Von Stockar, Yang and Wilke (3). A key innovation of this process is the use of cell recycle to enhance the produc-
Figure 3.2. Flow Diagram of Process of Wilke, Von Stockar and Yang (3).
tivity of T. reesei QM9414 to 16 IU/l/hr. A steady state cell density of seven grams per litre is obtained by recycling 65% of the cells leaving the last induction stage. This technique makes enzyme precipitation unnecessary to achieve enzyme concentration of 3.5 F.P.A. in the hydrolysis reactor.

Recovery of cellulase adsorbed onto unreacted solids is eliminated from this scheme. Yang (4) was unable to measure cellulase activity in supernatants obtained by washing unreacted hydrolysis solids. Cellulase is recovered from the product stream by contacting with fresh solids in 3 countercurrent stages to regain 35% of the enzymes employed. The use of only three equilibrium contacting stages was shown by Yamanaka (5) to be sufficient to recover 98% of the enzyme in solution.

This process eliminates the use of ball milled quality substrate and assumes that hammermilling provides -20 mesh newsprint. Savings in milling cost are offset by lower hydrolysis conversion. Fifty percent of cellulose is hydrolyzed in 40 hours with a newsprint concentration of 4%. Hydrolysis is carried out at 45° C rather than at 50° C to reduce enzyme denaturation.

3.1.3. Corn Stover Hydrolysis Process

The process described in Section 3.1.2 was modified by Yang and Wilke (6) to utilize corn stover, and integrated
with an ethanol manufacturing scheme. Figure 3-3 presents a material flow diagram of this process. Substrate is hammer-milled and undergoes dilute acid pretreatment to extract a pentose stream. Enzyme recovery is carried out in two mixer-filter stages, recovering 58% of the cellulase employed. Hydrolysis of a 5% corn stover suspension yields 40% conversion of cellulose in 40 hours. A 2.6% glucose stream is obtained by recycling one half of the product stream back to the hydrolysis reactors.

Enzyme make-up is produced in a two stage continuous fermentation of *T. reesei* QM9414 recycling 80% of the exit stream cells to the first stage. An overall dilution rate of 0.027 hr.\(^{-1}\) is employed. Carbon source is identified as delignified cellulose, but the type is not specified.

Coupling of glucose production with ethanol fermentation requires concentrating the glucose stream to 11% prior to entering the ethanol fermentors. A seven effect evaporator is used for this purpose.

This process has been employed as a basis of comparison for evaluation of subsequent process improvement work because operating cost data is available for the various processing steps.

3.1.4. Corn Stover Process Optimization

Using a simple Michaelis-Menten kinetic model, Lindsey (7) performed an optimization of the process described in
Figure 3.3 Berkeley Process. Material Balance Flow Diagram for Integrated Processing Scheme (Wilke and Yang (6)).
section 3.1.3. The optimization evaluated substrate concentration of 2.5-10%, enzyme activities of 2.1-5.6 F.P.A and hydrolysis recycle fractions of 0-.75. Fixed unit costs were assumed for acid treated corn stover and cellulase produced with \textit{T. reesei} QM9414. Enzyme recovery was assumed to be constant at 40% of the utilized cellulase.

The reported optimum utilized 5% solid suspension with cellulase of 2.1 F.P.A. to obtain 39% conversion in 62 hours. This result points out the high cost of producing enzyme when compared to other variables and when the cost of corn stover is zero. The effect of changes in enzyme production costs, substrate cost, and enzyme recovery on the cost of producing glucose was investigated but the effect of these variables on the optimum process conditions was not shown.

The economic evaluation presented in this work is analogous in method to Lindsey's (7), however the range of operation is expanded and the search for an optimum accounts for variations in unit cost of producing enzymes and in pretreating the substrate, and uses enzyme recovery levels measured experimentally.

3.2. Process Evaluation

Wilke et al. (8) have presented a summary of reported process economics for production of ethanol from glucose by several enzymatic hydrolysis processes (Table 3-1). Com-
### TABLE 3-1 Process Cost Analysis

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol Capacity (10^6 U.S. gal/yr.)</td>
<td>25</td>
<td>10</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>Substrate</td>
<td>urban waste</td>
<td>corn stover</td>
<td>urban and pulp mill waste</td>
<td>corn stover</td>
</tr>
<tr>
<td>Substrate Cost</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$/ton substrate</td>
<td>6.06</td>
<td>30</td>
<td>15.75</td>
<td>30.00</td>
</tr>
<tr>
<td>$/gal EtOH</td>
<td>0.132</td>
<td>1.31</td>
<td>0.205</td>
<td></td>
</tr>
<tr>
<td>Total Capital ($ 10^6)</td>
<td>65.1</td>
<td>36.4</td>
<td>75.9 (1981)</td>
<td>34.3</td>
</tr>
<tr>
<td>Manufacturing Cost Without credits ($/gal)</td>
<td>1.22</td>
<td>3.11</td>
<td>1.65 (1983)</td>
<td>1.05 (with profit)</td>
</tr>
<tr>
<td>Manufacturing Cost With credits ($/gal)</td>
<td>0.94</td>
<td>2.73</td>
<td>0.749 (1983)</td>
<td></td>
</tr>
</tbody>
</table>

complete summaries of these evaluations and their assumptions are not available, therefore comparison on a uniform basis is not possible.

Evaluation of the Gulf/Arkansas and MIT processes was based on processing conditions not yet verified experimentally. Natick's process relies on 2-roll compression milling to achieve high cellulose conversion with perhaps optimistic milling costs. All process evaluations cited are subject to uncertainties in feedstock prices and marketability and selling price of byproducts such as yeast.

When adjusted for inflation, Yang's estimate of the price of glucose manufactured from corn stover costing $0-30/ton is 16-25 cents per pound. This price is based on a number of unverified assumptions or assumes technology not yet available:

1. Carbon and nitrogen sources for enzyme production are assumed to be inexpensive industrial feedstocks.

2. Hydrolysis is carried out in open air tanks for 40 hours without growth inhibitors or contamination.

3. Enzyme production employs agitated vessels with a volume of 500,000 gallons.

In view of these assumptions the estimated price of 16-25 cents per pound may be somewhat optimistic.

Economic evaluations with tentative processes such as
the ones covered in the last two chapters can be performed only if a large number of assumptions are made. The estimates generated, including the one provided in this work, are likely to carry an uncertainty of ±25% (9) if most of the processing conditions are defined, and can have substantially larger variances depending on the number and quality of the assumptions made.
References: Chapter 3


4. **Experimental Procedures**

4.1. **Corn Stover Composition Determination**

Prior to utilization, the composition of corn stover employed was determined following Wilke, et al. (1). A summary of this procedure is presented in Figure 4-1. Following milling and sieving, solids are extracted with benzene and alcohol to determine its soluble organics content. Solids are then exhaustively hydrolyzed with concentrated sulfuric acid to determine the carbohydrate content. The unhydrolyzed solids are burned, providing a determination of ash, measured as solids remaining after burning, and acid insoluble matter including lignin, measured as the weight loss of solids.

The lignin content is found by washing some benzene-alcohol extracted solids with water, exhaustively hydrolyzing the solids and burning the residue. The solid remaining after burning is ash and lignin is measured as the weight loss upon burning. A complete description of the solids composition determination procedure is provided in Appendix A.1.

4.2. **Substrate Pretreatment**

4.2.1. **Dilute Acid Pretreatment**

Prior to utilization in hydrolysis experiments, corn stover was milled to 2 mm. particle size in a hammer mill
Figure 4-1  SCHEME FOR SOLIDS ANALYSIS

SOLID MATERIAL

wiley mill, sieve, dry at 105°C

40-60 MESH FRACTION

benzene/ethanol extraction

EXTRACTIVES CONTENT

exhaustive extracted material hydrolysis

Liquor ---------> GOP, GLC methods content

dry weight

RESIDUE ---------> TOTAL ACID & ash INSOLUBLES (including lignin)

H₂O wash

H₂O WASH FACTOR

H₂O WASHED MATERIAL

exhaustive hydrolysis

discard liquor

dry weight

RESIDUE ---------> LIGNIN CONTENT & ash

1 After Wilke, C.R., et al. (1)
and treated with sulfuric acid to hydrolyze hemicellulose following a procedure from Wilke, et al. (2). Two hundred and fifty (250) grams of corn stover were added to three liters of boiling sulfuric acid contained in a 5 liter round bottom, three neck flask heated with a glass-col heater. The mixture is stirred at 40–50 rpm and maintained at 100 °C for 5.5 hours. Water loss due to evaporation was prevented by fitting a water cooled condenser to the flask vent.

On selected pretreatment runs, samples were periodically collected and analyzed for glucose and xylose concentration using a liquid chromatograph.

After 5.5 hours, the solid suspension was filtered under vacuum in a Buchner funnel. The solids were resuspended in 3 liters of water and filtered twice prior to being neutralized to pH 5 with 10 N NaOH. Following neutralization, the solid suspension was filtered, washed with 2 liters of water and filtered to near dryness. Pretreated solids were allowed to dry to ambient moisture and pretreatment liquors and wash liquors were discarded. The composition of pretreated corn stover was determined using the procedure outlined in section 4.1.

4.2.2. Explosive Steam Decompression

Corn stover samples were shipped to the Iotech Corporation, Ontario, Canada for pretreatment in their explosive
steam decomposition apparatus. Exact conditions of pretreatment were not revealed by Iotech, instead ranges of processing variables were supplied. Corn stover was contacted with steam at a pressure of 500-1000 psi for times ranging from 5 seconds to 5 minutes in a pressure chamber prior to being released to atmosphere (3). Figure 4-2 presents a schematic diagram of Iotech's pretreatment apparatus.

Pretreated material was received frozen and was analyzed for moisture content, soluble lignin, and for glucose and xylose produced during pretreatment.

4.2.3. Acid-Base Pretreatment

Air dried acid treated corn stover was added to one liter of 0.25 M sodium hydroxide boiling in a 3 liter flask. Corn stover suspension was 10% by weight. The reaction flask vent was fitted with a water cooled condenser to prevent water loss and with an ascarite carbon dioxide absorber to prevent carbon dioxide from dissolving into the sodium hydroxide solution.

After 3 hours, the solids were filtered, washed, neutralized, re-filtered and allowed to dry to ambient moisture.

4.3. Enzyme Production
Figure 4.2 Schematic Diagram of the Iotech Corporation Explosive Steam Decompression Apparatus.
4.3.1. Cellulase Production for Use in Hydrolysis Experiments

Cellulase was produced in batch fermentation utilizing T. reesei Rut C-30 following Tangnu, et al. (4). Viable cultures of T. reesei were maintained on refrigerated potato dextrose agar slants (PDA). Five hundred ml. Erlenmeyer flasks containing 200 ml. of nutrient medium, including 1% glucose, were inoculated from PDA slants and incubated for 96 hours at 28° C in an orbital shaker. Cultures in these flasks were used as inoculum (10%) for flasks containing 200 ml. nutrient medium, including 1% Solka Floc. Upon incubation for 5-6 days in an orbital shaker at 28° C this culture served as inoculum for a fermentor.

Cellulases were produced by batch fermentation in a 14 liter New Brunswick Scientific Company fermentor with working volume of 10 liters. The nutrient medium employed is shown in Table 4-1. Carbon source was Solka Floc at concentration of 50 g/L. Temperature and pH were controlled at 28° C and pH of 5 or greater, respectively. Agitation was maintained at 300-400 rpm and air was supplied at 0.2 vvm.

Samples were periodically collected and analyzed for overall activity toward filter paper (FPA), CMC ase activity (Cx), activity toward cotton (C₁), β-glucosidase activity and soluble protein. A summary of these assay procedures are provided in Appendix A.3,4,5,6,8.
<table>
<thead>
<tr>
<th>Components (g/l)</th>
<th>Cellulose</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 g/L</td>
<td>50 g/L</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.4</td>
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</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.0</td>
<td>3.8</td>
</tr>
<tr>
<td>MgSO₄</td>
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<td>0.3</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Peptone</td>
<td>1.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Tween-80 (ml)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Trace elements (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

1 After Tangnu, et al. (4)
After 6-7 days the cellulase solution was harvested. Mycelia were removed by filtering the raw culture through glass wool. The enzyme solution was buffered to a pH of 5 by adding 0.05 M acetate buffer and 0.02% sodium azide was added to prevent microbial growth. The solution was refrigerated until used.

4.3.2. Shake Flask Studies with Steam Exploded Corn Stover as Substrate

Corn stover pretreated by steam explosion was suspended in water to form a 10% suspension. The solids were alternately filtered and resuspended four times until the filtrate lost most of its pigment. Twenty g/L of this material, 20 g/L of unwashed material and 10 g/L Solka Floc served as carbon source for different shake flasks. Media for these flasks were prepared as described in section 4.3.1. Flasks were incubated in a shaker bath at 28° C. Samples were withdrawn from these flasks and were analyzed for cellulolytic activity by the methods described in Appendix A.

After 3 days, an inoculum was withdrawn from the shake flasks and was used to seed new shake flasks prepared as previously described. This procedure was repeated until 3 generations of shake flasks were incubated. Sampling on each flask continued for periods of 8 days.

4.3.3. Fermentation with Corn Stover as Carbon Source

Batch fermentations employing acid-treated corn stover,
acid-base treated corn stover or water washed steam exploded corn stover were conducted on a 3 liter scale. Medium and fermentation conditions used were described in section 4.3.1. Corn stover was added at a concentration of 20 g/L, providing a cellulose concentration of approximately 10 g/L. Samples were analyzed for filter paper activity during the course of the fermentation.

4.4. Hydrolysis Experiments

4.4.1. Hydrolysis of Acid Treated Corn Stover

Batch hydrolysis experiments were conducted for a period of 24 and 48 hours employing substrate concentrations varying from 5 to 25% by weight and enzyme activities ranging from 0.5 to 7 IU/ml. Experiments were carried out in 600 ml. Erlenmeyer flasks containing 150 ml. of cellulase solution. The contents were stirred at approximately 150 rpm and were maintained at a constant temperature of 45° C and pH of 5.

In experiments with substrate concentrations exceeding 10% by weight, stepwise addition of substrate was employed. After an initial solids loading of 5 to 10%, the reaction was allowed to proceed for one-half hour at which point an increment of corn stover was added. Additional substrate increments enabled 25% total substrate concentrations to be achieved in 3 hours.

During the course of the reaction samples were
withdrawn and analyzed for reducing sugars using the DNS method (Appendix A.2). Selected samples were analyzed for specific sugars using a high pressure liquid chromatograph and the overall activity of the enzyme remaining in solution was measured using the filter paper assay (Appendix A.4). A limited number of samples were also analyzed for activity toward cotton and CMC and for β-glucosidase activity. Procedures followed for these assays are listed in Appendix A.4, 5, 6.

Enzymes present in hydrolysates were precipitated with acetone prior to being measured for activity. This was necessary because high reducing sugar concentrations found in hydrolysates created high sugar backgrounds, and interfered with measurement of sugars produced during the test. A description of this procedure is presented in Appendix A.7.

4.4.2. Hydrolysis with Steam Exploded Corn Stover

Batch hydrolysis experiments were conducted for a period of 48 hours employing freeze dried substrate at concentrations of 5 to 35% and enzyme activities of 1 to 7 IU/ml. Experiments were carried out in 600 ml Erlenmeyer flasks containing 150 ml of cellulase solution. The contents were stirred at approximately 150 rpm and were maintained at a constant temperature of 45°C and pH of 5. Stepwise addition of substrate to achieve high solids concentration was not employed.
During the course of the reaction, samples were withdrawn and analyzed for glucose, xylose and cellobiose using a high pressure liquid chromatograph. Selected samples were analyzed for filter paper activities after precipitation with acetone (5).

4.5. Miscellaneous

4.5.1. Hydrolysis Spent Solids Washing

Hydrolysis suspensions obtained by hydrolyzing 5, 15, and 25% initial suspensions of acid treated corn stover for 24 hours were vacuum filtered in a Buchner funnel. Hydrolysate liquid was assayed for reducing sugars using the DNS method and the total volume collected was recorded. One inch thick plugs of the unhydrolyzed solids were washed with distilled water. Each wash, consisting of a volume of water retained by the cake, was collected and analyzed for reducing sugars. A total of five cake volume washes were employed.

4.5.2. Relative Filtration Rates of Hydrolysis Suspensions

Hydrolysis suspensions obtained by hydrolyzing 5-25% initial solids suspensions for 24 hours were filtered under vacuum in a Buchner funnel. The filtrate volume collected during two minutes of filtration through a 1/2 inch thick, 12.5 cm. diameter solids bed was measured.
References: Chapter 4


5. Experimental Results and Discussion

5.1. Substrate Composition and Pretreatment

5.1.1. Corn Stover Composition

Table 5-1 presents a summary of the composition of corn stover obtained from the University of California, Davis. A glucose content of 41% is typical of corn stover, a xylose content of 19% is higher than normal. Preliminary work reported in this chapter also employed corn stover obtained from Purdue University, which contained 40% glucose and 16% xylose equivalents. The composition reported for California corn stover does not account for 14% of the material. This is due to dirt and proteins which were not measured.

### TABLE 5-1 Corn Stover Composition

<table>
<thead>
<tr>
<th>% Carbohydrate</th>
<th>% Sugar Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>36.7 Glucan</td>
<td>40.7 Glucose</td>
</tr>
<tr>
<td>17.0 Xylan</td>
<td>19.3 Xylose</td>
</tr>
<tr>
<td>2.2 Arabinan</td>
<td>2.5 Arabinose</td>
</tr>
<tr>
<td><strong>Σ=55.9</strong> Carbohydrate</td>
<td><strong>Σ=62.5 Sugar Equivalent</strong></td>
</tr>
</tbody>
</table>

11.4 Lignin
18.5 Ash (= 21.5 as actual silicates, obvious presence soil)
6.5 Ethanol/Benzene Extractives
0.4 Acid Insolubles
N.D. Protein,

**Σ=95.7**

1. University of California, Davis
Fall, 1980 crop
5.1.2. Dilute Acid Pretreatment

Figure 5-1 presents a typical sugar concentration versus time profile for dilute acid pretreatment of corn stover. Typically 60-70% of available xylose is obtained along with 5-6% of available glucans. Xylose is produced continuously during the 5.5 hour period, with rates slowing after 4-5 hours. Glucose production is finished in 2 hours.

Following pretreatment, 60% of the original material is recovered as solids. The composition of corn stover after pretreatment is given in Table 5-2. Xylose composition has been reduced to 10%, glucose composition has been increased to 57% by removal of xylose and acid soluble material.

Xylose conversions observed were 10-15% lower than those reported by Wilke, et al. (1). A discrepancy of this magnitude is not unusual. Substrate crystallinity, lignin content and porosity varies from crop to crop.

Indiana corn stover employed in preliminary work contained 57% glucan and 10.2% xylan after pretreatment.

5.1.3. Steam Explosion Pretreatment

Steam exploded corn stover received from the Iotech Corporation contained 65% by weight water. Of the original solids, 33% were soluble in benzene alcohol, 24% of lignin was soluble in 80°C water, 40% of available hemicellulose and 11% of available cellulose was converted to xylose and
Figure 5.1. Reaction Profile During Acid Treatment of Corn Stover.
### TABLE 5-2  Pretreated Corn Stover Composition¹
Assay (100% Dry)

<table>
<thead>
<tr>
<th>% Carbohydrate</th>
<th>% Sugar Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>51.3 Glucan</td>
<td>57% Glucose</td>
</tr>
<tr>
<td>8.8 Xylan</td>
<td>10% Xylose</td>
</tr>
</tbody>
</table>

Σ=60.1 Carbohydrate  
Σ=67% Sugar Equivalent

15.1 Lignin
5.4 Ash (~ 7.3 as actual silicates)
6.0 BZ/EtOH Extractives
N.D. Protein

Σ= 88.5*

* Balance comprised of the unknown value for (dirt/oxides) and protein content, not determined.

---

¹ Acid treated corn stover, University of California, Davis. Fall, 1980 crop.
Chapter 5

The moisture content observed was consistent with typical values reported by Iotech (2). This large amount of moisture stems from steam condensing on the solids during the later stages of pretreatment. The amount of water soluble lignin measured is consistent with typical values reported by Iotech. Comparison of xylose and glucose conversions is postponed until section 5.4 where enzymatic hydrolysis data is discussed.

5.2. Cellulase Production with T. reesei (Rut-C-30)

5.2.1. Batch Cultures Employing Solka Floc as Carbon Source

Results from batch production of cellulase obtained by this work and by Tangnu (3) and Wiley (4) are presented in Table 5-3. Batches conducted in 1979 and in 1980 employed cultures from different slants supplied by Rutgers University. Those batches grown from the 1979 slant produced higher levels of component activities and soluble protein.

Wald (5) analyzed this data on the basis of specific activity production, measured as the ratio of a given component activity and the amount of soluble protein present in the culture, and observed that the ratio of component activity to soluble protein was reasonably constant for the data presented in Table 5-3. From this, it may be observed that lower activities obtained with the 1980 slant were linked to reduced protein synthesis by the strain. An
<table>
<thead>
<tr>
<th>Ref</th>
<th>Date</th>
<th>FPA (U/ml)</th>
<th>C1</th>
<th>CMC</th>
<th>Cellulase</th>
<th>Soluble Protein (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3)</td>
<td>7/79</td>
<td>1.4</td>
<td>1.2</td>
<td>313</td>
<td>23</td>
<td>22</td>
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<tr>
<td>(4)</td>
<td>12/79</td>
<td>9.4</td>
<td>0.7</td>
<td>150</td>
<td>14</td>
<td>12</td>
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<tr>
<td>this work</td>
<td></td>
<td>4/80</td>
<td>5.6</td>
<td>0.2</td>
<td>103</td>
<td>10.3</td>
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<td></td>
<td>9/80</td>
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<td>0.4</td>
<td>140</td>
<td>8.6</td>
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<tr>
<td>(4)</td>
<td>11/80</td>
<td>7.0</td>
<td>0.4</td>
<td>193</td>
<td>7.9</td>
<td>14</td>
</tr>
</tbody>
</table>

All data from 10 liter batch cultures, 5% Solka Floc, 144 hours.
exception to this observation is the 11/80 run which contained a high level of soluble protein and low ratios of FPA, $C_1$ and cellobiase. Production of cellobiase in batch culture showed a consistent decrease with slant culture age for experiments carried out with both cultures.

Table 5-4 presents a summary of cellulase production results obtained by Gulf Oil Chemical Co. (6) during pilot scale batch fermentation of T. reesei Rut-C-30. High activity is again associated with high soluble protein production, although some exceptions are evident. There appears to be significant variability in the ratio of cellulase components produced from batch to batch. Reduction of cellobiase production with time is also apparent from these data.

Consistent production of high activity cellulase solutions has not been demonstrated thus far. Variability in reported data may be linked to oxygen transport limitations. Batches producing low enzyme activity were observed to produce a green pigment. During shake flask studies, it was possible to remove the green coloring from cultures by increasing the degree of agitation (7). Decrease in activity with culture age may indicate strain instability. Further work is needed to understand the metabolic behavior of Rut-C-30 and to define operating conditions which produce cellulase at consistent activities and ratios.
<table>
<thead>
<tr>
<th>Run</th>
<th>FPA</th>
<th>C₁</th>
<th>Cₓ</th>
<th>B-g</th>
<th>Soluble Protein (g/L)</th>
<th>FPA/SP</th>
<th>C₁/SP</th>
<th>Cₓ/SP</th>
<th>B-g/SP</th>
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<td>0.08</td>
<td>82</td>
<td>--</td>
<td>13.5</td>
<td>0.40</td>
<td>0.01</td>
<td>6.1</td>
<td>--</td>
</tr>
<tr>
<td>20</td>
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<td>1.0</td>
<td>273</td>
<td>--</td>
<td>23</td>
<td>0.51</td>
<td>0.04</td>
<td>11.9</td>
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<tr>
<td>21</td>
<td>10.0</td>
<td>1.4</td>
<td>196</td>
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<td>17.3</td>
<td>0.58</td>
<td>0.08</td>
<td>11.3</td>
<td>--</td>
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<tr>
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<td>8.6</td>
<td>1.0</td>
<td>199</td>
<td>21.4</td>
<td>23</td>
<td>0.37</td>
<td>0.04</td>
<td>8.6</td>
<td>0.9</td>
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<td>83</td>
<td>11.0</td>
<td>10.5</td>
<td>0.24</td>
<td>0.04</td>
<td>7.9</td>
<td>1.0</td>
</tr>
<tr>
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<td>6.5</td>
<td>0.6</td>
<td>79</td>
<td>6.5</td>
<td>18</td>
<td>0.36</td>
<td>0.03</td>
<td>4.4</td>
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<tr>
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<td>0.92</td>
<td>106</td>
<td>8.9</td>
<td>25.1</td>
<td>0.41</td>
<td>0.04</td>
<td>4.2</td>
<td>0.4</td>
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<tr>
<td>40</td>
<td>9.3</td>
<td>--</td>
<td>82</td>
<td>7.7</td>
<td>17.4</td>
<td>0.53</td>
<td>--</td>
<td>4.7</td>
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</tr>
<tr>
<td>43</td>
<td>4.2</td>
<td>0.2</td>
<td>48</td>
<td>5.0</td>
<td>11.7</td>
<td>0.36</td>
<td>0.02</td>
<td>4.1</td>
<td>0.4</td>
</tr>
<tr>
<td>44</td>
<td>7.1</td>
<td>0.33</td>
<td>61</td>
<td>6.7</td>
<td>16.4</td>
<td>0.43</td>
<td>0.02</td>
<td>3.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

1. *T. reesei* Rut-C-30, 144 hours, 5% Solka Floc.
5.2.2. Cellulase production with Steam Exploded Corn Stover as Substrate

Figure 5-2 presents time versus cellulase activity profiles for shake flask studies employing Solka Floc and steam exploded corn stover as carbon sources. Activities reported serve as relative comparison only since the cellulase produced during shake flask incubation is in a region where assay sensitivities are poor. T. reesei appeared to grow well on washed steam exploded corn stover and produced cellulase activities comparable to those produced with solka floc. Similar fermentations with unwashed steam exploded corn stover produced no measurable cellulase activity even after three generations of shake flasks. Streaked PDA plates from all shake flasks showed no signs of contamination. PDA plates streaked with cultures grown on unwashed corn stover showed a lag period prior to resuming growth.

Further work is needed to determine the proper adaptation technique to allow T. reesei to grow on unwashed steam exploded corn stover. Cellulase production is inhibited by a soluble compound found in the corn stover after steam explosion. Lignin or soluble acids are possible inhibitors found in the stover. The presence of glucose in high concentrations may also act as a repressor.

5.2.3. Batch Fermentation of T. reesei with Corn Stover

Table 5-5 presents a summary of 3 liter batch fermenta-
Figure 5.2. Production of Cellulase in Shake Flask Studies with Corn Stover.
tions conducted using various forms of pretreated corn stover as carbon source. Two-thirds of the activity produced with Solka Floc as substrate was obtained when acid-base treated corn stover was used. Water washed steam exploded corn stover produced approximately half the activity measured when Solka Floc was used. Negligible activity was produced when acid treated corn stover was employed, demonstrating that delignification is necessary to allow T. reesei to grow on waste cellulose.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Filter Paper Activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solka Floc</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Acid treated Corn Stover</td>
<td>20 g/L</td>
</tr>
<tr>
<td>Acid-Base treated Corn Stover</td>
<td>20 g/L</td>
</tr>
<tr>
<td>Steam Exploded Corn Stover, Water washed</td>
<td>20 g/L</td>
</tr>
</tbody>
</table>

1. with S.K. Tangnu

5.3. Enzymatic Hydrolysis of Acid Treated Corn Stover

5.3.1. Glucose Production

Figure 5-3 presents typical reducing sugar
Figure 5-3 Reducing Sugar Production Versus Time during enzymatic hydrolysis.

Substrate: Acid treated California Corn Stover
Enzyme: 5IU/ml Cellulase From Rut-C-30 (11/80)
concentration versus reaction time curves for batch hydrolysis of acid treated corn stover. Hydrolysis was conducted for a period of 48 hours using cellulase with 5 IU/ml activity and California corn stover at initial substrate concentrations of 5-25%. Reducing sugar concentrations in the range of 90-100 g/l are observed when the stepwise addition technique described in Chapter 3 is employed to achieve substrate concentration of 25%. Following a rapid reaction rate during the first few hours, reducing sugar production proceeds slowly at a nearly linear rate from 8 to 48 hours. Appendix B.1 presents reducing sugar concentrations versus time profiles for hydrolysis conducted with substrate concentrations of 5-25% and enzyme activities of 0.5 to 7 IU/ml. These profiles, along with information regarding component sugars produced form a basis for the economic evaluation described in Chapters 6, 7 and 8.

Figure 5-4 shows component sugar concentrations versus time profiles for hydrolysis conducted with specific enzyme activity of 47.5 IU per gram of corn stover. Production of glucose proceeds slowly after 8 hours and appears nearly complete after 24 hours. Xylose production proceeds slowly at first and is complete in 12 hours. No cellobiose is formed under the conditions given.

Figure 5-5 shows component sugar concentrations versus time profiles for hydrolysis conducted with specific enzyme activity of 7.5 IU per gram of corn stover. Production of
Figure 5.4 Hydrolysis with High Cellulase Specific Activity.
Figure 5.5 Hydrolysis with Low Cellulase Specific Activity.
glucose proceeds more gradually initially and does not slow as dramatically as the reaction presented in Figure 5-4. After 24 hours, hydrolysis does not appear complete. Xylose production again stops after 12 hours. Cellobiose in solution undergoes a maximum early in the reaction and decreases to a fixed value after sixteen hours. The behavior of this reaction suggests a limitation on reaction rate by both overall activity and cellobiase activity. Rates may also be slowed by presence of glucose and cellobiose in high concentration. Appendix B.2 presents a summary of reaction profile studies.

Figure 5-6 presents a summary of glucose conversions at 48 hours as a function of substrate concentration and enzyme activity. Conversion decreases with increasing substrate concentration and increases with higher enzyme activity. The maximum conversion observed was 53%.

Figure 5-7 shows glucose conversion versus substrate concentration at different enzyme activities and reaction times. Reaction conditions with high ratio of enzyme to substrate lead to the same maximum conversion at a faster rate than reaction conditions with low ratio of enzyme to substrate. This suggests that enzyme activity has a major effect on reaction rate, not on the maximum conversion obtainable with a given substrate.

Figure 5-8 shows glucose conversion versus specific cellulase activity per gram of corn stover at 48 hours.
Figure 5.6  Glucose Conversion vs Substrate Concentration and Enzyme. Acid Treated Corn Stover (California) with Cellulase from Rut C-30 (11/80).
Figure 5.7. Effect of Reaction Time on Hydrolysis Conversion.
Figure 5.8  Glucose Conversion Versus Specific Activity.
Acid Treated Corn Stover (California), Cellulase from Rut-C30 (11/80)
Addition of cellulase beyond 30 IU per gram of substrate produces no significant increase in glucose conversion.

5.3.2. Xylose Production

Figure 5-9 shows xylose concentration produced during enzymatic hydrolysis with different corn stover concentrations. Xylose concentration is directly proportional to substrate concentration and independent of enzyme activity utilized, indicating that xylanase was not present in limiting amounts. Xylose conversion was 40% for all experiments conducted.

5.3.3. Cellobiose Production

Figure 5-10 shows cellobiose concentration observed after 48 hours of hydrolysis versus specific cellobiase activity. Cellobiose accumulation results when specific cellobiase activity is less than 50 IU per gram of substrate. This suggests that a cellobiase to overall activity ratio of 2:1 would provide best hydrolysis results. Figure 5-11 presents glucose conversion versus specific cellulase activity for hydrolysis conducted at cellobiase to overall cellulase activity ratios of 1:1.1 and 1:1.9. Although maximum glucose conversion achieved is approximately the same, the runs with higher cellobiase activity require less overall cellulase activity to accomplish the same results.
Figure 5-9 Xylose Production During Enzymatic Hydrolysis.

Acid Treated Corn Stover (California) Cellulase from Rut-C-30 (11/80).
Figure 5-10. Cellulose Concentration versus Specific Cellulase Activity.
Figure 5.11. Glucose Production Versus Specific Cellulase Activity for Different ratios of cellobiase to cellulase.
5.3.4. Enzyme Recovery

Figure 5-12 presents enzyme remaining in solution, as percent of original activity, as a function of substrate concentration and initial activity. For a given enzyme activity, enzyme remaining in solution decreases with increasing solids concentration. This behavior is expected since cellulase adsorbs onto cellulose. For a given substrate concentration, increasing the amount of cellulase used increases the amount of activity left in solution. However, this increase diminishes with each greater increment of cellulase employed, implying that an adsorption isotherm is present. Figure 5-13 presents a plot of adsorbed units IU/g in solution IU/ml, which confirms the existence of an adsorption equilibrium isotherm.

Figure 5-14 presents a plot of component activity remaining in solution versus hydrolysis time. Overall activity declines rapidly during the first hour of hydrolysis and continues to decrease gradually during the course of the reaction. Even though hydrolysis was proceeding, release of enzymes back into solution was not observed. This behavior is consistent with observations made by Castanon (8) on adsorption of cellulases onto newsprint. As shown by previous workers (8, 9), B-glucosidase remains in solution to a much greater extent than any other component and is adsorbed to the greatest extent.
Figure 5-12. Enzyme Remaining in Solution After 24 hrs. of Hydrolysis.
Figure 5.13. Apparent Adsorption Isotherm of Bound Enzyme (I.U./g) vs. Free Enzyme (I.U./ml).
Figure 5.14. Original Activity Remaining in Solution Versus Time.
5.3.5. **Performance Comparison of Cellulase from QM9414 and Rut-C-30**

Figure 5-15 compares hydrolysis of corn stover using cellulase from *T. reesei* strains QM9414 and Rut-C-30. Hydrolysis with strain Rut-C-30 displays a faster initial reaction rate. After 10 hours, reaction rates are roughly equivalent. Final conversion is 15% higher for the case employing cellulase from strain Rut-C-30. Further comparison between the two strains, accounting for all manufacturing variables, is covered in Chapter 8.

5.4. **Hydrolysis of Steam Exploded Corn Stover**

5.4.1. **Glucose Production**

Figure 5-16 shows a reaction profile for hydrolysis conducted with steam exploded corn stover at low specific enzyme activity. Although conversion to glucose was 62% in 48 hours, glucose production proceeds at a slow rate throughout the course of the reaction. This may be explained in part by the high cellobiose and glucose concentration present during the early stages of reaction and by the use of relatively dilute enzyme. Steam exploded corn stover used was freeze dried to remove excessive moisture. Workers (10) hydrolyzing steam exploded wood have observed that freeze drying the substrate slowed the rate of hydrolysis.

Figure 5-17 shows a similar experiment conducted at
Figure 5.15. Comparison Hydrolysis with Cellulase for Different Strains of T. reesei.

With Cellulase from:
- ○ Rut-C-30
- △ QM 9414
Figure 5.16 Hydrolysis of Steam Exploded Corn Stover at Low Specific Cellulase Activity.
Figure 5.17 Hydrolysis of Steam Exploded Corn Stover at High Specific Cellulase Activity of 63 IU/g.
specific cellulase activity of 63 IU/g and substrate concentration of 10%. With sufficient enzyme activity available and lower initial glucose and cellobiose concentration, the reaction profile appears similar to those obtained with acid pretreated corn stover. Appendix B.3 provides a summary of hydrolysis conducted with steam exploded corn stover.

Figure 5-18 shows glucose conversion versus specific cellulase activity for hydrolysis conducted with steam exploded corn stover for 48 hours. Maximum conversion is 82%, requiring 30 IU/g specific cellulase activity. Although conversions are greatly increased by using steam exploded corn stover instead of acid treated corn stover, the amount of enzyme required to achieve maximum conversion in a given amount of time is roughly the same.

5.4.2. Enzyme Adsorption

Figure 5-19 compares the enzyme adsorption isotherm for steam exploded corn stover and cellulase from Rut-C-30 with a similar isotherm obtained with acid treated corn stover. The large amount of enzyme adsorbed onto cellulose in steam exploded corn stover, suggests that its surface area was greatly increased during pretreatment.

5.4.3. Xylose Production

Sixty to seventy percent of available pentoses were available after hydrolysis and pretreatment. This is roughly equivalent to the pentose conversion during dilute
Figure 5.18. Glucose Conversion at 48 hours Versus Specific Cellulase Activity. Steam Exploded Corn Stover Cellulase from Rut-C-30 (11/80).
Figure 5.19 Steam Exploded Corn Stover Cellulase Adsorption Isotherm.
acid pretreatment, and within the range of conversions reported by Iotech.

5.5. Miscellaneous Process Development Studies

5.5.1. Hydrolysis Solids Washing

Figure 5-20 shows the amount of total sugar recovered from hydrolysis after washing the spent solids with water. Typically 2-3 washes equal to the water retained in the filter cake were necessary to recover all sugar produced. Filter cakes from hydrolysis typically contained only 30% solids. This behavior suggests that unless a different method of filtration is employed for dewatering solids, benefits of high glucose concentration obtained from operating at high solids content will be diminished by dilution from washing.

5.5.2. Relative Filtration Rates

Figure 5-21 shows relative filtration rates obtained with solids from hydrolysis employing 5-25% solids concentration. Under equivalent surface area, cake thickness, pressure, and $\alpha'$ (specific cake resistance constant), the Carman equation predicts filtration rates will vary inversely proportional to the reciprocal solids concentration (figure 5-21). Observed rates are in qualitative agreement with predicted results with deviation probably due to $\alpha'$, which is a function of particle size. Hydrolysate slurries may have different particle size distributions due
Figure 5.20. % Sugar Recovered Versus Wash Water Used.
Relative Filtration Rate (%)

- Measured rate
- Predicted by:

\[
\frac{dV}{Ad\theta} = \frac{A p^{1-s}}{\mu a'(w/A)} \text{ for constant } a'
\]

Substrate Concentration (wt.%)

Figure 5.21. Relative Filtration Rate for Corn Stover Slurries Versus Substrate Concentration.
to varying degrees of conversion or different extents of attrition caused by particle collision in different slurry concentrations.

5.6. **Kinetic Model For Acid Treated Corn Stover Hydrolysis**

A kinetic model developed by Wald, et al., (5) for enzymatic hydrolysis of acid pretreated agricultural residues was evaluated as a possible model to describe corn stover hydrolysis. The model incorporates concepts of heterogeneous catalysis for the cellulose to cellobiose reactions and Michaelis Menten kinetics for the homogeneous cellobiose to glucose reactions. The principal assumptions used in formulating the model are as follows:

1. Cellulose is assumed to be composed of two regions, amorphous and crystalline, exhibiting different properties and reaction rates.

2. The cellulase enzyme system is adequately described by the filter paper and cellobiase activity measurement.

3. Adsorption of cellulase onto cellulose is fast and equilibrium exists at all times.

4. The reaction network shown in Figure 5-22 applies.

5. Degradation of cellulose is modelled as a shrinking shell containing an amorphous shell and a crystalline core. Particle size is assumed to follow a distribution function, measured experimentally.
Figure 5-22 Cellulose Hydrolysis Reaction Network and Reaction Scheme (5)

A. Network:

\[
\text{Amorphous Cellulose} \xrightarrow{f(\text{FPA})} \text{Cellobiose} \xrightarrow{f(\text{cellobiase})} \text{Glucose}
\]

B. Reaction Scheme:

\[
\begin{align*}
E_C + S_A & \xrightleftharpoons[\kappa_1 \kappa^{-1}]{\kappa_2 \kappa^{-2}} \eta A \xrightarrow{\kappa_4 \kappa^{-4}} E_C + G_2 \\
E_C + S_B & \xrightleftharpoons[\kappa_3 \kappa^{-3}]{\kappa_4 \kappa^{-4}} \eta C \xrightarrow{\kappa_4 \kappa^{-4}} E_C + G_2 \\
E_A + G_2 & \xrightleftharpoons[\kappa_5 \kappa^{-5}]{\kappa_4 \kappa^{-4}} \eta AG_2 \\
E_B + G_2 & \xrightleftharpoons[\kappa_6 \kappa^{-6}]{\kappa_7 \kappa^{-7}} E_{BG_2} \xrightarrow{\kappa_7 \kappa^{-7}} E_B + 2G \\
E_B + G & \xrightleftharpoons[\kappa_8 \kappa^{-8}]{\kappa_8 \kappa^{-8}} E_{BG} \\
E_{BG_2} + G & \xrightleftharpoons[\kappa_9 \kappa^{-9}]{\kappa_9 \kappa^{-9}} E_{BG_2G}
\end{align*}
\]

\[G = \text{Glucose} \quad S_A = \text{Amorphous Cellulose} \quad S_C = \text{Crystalline Cellulose} \quad E_C = \text{Cellulase} \quad E_B = \text{Cellobiase} \quad G_2 = \text{Cellobiose} \]
6. Shear and thermal denaturation of cellulase is neglected.

7. Cellulase adsorption is a function of available cellulose surface area.

8. Glucose formation by exo-B-1,4-glucan glucanohydrolase directly from cellulose is neglected.

9. Cellobiose acts as an inhibitor by a non-competitive mechanism.

10. The reaction scheme presented in Figure 5-22 applies.

Figure 5-23 shows a comparison between predicted and observed hydrolysis reaction results. At high cellulase activity per gram of corn stover, the model predicts cellobiose and glucose reaction profiles closely. Glucose produced at 24 hrs. is underpredicted by approximately 6%. At low cellulase activity per gram of corn stover, qualitative description of glucose concentration versus time is obtained; however, the model overpredicts glucose production by 10%. Cellobiose concentrations after 8 hours reaction time are consistently underpredicted, possibly due to the assumption that cellobiase is not denatured during the course of the reaction. During this evaluation, inhibition and initial rate constants were not optimized; values numerically obtained by Wald (5) for rice straw hydrolysis were employed. A better fit may be obtained if these constants are numerically optimized and if initial reaction rates are
Figure 5-23. Kinetic and Model Prediction of Corn Stover Hydrolysis Results.
measured for corn stover hydrolysis.
References: Chapter 5


7. Hendy, N., Department of Chemical Engineering, University of California, Berkeley. Personal communication.


10. Sciamanna, A.F., University of California, Berkeley, Department of Chemical Engineering. Personal communication.
Chapter 6

6. Process Design and Evaluation

This chapter presents a summary of operating conditions, equipment specifications and other assumptions made to enable preliminary design of an enzymatic hydrolysis processing scheme. The purpose of this design is to specify processing variables with sufficient accuracy to enable comparative evaluation of the cost of producing glucose under various conditions. The design presented should be regarded as tentative and its use for definitive cost estimation is not recommended.

6.1. Basic Design and Alternatives

6.1.1. General Basis

The following is a summary of assumptions and design conventions applying to all processing schemes and conditions evaluated:

1. Daily production is 214 tons per day of glucose to enable coupling with a 10^7 gallon per year ethanol production facility.

2. Product concentration is 10%, the glucose concentration required in conventional ethanol fermentation processes.

3. Solids are burned for power generation.
4. No design or evaluation of byproduct processing is included.

5. No design or evaluation of waste treatment is included.

6. No storage for corn stover is provided.

6.1.2. Base case process

A block flow diagram of the processing scheme used as a basis is presented in Figure 6-1. The process is similar to a design by Yang (1) presented in Chapter 3. Product stream recycle was eliminated from the process as Lindsey (2) showed that recycle did not reduce processing cost. Enzyme production is conducted batchwise since at the time of this evaluation, data on continuous growth of T. reesei Rut-C-30 is scarce.

6.1.3. Processing Alternatives

As alternatives to the design presented in section 6.1.2 the following processing methods were evaluated:

1. Ultrafiltration was employed as an alternative to equilibrium contacting for enzyme recovery.

2. Continuous schemes, with and without cell recycle, for T. reesei Rut-C-30 fermentation were designed as alternatives to batch fermentation.

3. Steam explosion was considered as a method of pretreatment and hydrolysis results employing steam exploded
Figure 6-1: ENZYMATIC HYDROLYSIS PROCESS: BLOCK FLOW DIAGRAM

- **CORN STOVER** → **MILLING** → **ACID TREATMENT** → **LIQUID TO XYLOSE UTILIZATION**
- **GLUCOSE 214 TON/DAY** → **ENZYME RECOVERY**
- **SOLIDS** → **HYDROLYSIS** → **SOLIDS TO FURNACE**
- **NUTRIENTS AND CELLULOSE** → **BATCH ENZYME PRODUCTION**
- **H₂SO₄**

XBL808-5701A
Chapter 6

materials were considered.

6.2. Equipment Sizing Criteria

The following is a summary of methods used in sizing process equipment. All equipment was sized using approximate methods which do not consider mechanical detail. This section covers the design of equipment by type. Details of how each type of equipment is specified in each processing step are provided in section 6.3 and in Appendix C where a listing of the design algorithm and documentation is provided. All equipment is sized as large as possible without exceeding the maximum size at which the piece of equipment would be commercially available. When maximum equipment sizes are reached, equal size multiples are specified.

6.2.1. Size Reduction

Pulverizers were sized based on the mass flowrate of corn stover passing through the process each hour:

\[ \text{MILLNO} = \frac{\text{MILLRATE}}{\text{MILLMAX}} \]

The number of mills is equal to the mass flowrate lb/hr divided by the maximum mill capacity lb/hr. The maximum mill rate is 20,000 lb/hr; larger pulverizers may be manufactured but data are not available.

Motor size is estimated in horse power at 0.01 horse power per mass flowrate of capacity (3):
MILLHP = MILLRT x 0.01

Milled product is recovered in a cyclone separator sized based on the volumetric flowrate of material through the mill. A density of 25 lb/ft$^3$ of corn stover is assumed, product stream is assumed to be 25% by volume corn stover.

6.2.2. Mixing

Agitators were assumed to be electrically driven pitched blade turbines with two impellers and standard geometry. Agitators were sized by a procedure presented by Gates, et al. (4) using solid suspension as a criterion. Assuming equal height and diameter, the diameter of the vessel housing the agitator is computed:

$$V = \frac{\pi}{4} \left( Z \right)^2 \left( \frac{\pi}{4} \right) T^3$$

$$T = \left( \frac{4V}{\pi} \right)^{1/3}$$

where $Z$ is the vessel height (ft), $T$ is the vessel diameter (ft), and $V$ is the vessel volume, ft$^3$. Assuming an impeller to diameter ratio of 0.3, and a scale of agitation of 7 (see Gates, et al.), the value of the dimensionless parameter $\phi$ is found using the graph in Figure 6-3.

The shaft speed $N$ (RPM) is found as follows:

$$\phi = \frac{N^{3.75}D^{2.81}}{ud}$$

$$N = \left( \frac{\phi ud}{D^{2.81}} \right)^{1/3.75}$$
Particle size determines terminal settling velocity of solids in low-viscosity liquid.

Agitators capable of scale level 6 will provide concentration uniformity of solids to 95% of the fluid-batch height and be suitable for slurry drawoff up to 80% of fluid-batch height.

Figure 6.3. Agitator Sizing Specifications.
where $D$ is the impeller diameter (ft), and $ud$ is the solid settling velocity (ft/min) estimated from the solids particle size using the graph in Figure 6-3 (lower).

The agitator horse power is estimated as follows:

$$D = \left\{ \frac{394 \frac{Hp}{\eta N^3(Sg)_{SL}}}{0.2} \right\}$$

$$Hp = \frac{D^5 \eta N^3(Sg)_{SL}}{394}$$

where $Hp$ is the agitator horse power, $D$ is the impeller diameter (ft), $\eta$ = the number of impellers, $N$ is the RPM, $Sg$ is the specific gravity of the slurry (SL).

For mixing nutrients and soluble raw materials agitators were not sized. The size of the mixing tank required was determined and cost correlations for mixing tanks including agitation were used (5).

### 6.2.3. Heat Transfer

Heat exchangers are assumed to be floating head, shell and tube heat exchangers with 3/4 inch O.D. X 1 inch square pitch and 16 foot bundle length rated to 150 psi (6). Heat transfer areas were calculated using the equation:

$$Q = UA \Delta tm$$

$$A = \frac{Q}{U \Delta tm}$$

where $Q$ is the heat load (BTU/hr), $\Delta tm$ is the log mean temperature difference and $U$ is the overall heat transfer coef-
efficient (BTU/hr ft$^2$ °F). Typical heat transfer coefficients were obtained from Perry, et al. (7). Table 6-1 presents a summary of these coefficients.

Evaporators are specified as short tube vertical, rated for a maximum pressure of 30 psi. Using typical heat transfer coefficient data for water in short tube evaporators from Perry, et al. (8), an average heat transfer coefficient of 505 BTU/ft$^2$ °F is estimated for a 5 effect evaporator operating with a 20°F temperature difference in each stage. This temperature difference, average $U$, and useful heat transfer area of 80%, leads to an evaporation rate per square foot of transfer area of 8 lb/ft$^2$ hr, a value reported as typical for sugar concentration (9). In sizing evaporators, boiling point rise due to glucose is neglected. This is reasonable as long as sugar is concentrated to 10% by weight or less.

For the purpose of estimating the cost of evaporation the total area required is calculated:

$$\text{AREAT} = \frac{\text{WEVAP} \times 1}{8\text{lb/hr-ft}^2} \times \frac{1}{0.8}$$

where AREAT is the total evaporator area (ft$^2$), WEVAP is the water evaporated each hour (lb/hr). The cost of evaporation is estimated by specifying 5 evaporators of area equal to one fifth of the total. This method provides an estimate of overall evaporation required. Actual design of an evaporation system would not normally specify equal areas and
<table>
<thead>
<tr>
<th>Shell Side</th>
<th>Tube Side</th>
<th>Design U</th>
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<td>water</td>
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</tr>
<tr>
<td>jacket water</td>
<td>water</td>
<td>230-300</td>
</tr>
<tr>
<td>steam</td>
<td>water</td>
<td>400-1000</td>
</tr>
<tr>
<td>demineralized water</td>
<td>water</td>
<td>300-500</td>
</tr>
</tbody>
</table>

After Perry, R.H., et al. (7)
Steam consumption is estimated as the ideal requirement:

\[
\text{Steam} = \frac{\text{water evaporated}}{\text{effects}}
\]

where both steam and water evaporated are given in lb/hr and effects is set to 5. Data available for sugar concentration show this estimate to be within 15% of observed heat loads.

Heat transfer surfaces related to maintaining a fixed temperature within a reactor were not calculated. It is assumed that reactors equipped with coils or jackets have sufficient heat transfer surface.

6.2.4. Vessels

Reactors vessels were sized in gallons and were assumed to have a working volume of 80% of nameplate capacity. Continuous reactors are sized as:

\[
\text{VOLUME} = \frac{\text{VOLUME RATE} \times \text{RESIDENCE TIME}}{0.8}
\]

where VOLUME is in gallons, VOLUME RATE is in lb/hr and RESIDENCE TIME is in hours. To calculate volume flowrates from mass flowrates, density equal to water is assumed. Batch tanks are sized based on a cycle time which includes cleaning and discharge rather than residence time.

Storage tanks are sized to provide two weeks of storage for raw materials. Tanks are sized as follows:
VOLUME = MASS RATE * DAYS * 1/p

where VOLUME is in gallons, MASS RATE in lb/day, and p in lb/gallon. The bulk density of inorganic salts was assumed to be 125 lb/ft$^3$, sulfuric acid density 111 lb/ft$^3$, and delignified cellulose density 50 lb/ft$^3$ (10).

6.2.5. Materials Transfer

Solids are fed to process equipment using screw conveyors measuring 12 inches in diameter by 20 feet in length. This provides an equivalent conveying capacity of 50 tons/hr of grain (11) per conveyor and is sufficient for the range of flowrates considered.

Pumps are specified as all purpose, single or dual stage and sized on the basis of gallon per minute flowrate. A pumping head of 50 feet of water is employed for estimating the horsepower of the pumps. Horse power requirements are estimated using data from Peters and Timmerhaus (12). All pumps are specified in duplicate.

6.2.6. Separation

Rotary vacuum filters are employed for continuous filtration of solids. Filter area is specified using the following equation:

\[ \text{AREA} = \frac{\text{FLOWRATE}}{\text{FILTER RATE}} \]
where FILTER RATE is the filtration rate in gallons/ft$^2$ hr, FLOWRATE is the liquid stream volumetric flowrate gallons/hr, and AREA is in square feet. Filtration rate for 5% corn stover suspensions were estimated by Yang (1) at 166 gal/ft$^2$hr. Flowrates for other corn stover concentrations were estimated with the data presented in Chapter 5. Fermentation broths were filtered at 200 gallons/ft$^2$hr (1).

Centrifuges were assumed to be solid bowl continuous. An estimate of the horse power requirement was obtained using a relation obtained from typical filtration power requirements:

\[
chp = 0.080869 \times (GPM)^{1.17658}
\]

where chp is the horse power, GPM is the volumetric flowrate of centrate (14).

Ultra Filtration membranes are sized based on pilot plant filtration data for ultrafiltration of cottage cheese whey obtained by Romicon, Inc. (17):

\[
F = -4.889 \ln C + 29.74
\]

where F is the membrane flux in gallons per ft$^2$ per day, C is the percent protein concentration.

Average flux is calculated as (18):

\[
FLUXAVE = FLUX2 + (FLUX1 - FLUX2) 0.667
\]
where FLUX2 is the flux at the concentrated end and FLUX1 is the flux at the dilute end of the membrane. Continuous flux using three stages was measured by Romicon as (18):

\[ \text{FCONT} = 0.87 \times \text{FAVE} \]

where FCONT is the continuous flux and FAVE is the average flux. Power consumption for the ultrafiltration system is assumed to be 0.02 Kw per ft^2 of membrane (19).

6.2.7. Miscellaneous

Air to fermentors is supplied by two stage air cooled reciprocating compressors rated for 100 psi discharge (15). Compressors are sized based on rated capacity in ft^3/minute. Size requirements are estimated from the V.V.M. (volume per volume per minute) requirements established for this fermentation by Tangnu (16).

The solids sterilizer is assumed to be a 9 foot by 160 foot modified rotary kiln specified by Yang (20) for previous process evaluation. This size sterilizer was maintained constant for all evaluations performed.

6.3. Process Components, Description and Assumptions

6.3.1. Milling

Incoming corn stover is milled in pulverizers to provide 2 mm. particle size material to the process. Pulverizers typically perform at this particle size specification or
smaller particle size, depending on the hardness of the material milled (21). Preliminary milling of corn stover is assumed to take place at its point of collection and stover fed to the mill is assumed to range between 1 and 0.25 inches. Any costs associated with this milling are assumed to be part of the substrate cost. Savings may result from specifying crushers instead of pulverizers as size reduction equipment. However, product particle sizes from two stage crushers are usually larger than those required in this application (22).

6.3.2. Pretreatment

Dilute Acid:

Figure 6-2 presents a flow diagram of the dilute acid pretreatment process design. Milled corn stover is fed to a continuous stirred tank reactors with a screw conveyor. Flowrate through the vessels maintains a total residence time of 5.5 hours. The reactor is assumed to be constructed of 304 s.s., to be jacketed and agitated and to have a maximum size of 200,000 gallons. Vapors generated during operation are recycled using a reflux condenser. Agitation is designed to suspend solids with a Chemineer (4) scale of agitation of 7.

The solid suspension leaving the reactor vessels is filtered in a rotary vacuum filter and washed with water at 45°C. Fifty percent (50%) of the liquid stream is recycled
Figure 6.2. Dilute Acid Pretreatment Flow Diagram.
to the reaction vessels. The remainder is passed through a heat exchanger to preheat the processes makeup acid stream. Sulfuric acid is mixed with water, preheated with the product stream and brought up to 100°C prior to entering the reaction vessel.

The following assumptions are included in the design:

1. Equipment is constructed of 304 s.s.
2. No loss of conversion is assumed when acid is recycled.
3. For the purpose of sizing reactors, residence time used during batch operation is used. Reactors are operated at 80% of capacity.
4. Displacement washing of solids takes place during filtration.
5. Heat of mixing of the sulfuric acid and water is small and is neglected.
6. No costs or credits are associated with the xylose stream produced.
7. Solids composition upon pretreatment is as discussed in Chapter 3.

Steam Explosion:

Preliminary design of a steam explosion pretreatment facility was not conducted as operating conditions were not
known with sufficient certainty to enable estimating steam consumption and equipment sizes. For the purpose of evaluation, a fixed pretreatment cost per ton of corn stover was employed. This method places a limitation on evaluation as overall steam requirements and thus the cost of generating steam cannot be estimated. Using a fixed pretreatment cost also prevents evaluation of scale-cost relationships.

6.3.3. Hydrolysis

Figure 6-3 presents a flow diagram of the hydrolysis reaction process design. Incoming solids are fed to one of three initial reaction feed stages in series in a manner analogous to the sequential solids addition technique described in Chapter 4. Following the feed section, the solid suspension enters a series of 3 reaction stages. Temperature is assumed to be 45°C, pH is 5.0. Residence time requirements for stirred tanks in series were determined graphically by plotting reaction rate versus cellulose concentration and obtaining the number of stages required to reach a desired batch conversion. Figure 6-4 presents an example of this technique. Reaction rates after 8 hours are nearly constant and no advantage is obtained when more reaction stages are included.

Reactors are constructed of carbon steel rated for atmospheric use, are agitated, unheated and have a maximum size of 100,000 gallons. Stages requiring more than 100,000 gallons of capacity use tanks of equal size in parallel.
Figure 6.3. Hydrolysis Flow Diagram.
\[ r_i = \text{Arctan} \left( \frac{1}{T_R_i} \right) \]

\( r \) is the reaction rate = g/l hr.
\( c \) is the corn stover concentration: g/l
\( T_R \) is the residence time, hr.

Figure 6.4 Graphical Construction for CSTR in Series\(^1\).

\(^1\)After Butt (22)
Eighty percent (80%) of rated capacity is used as working volume.

Agitators are sized using a Chemineer (4) procedure for agitating solid suspensions assuming a scale of agitation of 7. Early feed stages where viscosity is high will behave as a viscous fluid rather than a solids suspension. This is neglected.

Suspensions leaving the hydrolysis vessel are filtered and washed in rotary drum filters. Solids are sent to a furnace for steam and electricity generation. The product stream is sent to the enzyme recovery stage.

The following processing assumptions are inherent in the design:

1. Sugars entering with solids do not inhibit initial reaction rates.

2. Recovered enzymes are not denatured to any extent. Enzyme components are not present in limiting amounts as a result of recovery.

3. Ninety-nine percent (99%) of sugars in the product stream are recovered.

4. No growth inhibitors or buffers are added to the reactors, and hydrolysis remains uncontaminated.
5. Channeling, bypassing or other short circuiting of flows is ignored in sizing reactors.

6.3.4. Enzyme Recovery

Equilibrium Contacting:

Figure 6-5 presents a schematic of an equilibrium contacting enzyme recovery process. Solids from pretreatment and hydrolysis product stream are countercurrently contacted in two stages consisting of a mixing tank and a rotary vacuum filter. Each stage provides 1/2 hour of residence time. Tanks are carbon steel mixing tanks rated for atmospheric pressure. Agitators are sized based on 10% solids suspensions. All equipment is of carbon steel construction.

The following processing assumptions apply:

1. No reaction takes place during equilibrium contacting.

2. Ninety-eight percent (98%) of enzymes are assumed recovered (33) provided an adsorption isotherm limit is not reached. If enzyme is present in excess of the maximum capable of being adsorbed by the solids, the excess is lost.

3. If solids suspension is greater than 10%, the solid is divided into two streams and enzyme stream is contacted with each solid stream in series.
Figure 6.5. Equilibrium Contacting Enzyme Recovery Step.
4. Water leaving the process with solids contains an equilibrium amount of sugars.

Ultrafiltration:

Preliminary evaluation of ultrafiltration as a means of recovering protein was conducted using pilot plant flux versus concentration data for ultrafiltration of cottage cheese whey obtained by Romicon, Inc. (17). (Figure 6-6) Three ultrafiltration stages are assumed to achieve 87% of the average batch ultrafiltration flux (18). Cheese whey has a molecular weight of 30,000, therefore the XM50 membrane used in cottage cheese protein ultrafiltration would retain over 85% of all protein.

The following processing assumptions apply:

1. No protein denaturation as a result of operating the ultrafiltration system occurs.

2. Ninety percent (90%) of all activity is recycled to the hydrolysis reactor.

3. Recycled enzyme is not deficient in any component as a result of enzyme recovery.

4. No product inhibition occurs as a result of recycling part of the glucose stream.

5. Membranes are on stream 66% of the time.
Figure 6-6. Ultrafiltration of Cottage Cheese Whey Flux Vs. Protein Concentration. After Breslau, et al. (18).
6.3.5. Enzyme Production

Batch:

Figure 6-7 presents a schematic diagram of a batch cellulase production process. The medium described in Chapter 3 is mixed and sterilized continuously prior to entering a batch fermentor. Solids are assumed sterilized in a rotary kiln, not designed. Inoculum is provided from a series of fermentors each having a working volume equal to 10% of the working volume of the next largest. The largest inoculum fermentor has 10% of the volume required for a fermentor.

Fermentors are assumed constructed of 304 s.s., rated for 2.5 atmospheres pressure to enable steam sterilization, have a maximum volume of 100,000 gallons, have coils, and are agitated. Fermentation cycle time is 156 hours, 144 hours for fermentation and 12 hours for charging, discharging and sterilization. Air is fed at a rate of 0.2 vvm. Agitation is provided to suspend the mycelia and 5% cellulose. Fermentation temperature is maintained at 25°C, pH is held constant at 5 pH units.

Fermentation broths are filtered in a rotary vacuum filter. The mycelia is burned and the cellulase broth is stored in a surge vessel providing cellulase continuously to the process. Filtration rates used are those estimated by Yang (1).

The following processing assumptions apply.
Figure 6-7. Batch Cellulase Production, Flow Diagram.
1. Cellulase is produced at an activity of 12 IU/ml consistently.

2. Unspecified inexpensive delignified cellulose and nitrogen sources are substituted for Solka Floc and protease peptone at the same ratios as were used in the laboratory work. Raw material costs are shown in Table 6-8.

3. No oxygen transport limitation is present in the fermentor.

Continuous Fermentation:

Process design for continuous fermentation is similar to the batch flowsheet shown in Figure 6-7 except support equipment such as feed sterilization and harvesting equipment may operate continuously and is smaller. Fermentation takes place in two stages having a dilution rate of 0.02 and 0.04 hr\(^{-1}\) respectively and produces cellulase of activity 3.5 IU/ml (16). Other fermentation conditions are similar to the batch process and medium used is that reported for continuous cellulase production in Chapter 3.

Continuous Fermentation with Cell Recycle:

Continuous cellulase production using recycled mycelia of \textit{T. reesei} Rut-C-30 has not been investigated. A preliminary evaluation of potential savings resulting when processing with cell recycle was conducted by analogy using
Chapter 6

Data on cell recycle with *Trichoderma reesei* QM9414 (24). Table 6-2 presents a summary of this estimate.

This processing scheme is similar to the continuous process described earlier, except a centrifuge is added to separate cell mass and cellulose from the fermentor broth.

The following assumptions apply to conducting this design:

1. Sixty-six percent (66%) of mycelia leaving the enzyme production reactors are recycled.
2. Recycled cells are viable and have a productivity of 70% of the productivity of non-recycled cells.
3. Cell recycle produces cellulase at 5.5 IU/ml with the same component makeup as non-recycle schemes.
4. Cellulose is recycled along with mycelia resulting in a 30% reduction of substrate cost.

6.3.6. Evaporation

The product stream is concentrated to provide a 10% glucose solution using a 5 effect evaporator. The evaporators are assumed to be short tube vertical constructed of stainless steel, the type used in the sugar industry for concentrating molasses (9). For the purpose of preliminary evaluation, the following assumptions apply:
TABLE 6-2  Estimate of Cellulase Productivity Using Cell Recycle

From Yang, et al. (24) QM9414 Data:

<table>
<thead>
<tr>
<th>Dilution Rate (hr(^{-1}))</th>
<th>S</th>
<th>Productivity X</th>
<th>Specific Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell recycle:</td>
<td>0.02</td>
<td>7.5</td>
<td>0.142</td>
</tr>
<tr>
<td>recycle fraction:</td>
<td>1- (\varphi = \frac{2.3}{6.9}), (\varphi = 0.67)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**productivity of recycled cells:**

\[
Y_F X_F + Y_R X_R = \overline{YX}
\]

\[
0.084 \times 2.3 + Y_R \times 4.6 = 0.043 \times 6.9
\]

\[
Y_R = 0.0225 \text{ or 73% of original}
\]

From Tangnu, et al. (16) Rut-C-30 Data:

Productivity: 0.0144 IU/mg hr, \(X=5\) g/L

Dilution Rate: 0.02 hr\(^{-1}\)

IU/ml with cell recycle: (0.0144 IU/mg hr) *

\[
[5 \text{ g/L} + 0.73] \times 10 \text{ g/L}] \times \frac{1}{(0.02 \text{ hr}^{-1})}
\]

\[
= 5.3 \text{ IU/ml}
\]
1. Boiling point elevation due to sugars is neglected.

2. Steam requirement is ideally calculated by dividing the total steam load by the number of effects.

3. For the range of operation, 8 pounds of water are evaporated per hour per square foot of surface area.

6.3.7. Storage

Storage is provided to maintain a two week inventory of fermentation nutrients and sulfuric acid. Storage vessels are assumed constructed of carbon steel. No storage capability is provided for corn stover.

6.4. Process Evaluation

6.4.1. Capital Cost

Capital Equipment costs for each processing step are estimated using equipment cost data presented in Table 6-3. Equipment cost formulas take the general form:

\[ \text{COST} = \text{MSI} \times A \times (\text{SIZE} \times B)^C \]

where A, B, C are constants and MSI is the Marshall and Swift Index. This evaluation employed an MSI Index of 707 corresponding to March of 1981.

Direct fixed capital is estimated using multipliers outlined by Peters and Timmerhaus (25). A summary of multipliers used for each processing section are listed in Table
Table 6-3 Equipment Cost Summary

cost equation  Cost = MSI * A * (Size * B) ** C

<table>
<thead>
<tr>
<th>Item</th>
<th>Size</th>
<th>Maximum Size</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pulverizer</td>
<td>lb/hr</td>
<td>20,000</td>
<td>0.72</td>
<td>1.</td>
<td>0.4102</td>
<td>(3)</td>
</tr>
<tr>
<td>cyclone</td>
<td>ft³/hr</td>
<td>100,000</td>
<td>0.05</td>
<td>1.</td>
<td>0.5128</td>
<td>(26)</td>
</tr>
<tr>
<td>screw conveyor</td>
<td>--</td>
<td>--</td>
<td>11.42*MSI</td>
<td>--</td>
<td>--</td>
<td>(11)</td>
</tr>
<tr>
<td>Agitator s.s.</td>
<td>HP</td>
<td>400</td>
<td>364.75</td>
<td>0.00995</td>
<td>0.75</td>
<td>(27)</td>
</tr>
<tr>
<td>Agitator c.s</td>
<td>HP</td>
<td>400</td>
<td>215.20</td>
<td>0.00995</td>
<td>0.75</td>
<td>(27)</td>
</tr>
<tr>
<td>Mixing tank with agitator</td>
<td>gal</td>
<td>200,000</td>
<td>0.8960</td>
<td>1.</td>
<td>0.5314</td>
<td>(28)</td>
</tr>
<tr>
<td>Storage tank</td>
<td>gal</td>
<td>200,000</td>
<td>0.1807</td>
<td>1.</td>
<td>0.505</td>
<td>(28)</td>
</tr>
<tr>
<td>30¢ s.s. atmospheric tank</td>
<td>gal</td>
<td>200,000</td>
<td>236.22</td>
<td>50,000</td>
<td>0.6</td>
<td>(28)</td>
</tr>
</tbody>
</table>
Table 6-3 Equipment Cost Summary

cost equation \( \text{Cost} = \text{MSI} \times A \times (\text{Size} \times B)^c \)

<table>
<thead>
<tr>
<th>Item</th>
<th>Size</th>
<th>Maximum size</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>304 s.s. fermentor</td>
<td>gal</td>
<td>100,000</td>
<td>314.96</td>
<td>50,000</td>
<td>0.6</td>
<td>(27)</td>
</tr>
<tr>
<td>Carbon steel atmospheric tank</td>
<td>gal</td>
<td>200,000</td>
<td>0.1295</td>
<td>1.0</td>
<td>0.5664</td>
<td></td>
</tr>
<tr>
<td>Shell &amp; tube exchangers carbon steel</td>
<td>ft²</td>
<td>10,000</td>
<td>0.4991</td>
<td>1.0</td>
<td>0.5754</td>
<td>(6)</td>
</tr>
<tr>
<td>Shell &amp; tube exchanger s.s. tubes</td>
<td>ft²</td>
<td>10,000</td>
<td>0.7986</td>
<td>1.0</td>
<td>0.5740</td>
<td>(6)</td>
</tr>
<tr>
<td>Short tube vertical evaporator</td>
<td>ft²</td>
<td>10,000</td>
<td>6.24</td>
<td>1.0</td>
<td>0.520</td>
<td>(8)</td>
</tr>
<tr>
<td>Centrifugal compressor</td>
<td>ft³/min</td>
<td>10,000</td>
<td>0.5166</td>
<td>1.0</td>
<td>0.669</td>
<td>(31)</td>
</tr>
</tbody>
</table>
Table 6-3 Equipment Cost Summary

cost equation  Cost = MSI * A * (Size * B) ** C

<table>
<thead>
<tr>
<th>Item</th>
<th>Size</th>
<th>Maximum Size</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid bowl centrifuge</td>
<td>HP</td>
<td>&lt; 75</td>
<td>6.92</td>
<td>1</td>
<td>0.7125</td>
<td>(14)</td>
</tr>
<tr>
<td>Solid bowl centrifuge</td>
<td>HP</td>
<td>&gt; 75</td>
<td>2.68</td>
<td>1</td>
<td>0.923</td>
<td>(32)</td>
</tr>
<tr>
<td>Rotary vacuum filter</td>
<td>ft²</td>
<td>1000</td>
<td>9.61</td>
<td>1</td>
<td>0.5012</td>
<td>(32)</td>
</tr>
<tr>
<td>Centrifugal pump s.s.</td>
<td>GPM</td>
<td>4000</td>
<td>0.44</td>
<td>50</td>
<td>0.3368</td>
<td>(12)</td>
</tr>
<tr>
<td>Centrifugal pump c.s.</td>
<td>GPM</td>
<td>4000</td>
<td>0.27</td>
<td>50</td>
<td>0.3368</td>
<td>(12)</td>
</tr>
<tr>
<td>Electrical Motor</td>
<td>HP</td>
<td>200</td>
<td>0.05</td>
<td>1</td>
<td>0.5128</td>
<td>(33)</td>
</tr>
<tr>
<td>Rotary kiln</td>
<td>--</td>
<td>--</td>
<td>72.39*MSI</td>
<td>--</td>
<td>--</td>
<td>(20)</td>
</tr>
</tbody>
</table>
6-4. Factors were chosen to reflect the relative complexity of the various processing steps under consideration. The factor for evaporation is lower because the estimated evaporation cost included installation. The factors computed correspond approximately to typical values for fluid-solid processing facilities. Using these factors, direct fixed capital investment is calculated as follows:

$$ \text{DFC} = \text{FACTOR} \times \sum \text{EQUIPMENT COST} $$

The contribution of capital equipment to the cost of manufacturing is computed using the capital related factor presented in Table 6-5. Straight line depreciation is assumed. Taxes are not included as the plant being evaluated is assumed to be tax exempt. Capital related costs per year are calculated as follows:

$$ \text{COST PER ANNUM} = \text{FACTOR} \times \text{DFC} $$

Ultrafiltration cost estimation is carried out using a direct operating capital cost of $80 per square foot of installed membrane capacity (37). This estimate includes all typical charges associated with capital installation and does not require the use of a multiplier. A membrane replacement cost of $20 per square foot is assumed required every two years (37).

6.4.2. Labor

Labor charges are computed using a labor factor multi-
**TABLE 6-4 Cost Multipliers**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Equipment</td>
<td>1.</td>
<td>1.</td>
<td>1.</td>
<td>1.</td>
<td>1.</td>
<td>1.</td>
<td>1.</td>
<td>1.</td>
<td>1.</td>
</tr>
<tr>
<td>Installation</td>
<td>0.39-0.47</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Piping</td>
<td>0.15-0.45</td>
<td>0.15</td>
<td>0.15</td>
<td>0.45</td>
<td>0.45</td>
<td>0.25</td>
<td>0.45</td>
<td>0.45</td>
<td>0.1</td>
</tr>
<tr>
<td>Instruments</td>
<td>0.05-0.15</td>
<td>0.05</td>
<td>0.05</td>
<td>0.15</td>
<td>0.05</td>
<td>0.05</td>
<td>0.15</td>
<td>0.15</td>
<td>0.10</td>
</tr>
<tr>
<td>Insulation</td>
<td>0.05-0.11</td>
<td>0</td>
<td>0</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Electrical</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Buildings</td>
<td>0.05-0.45</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Land &amp; Yard</td>
<td>0.15-0.18</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Auxiliary</td>
<td>0.4-1.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>2.1</td>
</tr>
<tr>
<td>SUBTOTAL</td>
<td>2.25</td>
<td>2.3</td>
<td>2.8</td>
<td>2.7</td>
<td>2.5</td>
<td>2.85</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Engineering  x1.25-1.4  x1.25  
Fees         x1.05-1.08  x1.05  
Contingency  x1.05-1.2   x1.1   

**TOTAL**          3.3    3.4    4.0    3.9    3.6    4.0    3.0

*From Peters and Timmerhaus (25) and Valle Riestra (35).*
TABLE 6-5  **Capital Related Factors**

(Cost per Annum = Factor x Fixed Capital)

<table>
<thead>
<tr>
<th>Item</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depreciation</td>
<td>0.1</td>
</tr>
<tr>
<td>Interest</td>
<td>0.06</td>
</tr>
<tr>
<td>Maintenance</td>
<td>0.06</td>
</tr>
<tr>
<td>Insurance</td>
<td>0.01</td>
</tr>
<tr>
<td>Plant Supplies</td>
<td>0.01</td>
</tr>
<tr>
<td>Taxes</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>0.24</strong></td>
</tr>
</tbody>
</table>

Valle Riestra, J.F. (35)
plied by the number of operators required and by labor costs. The number of operators required for each operation are listed in Table 6-6 (35). Table 6-7 presents a summary of the labor related cost factor. Base labor rate is $22,000 per year per operator. Four shifts per day are assumed.

6.4.3. Raw Materials

Table 6-8 presents a summary of raw materials costs. Medium concentration and sulfuric acid used are as specified in Chapter 4. Carbon source for fermentation is defined as delignified cellulose. Fermentation work has used ball milled Solka Floc as carbon source, a raw material too expensive for industrial use. It is assumed that some form of pretreatment may be developed to enable use of agricultural waste as fermentation carbon source. Corn steep liquor is used instead of proteose peptone used in laboratory experiments, as the cost of peptone is too high for industrial use. Corn steep liquor is assumed to replace peptone at the same concentration. However, preliminary work indicates that more corn steep liquor will be required (39). Use of raw materials is based on 330 Day/Year.

Steam explosion is assumed to cost $7.5 per ton of biomass processed (40). This cost is accounted for as a raw materials cost as no design is provided for this processing step. Corn stover is not accounted for as a raw material; its cost is treated as an independent variable. Current
<table>
<thead>
<tr>
<th>Operation</th>
<th>Labor (Operators/Shift)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis</td>
<td>2</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>2</td>
</tr>
<tr>
<td>Enzyme Recovery</td>
<td>1</td>
</tr>
<tr>
<td>Enzyme Production Batch</td>
<td>6</td>
</tr>
<tr>
<td>- Continuous</td>
<td>2</td>
</tr>
<tr>
<td>Milling</td>
<td>1</td>
</tr>
<tr>
<td>Evaporation</td>
<td>1</td>
</tr>
<tr>
<td>Storage</td>
<td>0</td>
</tr>
</tbody>
</table>

Shifts per day = 4
## TABLE 6-7 Labor Factor

<table>
<thead>
<tr>
<th>Item</th>
<th>Cost Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operating Labor</td>
<td>1.0</td>
</tr>
<tr>
<td>Fringe Benefits</td>
<td>0.22</td>
</tr>
<tr>
<td>Supervisor, Clerical</td>
<td>0.18</td>
</tr>
<tr>
<td>Operating Supplies</td>
<td>0.10</td>
</tr>
<tr>
<td>Laboratory</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>1.65</strong></td>
</tr>
</tbody>
</table>

Base Labor Rate: $22,000\(^1\) per person year.

1. Valle Riestra (35)
<table>
<thead>
<tr>
<th>Material</th>
<th>Cost ($/ton)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfuric Acid</td>
<td>58.</td>
<td>(37)</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>65.</td>
<td>(37)</td>
</tr>
<tr>
<td>Potassium Phosphate</td>
<td>180.</td>
<td>(37)</td>
</tr>
<tr>
<td>Magnesium Phosphate</td>
<td>230.</td>
<td>(37)</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>90.</td>
<td>(37)</td>
</tr>
<tr>
<td>Corn Steep Liquor</td>
<td>400.</td>
<td>(38)</td>
</tr>
<tr>
<td>Delignified Cellulose</td>
<td>100.</td>
<td>(1)</td>
</tr>
</tbody>
</table>
estimates of agricultural waste substrate costs range at $0-50 per ton.

6.4.4. Utilities

Utilities for all operating steps were accounted for individually and include steam, water and electricity consumption. Electricity assumed bought from a utility company is charged at 4.4 cents per Kw hour (13). If surplus steam is generated, it is converted to electricity at a cost of 1.6 cents per Kw hour (14). Process and cooling water is charged at 33 cents per 1000 gallons (13). Water removed from the product stream is recycled for a credit of the same value.

Steam is assumed generated from corn stover at the heating value of lignin: 12,700 BTU/lb (29). Solids leaving hydrolysis contain 2.33 pounds of water per pound of corn stover. After removing the heat required to vaporize this water a net 5000 BTU/lb of corn stover burned remains. The cost of boilers capable of burning corn stover is given in Table 6-9 (30). If additional steam is required to run the process, it is assumed supplied by coal costing $34 per ton (13). The cost of coal fired boilers is given in Table 6-9 (30). A higher boiler cost results in using coal instead of corn stover due to emissions control equipment required when burning coal.
TABLE 6-9  Steam Cost

Corn Stover Boilers:

<table>
<thead>
<tr>
<th>Range</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1x10^6 lb/day</td>
<td>Cost = MSI * 836.7 / lb/day steam $/1000 lb steam</td>
</tr>
<tr>
<td>&gt;1x10^6; &lt;8x10^6 lb/day</td>
<td>Cost = MSI * 2.68 [(lb/day)/1000]^0.85 / (lb/day)</td>
</tr>
</tbody>
</table>

Coal Boilers:

<table>
<thead>
<tr>
<th>Range</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1x10^6 lb/day</td>
<td>Cost = MSI * 1246 / lb/day</td>
</tr>
<tr>
<td>&gt;1x10^6; &lt;8x10^6 lb/day</td>
<td>Cost = MSI * 12.23 [(lb/day)/1000]^0.71 / (lb/day)</td>
</tr>
</tbody>
</table>
References: Chapter 6


5. Peters and Timmerhaus, p. 572.

6. Ibid., p. 669.


8. Ibid., p. 10.34.


12. Ibid., p. 556.

13. Ibid., p. 881.


26. Ibid., p. 599.


30. Murphy, T.K., University of California, Berkeley. Personal communication.


32. Ibid., pp. 587-589.

33. Ibid., p. 566.
34. Ibid., pp. 180-181.

35. Valle Riestra, F., Dow Chemicals, Inc. Personal communication.


38. Sciamanna, A.F. University of California, Berkeley. Personal communication.


This chapter presents a summary of the algorithm used to evaluate the cost of producing glucose under the various operating conditions presented in Chapter 5. A listing of this program along with documentation providing variable definitions and input/output information is provided in Appendix C.

7.1. Computer Algorithm Capabilities

7.1.1. General Organization

Figure 7-1 presents a schematic of the computer program's organization. The program is composed of subroutines nested in three levels corresponding to their function. Level 1 subroutines serve primarily to manipulate, distribute and summarize information. Level 2 routines contain process design information necessary to specify and cost equipment and are responsible for material, energy and equipment summaries. Level 3 routines perform equipment costing and sizing calculations and other similar functions.

7.1.2. Required Information and Operating Options

Information required to operate the program consists of:

1. Experimental data containing conversion and enzyme recovery versus time information.
Figure 7.1 Subroutine Organization
2. Composition and physical property information of substrate and reaction steps.

3. Design specification, cost data and economic evaluation data.

Information which would normally be varied from run to run or which will tend to change with frequency (items 1, 2) are designed to be read to the program each time it is run. A summary of these variables is presented in Table 7-1. Iteration to evaluate cost versus residence time relies on linear approximations of conversion and enzyme recovery versus time data. This is correct if the interval over which the iteration takes place is short and if the earliest residence time considered is 8 hours, after which reaction rates are nearly constant and slow. The validity of searching for optimum sugar production conditions while excluding initial reaction periods is discussed in Chapter 8.

Information which is related to the substrate under consideration, such as composition and other information specific to the experimental data discussed in Chapter 5 are grouped in the beginning of each subroutine. This information should be examined and revised whenever evaluation of a new set of data is required.

Design related information is found whenever it is used in the design subroutines. Evaluation of a different design will require either changing all code related to the design
### TABLE 7-1  Program Input Variables

<table>
<thead>
<tr>
<th>Name</th>
<th>Meaning</th>
<th>Dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Glucose conversion</td>
<td>Fractional</td>
</tr>
<tr>
<td>YXYL</td>
<td>Xylose conversion</td>
<td>Fractional</td>
</tr>
<tr>
<td>PGPRSU</td>
<td>% Glucose in reducing sugars</td>
<td>Fractional</td>
</tr>
<tr>
<td>FEAR</td>
<td>% Enzyme recovery</td>
<td>Fractional</td>
</tr>
<tr>
<td>TR</td>
<td>Residence time</td>
<td>hr</td>
</tr>
<tr>
<td>SCON</td>
<td>Initial substrate concentration</td>
<td>%</td>
</tr>
<tr>
<td>eHY01</td>
<td>Initial Enzyme strength</td>
<td>IU/ml</td>
</tr>
<tr>
<td>GPROD</td>
<td>Glucose Production Desired</td>
<td>ton/day</td>
</tr>
<tr>
<td>TRI</td>
<td>Minimum residence time</td>
<td>hr</td>
</tr>
<tr>
<td>TRMAX</td>
<td>Maximum residence time</td>
<td>hr</td>
</tr>
<tr>
<td>erslop</td>
<td>Enzyme recovery slope</td>
<td>---</td>
</tr>
<tr>
<td>erint</td>
<td>Enzyme recovery intercept</td>
<td>---</td>
</tr>
<tr>
<td>xslop</td>
<td>Conversion slope</td>
<td>---</td>
</tr>
<tr>
<td>yint</td>
<td>Conversion intercept</td>
<td>---</td>
</tr>
</tbody>
</table>
change or incorporating additional subroutines.

A summary of operating options for the computer program is presented in Table 7-2. To prevent excessive use of computer time, equipment list option is not allowed with iterative rows and request for varying both recycle fraction and residence time will default to varying only residence time. Sample outputs for each operating option are listed in Appendix D.

7.1.3. Information Distribution and Output

Information is passed between subroutines in level 1 and 2 by common statements. A summary of these statements is provided in Chapter 4. Information passes to and from subroutines in level 3 as arguments in a subroutine call statement. Economic summary data such as capital cost sum, utilities consumed and raw materials are stored in one-dimensional arrays where each process step is assigned a fixed array position. Table 7-3 presents a summary of economic sum arrays.

Output is generated as follows:

1. Input variables are printed in the main program.

2. Material, energy and equipment lists are printed in each processing step subroutine.
<table>
<thead>
<tr>
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<th>Definition</th>
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<td></td>
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<td></td>
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</tr>
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<td>tropt</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>----------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Capcos (7)</td>
<td>Capital cost sum</td>
<td>$</td>
</tr>
<tr>
<td>Uel (7)</td>
<td>Electrical consumption sum</td>
<td>Kw hr/day</td>
</tr>
<tr>
<td>Ust (7)</td>
<td>Steam consumption sum</td>
<td>lb/day</td>
</tr>
<tr>
<td>Uwa (7)</td>
<td>Water consumption sum</td>
<td>gallon/day</td>
</tr>
<tr>
<td>Ucw (7)</td>
<td>Cooling water consumption sum</td>
<td>gallon/day</td>
</tr>
<tr>
<td>Raw (7)</td>
<td>Raw material cost sum</td>
<td>$/year</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>Processing Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydrolysis</td>
</tr>
<tr>
<td>2</td>
<td>Enzyme recovery</td>
</tr>
<tr>
<td>3</td>
<td>Enzyme production</td>
</tr>
<tr>
<td>4</td>
<td>Enzyme recovery</td>
</tr>
<tr>
<td>5</td>
<td>Milling</td>
</tr>
<tr>
<td>6</td>
<td>Evaporation</td>
</tr>
<tr>
<td>7</td>
<td>Storage</td>
</tr>
</tbody>
</table>
Sample output is provided in Appendix D. Documentation in Appendix C presents a list of program line locations where a given variable is used, read or printed.

7.1.4. Limitations

The use of an empirical fit to manipulate experimental data limits the use of this program to ranges where data exist. Currently, an integrated kinetic model based on theory which adequately describes the hydrolysis reactions under investigation is not available. The availability of such a model would enable evaluation of a wider range of conditions through extrapolation. A possible improvement in the usefulness of this program could result by coupling the differential kinetic model developed by Wald (1) to this program and employing canned subroutines to solve the differential equations. This approach, although effective, will be cumbersome and costly for evaluating large ranges of operating conditions.

The accuracy of each of the processing schemes presented relies on the amount of information available on each processing step. Comparison between operating conditions is possible as the magnitude of the error for cases using the same cost and size criteria does not change. However, comparison between processing schemes even with the same set of experimental data is subject to variations in
uncertainty. One improvement would be to define the uncertainty of each cost data item separately and determine the error for each processing step statistically.

7.2. **Subroutine Outlines**

This section presents logic diagrams for key subroutines or type of subroutines found in the program. For a detailed treatment of subroutine composition refer to the program listing with comment documentation found in Appendix C.

7.2.1. **Main Program Subroutine**

Figure 7-2 presents a logic diagram for the program's main body. The program's main function is to check program constraints, and to increment any iterative variable. The program calls only one subroutine, Model. Model is responsible for calling the following subroutines:

1. Matbal (Material Balance)
2. Hydrol (Hydrolysis)
3. Enzrec (Enzyme Recovery)
4. Pretre (Pretreatment)
5. Enzprod (Enzyme Production)
6. Mill (Milling)
7. Store (Storage)
8. Evap (Evaporation)
9. Econ (Economics)

7.3. **Material Balance**

A material balance subroutine is responsible for specifying all streams entering and leaving the hydrolysis processing step. The subroutine corresponds to an algebraic
Figure 7.2  Main Program Logic Diagram
solution of the material balances presented in Figure 7-3 and 7-4. Specified conversion, enzyme recovery, desired daily production, and substrate initial concentration are specified for this purpose. Solution of this material balance enables sizing enzyme recovery, hydrolysis, enzyme production, pretreatment and evaporation routines. In addition to providing an algebraic solution, this routine prints a material balance summary.

7.3.1. Process Selection Routines

Subroutines Enzrec, Enzprod and Pretre serve to select desired enzyme recovery, enzyme production and pretreatment subroutines. Subroutines Enzrec and Pretre merely call the appropriate subroutine without performing any other function. The enzyme production subroutine prevents the program from violating material balance constraints. If the material balance prescribes an enzyme concentration (IU/ml) greater than the capability of the desired enzyme production scheme this will be detected and a warning issued. Figure 7-5 presents a summary of this subroutine.

7.3.2. Process Operation Subroutines

Figure 7-6 presents a logic diagram for subroutine Pra-cid, the dilute acid pretreatment subroutine. All subroutines used to determine operation requirements of process
**NOMENCLATURE**

- **S** = Corn Stover T/Day
- **W** = Water T/Day
- **G** = Glucose T/Day
- **E** = Enzyme IU/Day
- **FR** = Recycle Fraction
- **FFG** = Fraction of glucose lost in filter
- **FFE** = Fraction of enzyme lost in filter
- **Fe** = Fraction of enzyme recovered

---

Figure 7.3 Hydrolysis Material Balance for Equilibrium Contacting Enzyme Recycle.
Nomenclature:

- $S$ = Corn Stover T/Day
- $W$ = Water T/Day
- $E$ = Enzyme IU/Day
- $F_{Fe}$ = Fraction of enzyme lost in filter
- $F_{FG}$ = Fraction of glucose lost in filter
- $F_{UF}$ = Fraction of recycle from ultrafiltration

Figure 7.4. Hydrolysis Material Balance for Ultrafiltration Enzyme Recovers.
Figure 7.5 ENZPROD SUBROUTINE.
DEFINE CONSTANTS

RECEIVE INFORMATION FROM COMMON

COMPUTE ANY MATERIAL STREAMS

DEFINE SIZE PARAMETERS
CALL SIZE ROUTINES

CALL COST ROUTINES

DETERMINE UTILS AND MATERIAL REQUIREMENTS

ENERGY + MATERIAL LISTS

YES

PRINT ENERGY AND MATERIAL

NO

EQUIPMENT LISTS

YES

PRINT EQUIPMENT LIST

NO

RETURN

Figure 7.6 Subroutine PRACID Logic Diagram
steps are similar in logic to the dilute acid pretreatment subroutine outlined. These subroutines require a size basis which is supplied by either the main program, the material balance subroutine or another process step subroutine. Table 7-4 presents a summary of the primary size criteria used for each process subroutine. In addition to this information, a number of internal process specifications covered in Chapter 6 are required.

7.3.3. Support Subroutines

Support subroutines perform sizing and costing functions for equipment specified in Chapter 6. Figure 7-7 presents a logic diagram for typical costing and sizing routines. Organization of additional routines is similar, listings are provided in Appendix C.
<table>
<thead>
<tr>
<th>Subroutine</th>
<th>Size Basis</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis</td>
<td>Water flow (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td></td>
<td>Pretreated solids (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td>Enzyme Production</td>
<td>Water flow (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td></td>
<td>Enzyme required (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td>Equilibrium Contacting Recovery</td>
<td>Water flow (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td></td>
<td>Pretreated solids (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>Water flow (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td>Dilute Acid Pretreatment</td>
<td>Pretreated solids (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td>Milling</td>
<td>Untreated solids (ton/day)</td>
<td>Dilute Acid Iotech</td>
</tr>
<tr>
<td>Iotech (Steam Explosion)</td>
<td>Pretreated solids (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td>Evaporation</td>
<td>Water flow (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td></td>
<td>Glucose (ton/day)</td>
<td>Matbal</td>
</tr>
<tr>
<td>Storage</td>
<td>Material stored (ton/day)</td>
<td>Dilute Acid Enzyme Production</td>
</tr>
</tbody>
</table>
SUBROUTINE CONSIZ (Condenser)

RECEIVE DATA

CALCULATE AREA

DIVIDE AREA BY MAXIMUM AREA

< 1 ?

YES

SIZE = AREA

NUMBER = 1

NO

NUMBER = LARGEST INTEGER + 1

AREA = AREA TOTAL / NUMBER

RETURN

SUBROUTINE CSCON (Condenser Cost)

RECEIVE SIZE AND NUMBER

CALCULATE COST

SUM CONDENSER COST

LAST CONDENSER

YES

INCREMENT CAPLOS (i) AREAS

RETURN

Figure 7.7 Typical Sizing and Costing Routines.
References: Chapter 7

8. Economic Evaluation, Results and Discussion

This chapter presents a summary of economic data obtained with the computer algorithm described in Chapter 7 and hydrolysis results presented in Chapter 5. Data on which these results are based may be found in Appendix D.

8.1. Glucose Production Cost, Base Case

8.1.1. Production Cost Excluding Corn Stover Cost

Figure 8-1 presents glucose production cost versus substrate concentration and enzyme activity for the base case design presented in Chapter 6. Corn stover cost is neglected and B-glucosidase activity is 1.1 times cellulase FPA. Optimum production conditions are 20% substrate concentration and 3 IU/ml enzyme activity. These conditions correspond to a specific activity of 13 IU/g substrate, and do not coincide with conditions for optimum conversion. An activity of 13 IU/g corresponds to a glucose conversion of 38% and as can be seen in Figure 5-8, is where the slope of the conversion versus specific activity curve begins to decrease substantially.

Figure 8-2 presents similar data obtained with B-glucosidase activity 1.8 times the cellulase filter paper activity. A similar behavior is observed. Optimum conditions are again 20% substrate and 3 IU/ml enzyme activity, conversion is 44%. This conversion is higher due to the absence of cellobiose inhibition and results in the lowest
Figure 8.1  Glucose Production Cost Versus Substrate Concentration At Various Enzyme Activities. Basis: No Corn Stover Cost Cellulase, B-g : FPA, 1.1 : 1
Figure 8.2 Glucose Cost Versus Substrate Concentration At Various Enzyme Activities.
Basis: No Corn Stover Cost
Cellulase, B-g : FPA, 1.1 : 1
glucose cost observed, 12.5 cents per pound.

### 8.1.2. Production Cost Including Corn Stover Cost

Figure 8-3 presents data from Figure 8-1 except corn stover utilized is assumed to cost $30/ton. Optimum production is shifted to 15% substrate by weight and 5 IU/ml cellulase activity, corresponding to a conversion of 50%. Lowest glucose cost is 25 cents per pound, 40% of this cost is related to corn stover.

Figure 8-4 presents economic data from Figure 8-2 when corn stover is assumed to cost $30/ton. A similar pattern is observed, optimum conditions are shifted to higher conversion and higher specific activities. Corn stover cost contribution is approximately 40% of the glucose production cost. Lowest glucose cost observed is 22 cents per pound.

### 8.1.3. Contribution of Processing Operations to the Cost of Manufacturing Glucose

Table 8-1 presents the average contribution of various processing steps to the overall processing cost, excluding corn stover cost. Enzyme production contributes 40% of all manufacturing cost and is the costliest process to operate. Dilute acid pretreatment contributes 21%, hydrolysis 15%, evaporation 10% and all other steps combined, 14%. The highest manufacturing cost component is capital equipment, contributing 65% of all manufacturing expenses.
Figure 8.3 Glucose Cost Versus Substrate Concentration At Various Enzyme Activities.
Basis: Corn Stover $30/Ton
Cellulase, B-g : FPA, 1.1 : 1
Figure 8.4  Glucose Cost Versus Substrate Concentration At Various Enzyme Activities.

Basis: Corn Stover $30/Ton
Cellulase, B-g : FPA, 1.8 : 1
TABLE 8-1  Contribution of Processing Operation and Manufacturing Component to Glucose Cost

A. Operation % of Glucose Cost

<table>
<thead>
<tr>
<th>Operation</th>
<th>% of Glucose Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Production</td>
<td>41</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>21</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>15</td>
</tr>
<tr>
<td>Evaporation</td>
<td>13</td>
</tr>
<tr>
<td>Other</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 %</td>
</tr>
</tbody>
</table>

B. Manufacturing Component % of Glucose Cost

<table>
<thead>
<tr>
<th>Component</th>
<th>% of Glucose Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capital</td>
<td>65</td>
</tr>
<tr>
<td>Utilities</td>
<td>17</td>
</tr>
<tr>
<td>Raw Materials</td>
<td>11</td>
</tr>
<tr>
<td>Labor</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 %</td>
</tr>
</tbody>
</table>
8.1.4. Effect of Enzyme Activity on Cost

Figure 8-5 presents manufacturing component costs versus enzyme activity at fixed substrate concentration. The dominance of enzyme production costs when corn stover cost is neglected explains the optimum manufacturing conditions observed. Under these circumstances a large increment in the cost of producing enzymes is required to provide a small savings in the cost of substrate pretreatment, and evaporation. When corn stover cost is $30/ton (40% of the total cost) enzyme production contribution is only 25% of the total cost, thus an increase in yield results in a decrease in substrate costs as well as a decrease in pretreatment, and evaporation costs.

8.1.5. Effect of Substrate Concentration

Figure 8-6 presents production cost versus substrate concentration at fixed residence time and enzyme specific activity. Corn stover and pretreatment costs do not vary since conversion for all cases is constant. Enzyme production costs increase with increasing solids concentration due to the adsorption isotherm behavior described in Chapter 5. Hydrolysis and evaporation costs decrease with increasing solids concentration due to smaller reactor volume requirements and higher concentration product streams. It was not possible to evaluate most hydrolysis cases involving 25% substrate concentrations due to constraints in the volume of water allowed to enter the hydrolysis vessel from the enzyme
Figure 8.5. Effect of Increasing Enzyme Activity at Fixed Substrate Concentration and Residence Time. (10% Corn Stover)
Figure 8.6 Glucose Cost Versus Substrate Concentration
production unit. If cellulase concentrations higher than 12 IU/ml can be achieved, possible cost reduction may result by operating at the optimum specific activity and 25% solids suspension.

8.1.6. Effect of Residence Time

Figure 8-7 presents glucose cost versus residence time at fixed substrate concentration and enzyme activity. Corn stover, enzyme production, pretreatment and evaporation costs decrease with increasing residence time due to higher conversions at higher residence time. Hydrolyzation cost increases with increasing residence time requirements. Costs were reduced when the rate of glucose conversion was 0.2% per hour or higher. Glucose conversion rates higher than this value occur for residence times between 0 and 8 hours, therefore the presence of a cost optimum in this range is not likely.

8.1.7. Economics of Scale, Direct Fixed Capital

Figure 8-8 presents glucose cost and direct fixed capital cost versus plant size. Glucose cost remains nearly constant until production drops to 125 tons per day. Operation at 214 tons per day appears inefficient in view of the magnitude of the direct fixed capital involved. At a daily production of 214 tons/day, direct fixed capital is 60 million dollars.
Figure 8.7. Glucose Cost Versus Residence Time at Fixed Substrate Concentration and Enzyme Activity. 15%, 5 IU/ml
Figure 8.8 Glucose Cost and Direct Fixed Capital Versus Plant Size.
8.1.8. Cost of Ethanol Production

For corn stover cost ranging from 0-30 dollars per ton, the lowest glucose cost observed is 12-22 cents per pound. Employing current ethanol production technology (1) the cost of manufacturing ethanol from glucose produced by this process is $1.8-3.3 per gallon. At this price ethanol production from corn stover could not compete favorably.

8.1.9. Comparison with QM9414

A direct economic comparison between processes using QM9414 and Rut-C-30 for cellulase production is difficult because the economic study performed with QM9414 employed a different basis and used different cellulase activity units. The best case reported for the QM9414 process used 5% substrate and approximately 0.5 IU/ml to obtain a yield of 40%. Cost reported was 10 cents per pound of glucose excluding storage and evaporation. The study was performed with a Marshal and Swift Index of 469 (2). Updating costs for inflation to MSI=707 and adding typical evaporation and storage costs for a similar case, the price of glucose obtained is 19 cents per pound. Processing with Rutgers-C-30 results in a savings of approximately 33% when corn stover cost is excluded. At corn stover costing 30 dollars per ton processing with QM9414 results in a cost of 28 cents per pound, six cents per pound higher than the Rut-C-30 estimated cost.
8.2. Process Alternatives

8.2.1. Enzyme Production

Figure 8-9 presents a summary of enzyme production cost in $ / million units versus fermentation productivity in IU/1/hr. Under current processing conditions, batch production is most economical with cellulase cost in the order of $4-5 per million units. Continuous processing with cell recycle may approach batch production costs. In order to operate the base case plant design with either a continuous or a continuous cell recycle scheme it will be necessary to enhance cellulase concentration produced along with increased productivity. Current material balance constraints and optimum operation specifications would not permit production of cellulase in the 3.5 to 5.5 IU/ml concentration range. Estimates rely on the use of inexpensive carbon and nitrogen sources instead of laboratory grade materials. At current selling prices, Solka Floc and proteose peptone would add 7 cents to the cost per pound of glucose, a figure equivalent in magnitude to the entire cost of producing enzyme with cheap carbon and nitrogen sources.

8.2.2. Enzyme Recovery

Figure 8-10 presents a cost of operating an enzyme recovery system versus required size. Ultrafiltration costs are approximately $4 per 1000 gallons of capacity. This cost is approximately three times higher than the cost of
Figure 8.9 Cellulase Production Cost Versus Productivity for Various Production Methods.
Figure 8.10. Cost of Enzyme Recovery Versus Size for Ultrafiltration and Equilibrium Contacting.
processing a similar volume through an equilibrium contacting enzyme recovery scheme. Both methods are less expensive than producing an equivalent increment of fresh enzyme for all cases examined. Typically equilibrium contacting costs 1.5 to 0.5 dollars per million units recovered, ultrafiltration costs 0.5 to 3.8 dollars per million units and batch enzyme production 4-5 dollars per million units.

8.2.3. Steam Explosion

Table 8-2 presents a summary of hydrolysis conditions and glucose production cost for an economic evaluation conducted on hydrolysis data with steam exploded corn stover. As the cost of pretreatment is unknown and the range of hydrolysis experiments conducted is limited, an optimum manufacturing cost was not sought. The best cost observed was 10 cents per pound excluding corn stover cost and 15 cents per pound including corn stover cost at $30/ton. This represents a savings of approximately 30% over the base case. The glucose production costs observed lead to ethanol prices of 1.75-2.50 dollars per gallon, still not economical.
Table 8-2  Glucose Production Cost With Steam Exploded Corn Stover

<table>
<thead>
<tr>
<th>Substrate Conc. wt %</th>
<th>Enz. Activity IU/ml</th>
<th>Residence time (hr)</th>
<th>Glucose Cost $/lb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C.S. 0$/ton</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>48</td>
<td>12.3</td>
</tr>
<tr>
<td>10</td>
<td>3.5</td>
<td>48</td>
<td>13.0</td>
</tr>
<tr>
<td>15</td>
<td>3.5</td>
<td>48</td>
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</tr>
<tr>
<td>25</td>
<td>3.5</td>
<td>48</td>
<td>11.9</td>
</tr>
</tbody>
</table>
References: Chapter 8


9. Conclusions and Recommendations

9.1. Conclusions

9.1.1. Experimental

The following conclusions may be drawn from the experimental results discussed in Chapter 5:

1. Maximum conversion obtained with acid treated corn stover and cellulase from *T. reesei* Rut-C-30 was approximately 55%. In view of the wide ratio of cellulase activity investigated, further increases in yield with this process are unlikely.

2. A high level of B-glucosidase, in the range of 1.8 B-glucosidase units to FPA units was demonstrated necessary to reduce cellobiose accumulation to low levels.

3. Conversions in the order of 80 percent were obtained during enzymatic hydrolysis of steam exploded corn stover. This constitutes a significant increase over the conversions obtained with acid treated corn stover.

4. *T. reesei* Rut-C-30 may be grown to produce cellulase using water washed steam exploded corn stover. An adaptation period may enable the organism to grow on unwashed material.

5. Preliminary evaluation indicates that hydrolysis results obtained with corn stover may be described by a
kinetic model developed by Wald (Section 5.6). Good correlation will require measurement of initial rates and inhibition constants specific for corn stover.

9.1.2. Process Evaluation

The following observations were made from economic evaluation results presented in Chapter 8:

1. A process design which includes dilute acid pretreatment, equilibrium contacting for enzyme recovery and batch enzyme production leads to glucose production costs of 12-22 cents per pound for corn stover cost of 0-30 dollars per ton. Corn stover is the dominant cost at 30 dollars/ton accounting for 40% of the cost of glucose. Treatment of corn stover cost as an independent variable in evaluations is not possible since the process optimum is a strong function of the corn stover cost.

2. Comparison of the cost of producing glucose with the base case process or with a previous process using \textit{T. reesei} QM9414 shows that processing with Rut-C-30 leads to cost reduction in the order of 33%. This is primarily due to use of higher substrate concentration in hydrolysis, production of cellulase at higher activities and to a lesser extent increased corn stover conversion.
3. Enzyme production is the single most expensive processing step, accounting for 40% of the processing costs excluding substrate cost. Cellulase production alternatives, continuous and continuous cell recycle schemes, cannot currently compete with batch production due to lower productivity and more dilute product streams. Cell recycle may replace batch production, but further work is needed.

4. Ultrafiltration as a means of recovering enzymes from the hydrolysis product stream is considerably more expensive than the equilibrium contacting scheme employed in the base case. However, if equilibrium contacting enzyme recovery is not capable of preserving an acceptable cellulase component ratio, ultrafiltration is still less expensive than not recycling enzymes.

5. Preliminary evaluation of hydrolysis with steam exploded corn stover shows that glucose may be produced at a cost of 10-15 cents per pound for corn stover ranging from 0-30 dollars per ton. This represents a cost reduction of approximately 33%. Optimization of this process may lead to further cost reduction.

6. At current glucose production costs, ethanol may not be manufactured profitably in conjunction with this process. With dominance of corn stover costs, significant processing cost reductions may bring about only modest
reduction in the price of manufacturing ethanol from corn stover; for example, at a glucose cost of 22 cents per pound, 7 cents per pound is corn stover cost, 6 cents per pound is enzyme production cost. A reduction in the cost of producing enzyme of 50% leads to a glucose cost reduction of only 14%.

9.2. Recommendations

9.2.1. Experimental

1. Research in hydrolysis of steam exploded corn stover presents an opportunity for process improvement and should be pursued.

2. Research aimed at replacing expensive fermentation medium nutrients and improving productivity will be necessary to reduce enzyme production costs. The use of steam exploded corn stover as substrate constitutes a potential solution to the high cost of fermentation media currently employed.

3. Understanding of T. reesei Rut-C-30's metabolism will be necessary to enable production of cellulase in a consistent manner.

4. An understanding of the adsorption behavior of cellulase onto corn stover and of the performance of recycled cellulase is needed to evaluate the suitability of equilibrium contacting enzyme recovery. Enzyme
5. Determination of solids filtration behavior based on filtration theory and measurement of corn stover solution viscosities will improve economic evaluation estimates.

6. Evaluation of a substrate having a higher cellulose content than corn stover may be desirable to reduce costs.

9.2.2. Process Evaluation

1. An estimate of the cost of corn stover for use in a hydrolysis plant is necessary before meaningful process evaluation may be conducted.

2. Likewise, an independent estimate of the cost of pretreating corn stover by steam explosion is required if this method is going to be compared with existing forms of pretreatment.

3. A statistical approach to evaluating the cost of capital is recommended as it will enable assessment of uncertainties involved.
Appendix A: Analytical Procedures

A.1 Composition Determination for Cellulosic Materials

A.1.1. Milling and Sieving

One hundred grams or more of air dry material is ground in a Wiley mill through a 2 mm. screen. The ground material is sieved through 0.25mm and 0.35mm opening screens to collect the 0.25mm to 0.35mm fraction, which is used for subsequent analysis.

The material is dried in an 105°C oven for at least 4 hours or until no weight change occurs with further drying.

A.1.2. Extraction

An accurately weighed portion of the dry material is placed in a tared glass extraction crucible equipped with a coarse (40-60 μM pore size) fritted glass filter. The material is extracted in a Soxhlet apparatus with benzene/ethanol (2:1) for at least 6 hours or until no change in weight occurs after additional extraction. The material is sucked dry and placed in a 105°C oven to dry. The change in weight of the material during extraction is taken as the extractives content, i.e.
% Extractives =

\[
\frac{(\text{Gross wt. pre-extract}) - \text{Gross wt. post extract})}{(\text{Gross wt. pre-extract}) - (\text{Tare wt.})} \times 100
\]

Extraction Factor = \frac{100 - (\% \text{ extractives})}{100}

The extraction factor above is used to correct values of the assays performed on extracted material, subsequently.

A.1.3. Exhaustive Hydrolysis

A convenient amount of dry extracted material is accurately weighed and placed in a vial equipped with a teflon-lined screw top. 0.5 gm of material and a 9.5 dram vial are convenient sizes. One ml. of 72 w/w% \( \text{H}_2\text{SO}_4 \) is added per 0.1 gm of material. The slurry is mixed on a vortex mixer, allowed to stand 3 minutes and mixed again. The vials are placed in a 30°C bath for one hour during which time they are removed and mixed at least three times. The slurry is quantitatively transferred to a 250 ml Erlenmeyer flask using 28 ml \( \text{H}_2\text{O} \) per ml of 72 w/w% \( \text{H}_2\text{SO}_4 \) used. This dilution produces 4 w/w% \( \text{H}_2\text{SO}_4 \) solution for the secondary hydrolysis. The flasks are capped with watch glasses and placed in a preheated autoclave. The flasks are heated for one hour at which time the autoclave is slowly allowed to depressurize to prevent the samples from boiling over.
The hot solutions are individually filtered through dry porcelain filtering crucibles. The residues are washed repeatedly with hot H₂O. The crucibles are dried in the 105°C oven, cooled in a desiccator and weighed. They are then placed in a muffle furnace (600°C) and ashed for at least 6 hours or until no weight change occurs after additional heating. The ashed crucibles are cooled in a desiccator and weighed. The change in weight after ashing is taken as the total acid insoluble content of the extracted material. This value is multiplied by the extraction factor to determine the content in the unextracted material, i.e.

\[
\% \text{ Total Acid Insolubles} = \frac{(\text{Gross wt. residue & crucible}) - (\text{Gross wt. after ashing})}{(\text{wt. starting sample})} \times (\text{extraction factor}) \times 100
\]

The supernatants and collected washings are neutralized to pH 5-6 by addition of CaCO₃. The CaCO₃ must be added slowly since foaming is a problem. The neutralized solution is filtered free of CaSO₄ precipitate through a medium porosity fritted glass funnel. The precipitate is washed several times with H₂O. The total volume is accurately measured by dilution to a fixed volume in a volumetric flask. A portion of the supernatant is then centrifuged to remove any residual solid and saved for further analysis.
A.1.4. Carbohydrate Analysis

Liquors are analyzed by the glucose oxidase-peroxidase (GOP) and gas-liquid chromatography (GLC) methods. The GOP method is a highly specific assay for monomer glucose. Many variations of the method have been used (2,3). For our assay the reagent is made as follows: 1200 units of purified glucose oxidase (1 ml of Sigma Chemical Co., type V) and 10 mg peroxidase (Sigma Chemical Co., type II, 125-200 purpurogallin units per 1 mg) is added to 500 ml of a 1% solution (or 2.5 ml of 4%) of ortho-dianisidine dihydrochloride (Sigma Chemical Co.) in H₂O. Care must be taken when handling o-dianisidine, for it is a known carcinogen.

8 ml of this reagent is added to 2 ml of sample containing between 10 and 200 μg of glucose in a test tube. The tubes are incubated in a 37°C bath for one hour. The reaction is stopped by addition of 4 drops of concentrated HCl. The tubes are allowed to stand at least for 5 minutes before reading their absorbance at 420 nm. against a reagent blank. The color is stable for several hours. It is a good idea to do color blanks of each solution since there may be absorbance at 420 nm. by the solution alone. Also when working with new material one should "spike" these solutions with known amounts of standard glucose to see if the sensitivity changes, an indication that interference is occurring.

A standard curve should be made with each run. The standards should be made from pure glucose and made at least
a day in advance so that mutarotation can be accomplished (GOP is specific for B-D-glucose).

Once the glucose content of a hydrolysis liquor is known the value is converted to give the weight of the sugar in the starting solid. This is done by multiplying the concentration of glucose in the supernatant by the total volume of the supernatant, by a factor converting the weight of glucose as a monomer to its weight as a polymer, and by dividing by a factor to correct for loss of sugar during exhaustive hydrolysis. This gives the glucan content in the extracted material. Multiplying by the extractive factor gives the content in the original material, i.e.,

\[ \% \text{ Glucan (polymerized glucose)} = \left( \frac{\text{glucose, g/L} \times \text{polymerization factor} \times \text{volume supernatant, L}}{\text{weight of material assayed, gm} \times \text{extraction factor} / \text{recovery factor} \times 100} \right) \]

Table A-1 presents values of polymerization and recovery factors. Gas-liquid chromatography (GLC) allows quantification of each monomer sugar, including arabinose, xylose, mannose, galactose, and glucose. In order for sugars to be analyzed by GLC they must be free of H\textsubscript{2}O and made volatile by derivatization.

A volume of sugar solution containing from 1 to 10 mg of sugar is freeze dried to remove all H\textsubscript{2}O. The dry residue
TABLE A-1  Recovery and Polymerization Factors

**POLYMERIZATION FACTORS**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Polymerization Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>0.88</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.9</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.9</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.88</td>
</tr>
</tbody>
</table>

**RECOVERY FACTORS**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Recovery Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>0.924</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.957</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.950</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.916</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.866</td>
</tr>
</tbody>
</table>
is brought up in 1 ml of dimethylsulfoxide solvent, containing about 5 g/L 2-OH-pyridine and 2 g/L myo-inositol, in a 1 dram vial with a teflon lined screw top. The vial is placed in a 40°C oven and allowed to equilibrate at least 6 hours. After equilibration, 1 ml of hexamethyldisilazane and 1/2 ml of chlorotrimethylsilane are added to the vial which is mixed and allowed to stand for about 1 hour. Two phases are formed. The lower phase is discarded. To the upper phase is added an equal volume of H₂O. After mixing the two phases are allowed to separate and the lower phase is discarded. The upper phase is dried by adding anhydrous Na₂SO₄. The derivatized sugars are now ready for analysis.

A column from 1-3 feet in length containing a liquid phase of low to moderate polarity (e.g. SE30, OV-17, SF96) on a diatomaceous earth support (e.g. Chromasorb G or W) which has been acid washed and silylated should be used. We have had success with a low liquid coatings (1-3%). Using an Aerograph 1520 gas chromatograph, equipped with a temperature programmer it has been found that an initial temperature of 120°C to 150°C followed by a programmed temperature rise of 8°C per minute to be a good combination to give a short analysis time with good resolution. Carrier gas flows of 20-30 cc/min. were used.

The recorder peaks are measured with a disc integrator. We use a Leeds and Northrup Speedomax G Model S Recorder equipped with a Disc Chart Integrator-Model 203. The sugar
Appendix A

Derivative peaks are quantified in terms of the area of the internal standard peak (myo-inositol). For each run the sugar peak areas are divided by the area of the internal standard peak, i.e.,

\[
\text{Sugar peak ratio units} = \frac{\text{Area sugar peak} \times \text{attenuation}}{\text{Area internal standard peak} \times \text{attenuation}}
\]

This ratio is then compared to a standard curve made by analyzing standard sugar solutions by the above method and plotting standard sugar ratio units vs. weight of sugar.

Once the quantity of a sugar is determined by GLC it can be converted back to its weight in the starting material in much the same way as described for glucose in the GOP method, i.e.,

\[
\% \text{Polymerized sugar in solid} = \frac{(\text{sugar in solution, g/L})}{(\text{volume freeze dried, ml/l ml DMSO solvent})} \times (\text{polymerization factor}) \times (\text{volume supernate, L}) \times (\text{recovery factor}) \times (\text{weight of material assayed, gm}) \times \text{extraction factor} \times 100
\]

A.1.5. \text{H}_2\text{O Wash}

Material which has been extracted with benzene/ethanol is washed with hot \text{H}_2\text{O} to remove acid insoluble material other than lignin. This is done by placing a known weight of material in a tared glass extraction crucible into a
Appendix A

Soxhlet apparatus. Boiling chips and antifoam are added to the H2O in the distillation flask to prevent bumping or foaming. The material should be washed overnight. After drying in the 105°C oven the washed material is weighed, and an H2O extraction factor is determined as follows:

\[
\text{H}_2\text{O Wash Factor} = \frac{(\text{Gross wt. post wash}) - (\text{Tare wt.})}{(\text{Gross wt. pre-wash}) - (\text{Tare wt.})}
\]

A.1.6. Exhaustive Hydrolysis

Exhaustive acid hydrolysis is performed on the dry H2O washed material. The procedure is exactly as before, the only exception being that the liquor generated by the hydrolysis is neither neutralized or saved. The residue collected in the dry porcelain crucible is washed, dried, weighed, ashed, and weighed again. The change in weight after ashing is taken as the lignin content of the extracted, H2O washed material. Multiplication by the extraction and H2O wash factors gives the lignin content of the starting material, i.e.,

\[
\% \text{ Lignin} = \frac{(\text{Gross wt. dry crucible & lignin}) - (\text{Gross wt. ashed crucible})}{(\text{weight of dry starting material})} \times (\text{extraction factor}) \times (\text{H}_2\text{O wash factor}) \times 100
\]

The difference between the total acid insolubles content and
the lignin content gives the acid insolubles content of the starting material, i.e.,

\[
\text{% Acid Insolubles} = \text{(\% total acid insolubles)} - \text{(\% lignin)}
\]
A.2  Reducing Sugar Determination - DNS Method

Procedure:

A.2.1  0.5 ml of sugar or enzyme solution are transferred to a test tube.

A.2.2  1.5 ml of DNS reagent are added to the test tube. The mixture is mixed and covered with a steel cap.

A.2.3  The sample tube is boiled for 5 minutes then cooled.

A.2.4  To the test tube are added 10 ml of water.

A.2.5  Reducing sugars are determined colorimetrically at 600 nm and a 0.03 mm slit.

A.2.6  Standard solutions of glucose in the range of 0.5 to 2.5 mg/ml are run concurrently.
A.3 FPA (Filter Paper Activity) ANALYTICAL PROCEDURE (2)

A.3.1 Materials, Reagents, etc.:
1. 50°C bath.
2. 16 x 100 mm test tubes.
3. filter paper strips, 1x6 cm. (50 mg.); cut from #1 Whatman.
4. 0.05 M acetate buffer, pH 5.0.
5. Glucose standard solution, 2 mg/ml in d.H₂O.
6. DNS

A.3.2 Preliminary Preparation:
1. Roll filter paper strips into tight coils.
2. Dilute enzyme sample to give a final FPA of 0.3-0.5 mg/ml.
3. Dilute standard glucose solution to 2.0, 1.0 and 0.5 mg/ml concentrations.

A.3.3 Preparation of Test Tubes:
1. Activity Tubes.
   a) Add 1.0 ml of diluted enzyme to each of two test tubes containing 1.0 ml acetate buffer (this is 1:1 dilution = x 2)
2. Background Tubes (BKGD)
   a) Add 1.0 ml of diluted enzyme to a test tube containing 1.0 ml acetate buffer; cap tubes (enzyme BKGD).
   b) Add filter paper coil to tube containing 1 ml. H₂O + 1 ml. buffer (filter paper BKGD).
3. Standard Tube
   a) Add 1.0 ml. of standard to each of two test tubes containing 1.0 ml acetate buffer and cap tubes.

A.3.4 Incubation of Test Tubes:
1. Add a filter paper coil to each activity tube, so that the coil lies on its side completely submerged in the enzyme solution. Cap tubes.
2. Place all activity, background and standard tubes in a 50°C bath for 60 minutes.
3. Add 5 ml. DNS to each tube.
4. Vortex all tubes taking care to disintegrate filter paper in activity tubes.
5. Place all tubes in 100°C bath for 10 minutes, followed by cooling in ambient bath 3-4 minutes. Vortex.
6. Clear filter paper debris in activity tubes with a glass rod and transfer cleared solutions to new tubes.
7. Read absorbance at 600 nm., using d.H₂O as a blank.

A.3.5 Activity Calculation:

1. Plot absorbance vs. mg. glucose in standard tubes to obtain standard curve. (Figure A-1 shows a typical curve).
2. Determine mg. glucose in activity and BKGD tubes by use of standard curve.
3. Subtract mg. of enzyme and filter paper BKGD from mg. in activity tube to obtain net enzyme activity. Record as:

   \[ \text{FPA (x 1/dil)} \]

   e.g., 0.50 (x5 x2) if enzyme was initially diluted 1:4 (= x5) and net activity is 0.5 mg/ml.

**NOTE:** Dilution of the enzyme includes the dilution by addition of acetate buffer. If enzyme was originally diluted 1:9, the dilution is 1/20, since addition of buffer further dilutes the enzyme by 1/2.
Figure A.1. FPA Dilution Curve
Cellulase from T. reesei Rut-C-30
1 ml enz + 1 ml buffer + 1 x 6 cm Whatman #1 Filter Paper
50°C, 60 min static bath. Assayed by DNS, in situ.
A.4 Cellobiohydrolase (Activity Toward Cotton) ANALYTICAL

PROCEDURE (2)

A.4.1 The C₁ assay is identical to the FPA assay, with the following exceptions.

a) 50 mg weighed absorbent Red Cross cotton is used as substrate rather than filter paper rolled into compact ball.

A.4.2 a) Use FPA enzyme-BKGD for C₁-BKGD if both assays are performed concurrently. Differences in enzyme dilution for the two assays should be taken into account.

b) Use 50 mg. cotton in tube containing 1 ml H₂O + 1 ml buffer (cotton BKGD).

A.4.3 Incubation is for 24 hours at 50°C rather than 60 minutes. Rubber stoppers are used to prevent excessive evaporation during incubation.

A.4.4 The FPA standard curve is used to calculate C₁ activity.

A.4.5 Record as C₁ activity (x 1/dil) e.g., 0.75 (x10) if initial enzyme dilution was 1:4 and net activity is 0.775 mg/ml.

NOTE: To obtain approximately the same concentration of DNS sugars as is obtained in the FPA assay, enzyme for C₁ assay should be one half as concentrated as is for FPA.
A.5 B-1,4-Glucanase Activity (CMC ASE) ANALYTICAL ACTIVITY (2)

A.5.1 Materials, Reagents, etc.:

1. 50°C bath.
2. 16 x 100 ml. test tubes.
3. 2% CMC in acetate buffer.
4. DNS.

A.5.2 Preparation of Test Tubes:

1. Activity tube:
   a) Add 1.0 ml. CMC to a test tube.
2. CMC-BKGD tube:
   a) Add 1.0 ml. CMC and 0.1 ml. d.H₂O to a test tube and cap.

A.5.3 Incubation of Test Tubes

1. Place CMC-BKGD tubes in 50°C bath.
2. Turn timer on and quickly but accurately add 0.1 ml. enzyme to activity tube, swirl to mix and rinse dispenser tip several times in mixture. (This is a 1:10 dilution = x 10.)
3. Place the activity tube in the 50°C bath at the instant the timer reaches the first half minute.
4. At 30 minutes on the timer, remove tubes from bath and quickly 50 ml. DNS.
5. Place tubes in 100°C bath for 10 minutes, followed by cooling ambient bath 3-4 minutes. Vortex.
6. Read absorbance at 600 nm., using d.H₂O as a blank.

A.5.4 Activity Calculation:

1. The C standard curve is used to calculate mg/ml x glucose in the CMC-BKGD tube and activity tube. (Figure A-2).
Figure A.2. $C_x$ Dilution Curve.
1 ml enz + 1.0 ml 2% CMC
50°C, 30 min, static batch.
Cellulase from T. reesei Rut-C-30.
A.6 B-Glucosidase ANALYTICAL ACTIVITY (2)

A.6.1 Standards:

1. Serial dilute standard to 0.275, 0.55 and 1.1 mg/ml with d.H₂O.
2. Add 0.1 ml of each standard to 1.0 ml 0.025 M acetate buffer in separate test tubes to obtain 0.025, 0.050 and 0.100 mg/ml standards.
3. Subtract reagent blank (5 ml GOP + 1.1 ml 0.025 M acetate buffer for zero intercept.)

A.6.2 Enzyme Background (ENZ-BKGD):

1. Add 0.1 ml dilute enzyme and 1.0 ml acetate buffer to a test tube.

A.6.3 Cellobiose Background (CB-BKGD):

1. Add 1.0 ml cellobiose and 0.1 ml d.H₂O to a test tube.

A.6.4 Test Samples (Activity Tubes):

1. Add 0.1 ml dilute enzyme to 1.0 ml 1.25% CB in a test tube. (This is a 1:10 enzyme dilution = x 11.)

A.6.5 All of Above Tubes (use chronological procedure for activity tubes):

1. Place in 50°C bath for 30 minutes.
2. Add 5.0 ml GOP reagent.
3. Place in 35 to 40°C bath for 30 minutes.
4. Add 3 drops concentrated HCl to each tube while mixing by vortex.
5. Let stand for 45 minutes.
6. Read absorbance at 400 nm.

A.6.6 Activity Calculation:
1. The GOP standard curve is used to calculate mg/ml glucose in BKGD tubes and activity tubes. (Figure A-3). Subtract CB-BKGD and ENZ-BKGD from mg/ml in activity tube to obtain net enzyme activity. Record as:

Cellobiase activity (x 1/dil)

e.g., 0.090 (x 22) if initial enzyme dilution was 1:1 (= x 2) and net activity is 0.090 mg/ml.
Figure A.3. (β-g) Enzyme Dilution Curve.
40°C & 50°C, 15 & 30 minutes, static bath
0.1 ml enz + 1.0 ml 1.25% CB in Acetate Buffer
(CB#1). Assayed by GOP. Cellulase from T.
reesei Rut-C-30.
It is useful, for economic analysis, to determine how much of the original enzyme activity remains in solution after an enzymatic hydrolysis. A practical hydrolysis, however, produces DNS sugars in amounts which render the direct measurement of hydrolysate activities impractical. This situation can be remedied by adding an appropriate volume of acetone to the hydrolysate. Most of the enzyme will precipitate, while most of the DNS sugars will remain in solution.

The following acetone precipitation procedure is based primarily on the thesis research of Richard T. Ige, June 19774 (LBL-30577). Experience subsequent to this research has been incorporated.

Procedure:

A.7.1 Add 1 part enzyme and 3 parts acetone to a 15 ml Corex centrifuge tube. Gently invert the tube several times to mix. Allow the tube to sit at room temperature approximately 15 minutes.

A.7.2 Centrifuge at 10K rpm for 10 minutes.

A.7.3 Gently pour off and discard supernate.

A.7.4 Invert a cuvette dryer onto the Corex tube and apply vacuum until acetone appears to be removed (2-3 minutes). Over-drying or leaving tubes for extended periods (overnight) can result in difficulty of precipitate redissolution.
A.7.5 Immediately add appropriate volume of d.H$_2$O, containing 0.02% NaN$_3$, to Corex tube. Gently vortex and cap with parafilm or rubber stopper.

A.7.6 Allow precipitate to soak 2-3 hours, with gentle vortexing every 15-30 minutes.

A.7.7 Control curves should be obtained using the same enzyme as was used in the hydrolysis experiments. Control curves include an unprecipitated activity curve and an acetone precipitated activity curve. The curves are obtained at the same time and in the same manner as the hydrolysate samples.

The example relating initial DNS sugars vs. DNS sugars recovered by acetone precipitation should, likewise, not be used as a general standard, since this relationship has been found to be variable.

**Examples of IU recovery by acetone precipitation:**

- $I_P =$ initial activity, $I_R =$ activity recovered by acetone

**FPA:** 0.5 mg/ml = 0.0463 IU

- $I_R = 1.33 I_P - 0.1$, for $0.1 \leq I_P \leq 0.25$

**C$_1$:** 0.5 mg/ml = 0.00193 IU

- $I_R = 1.33 I_P - 0.01$, for $0.01 \leq I_P \leq 0.025$

**D$_X$:** 0.5 mg/ml = 0.0925 IU

- $I_R = 1.33 I_P - 4.0$, for $4.0 \leq I_P \leq 10.0$

**CD(-g):** 0.05 mg/ml = 0.00926 IU

- $I_R = 1.33 I_P - 0.177$, for $0.16 \leq I_P \leq 4.0$

(This example should not be used as a general standard.)
Example of DNS sugars recovered by acetone precipitation:

\[
\text{DNS sugars recovered} = 0.25 \times (\text{initial DNS sugars}) - 0.70
\]
A.8 Soluble Extra-Cellular Protein ANALYTICAL PROCEDURE

(2)

A.8.1 Initial Preparation of Reagent, Standard Tubes and Reaction Tubes:

1. Add 1 part NaK Tartrate solution to 100 parts CO₃ solution and mix well. (It is necessary to add NaK Tartrate before Cu⁺⁺.

2. Add 1 part Cu⁺⁺ solution to above and mix well, to complete SP reagent.

3. Dilution of bovine albumin stock solution: Serial dilution std to 1.0, 0.5 and 0.25 g/L in 1 ml aliquots.

4. Add 5 ml SP reagent to a number of spect. tubes appropriate to analyzing the required number of standards and samples. These tubes comprise the reaction tubes.

5. Dilute 2 N Folin reagent to 1 N in appropriate volume to complete assay.

A.8.2 Incubation of Standard and Sample Tubes

1. Add 1.0 ml of each standard and sample to separate reaction tubes, using a new dispenser tip for each. After addition of a standard or sample, swirl reaction tube to mix and wash dispenser tip two or three times in reaction tube. Vortex all tubes.

2. Allow all reaction tubes to set at room temperature for approximately 30 minutes.

A.8.3 Development of Reaction Tubes

1. While vortexing each reaction tube, quickly add 0.5 ml 1 N Folin phenol reagent, remove tube from vortex and quickly vortex again.
2. Allow tubes to set at room temperature for approximately 30 minutes.

3. Read absorbance at 700 nm. and plot standard curve.

4. Calculate SP in samples by use of standard curve.
References: Appendix A


APPENDIX B: HYDROLYSIS DATA
B.1 Reducing Sugar Concentration Vs. Time.
1. California Corn Stover-Acid Treated
2. Cellulase from T. reesei Rut-C-30

XBL819-6482
B.2.1. Component Sugar Concentration Vs. Time.
Cellulase 2.5 IU/ml T. reesei Rut-C-30 Indiana Corn Stover, Acid Treated.
Figure B.2.2. Component Sugar Concentration Vs. Time.

1. Cellulase from T. reesei Rut-C-30, 4.5 IU/ml.
2. Acid Treated Indiana Corn Stover.
Figure B.3.1  Sugar Concentration vs Time, Hydrolysis of Steam Exploded Corn Stover.
Figure B.3.2. Sugar Concentration Vs. Time, Hydrolysis of Steam Exploded Corn Stover.

2. Steam Exploded California Corn Stover, 15% by wt.
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