MASTER

FUNDAMENTAL STUDY OF THE MECHANISM
AND KINETICS OF CELLULOSE HYDROLYSIS
BY ACIDS AND ENZYMES

Progress Report
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ABSTRACT

This project deals with acidic and enzymatic hydrolysis of cellulosic materials. High lights of the first eight months of our project are as follows:

- (1) Essentially homogeneous C_1 , C_x , and Cellobiase enzyme have been isolated from Cellulase-Onozuka of Trichodeima viride origin. Besides the 3 major components, one protein of a molecular weight of 10,000 was purified, which has strong C_x activity and also another (M.W. 23,000) with strong C_1 activity.
- (2) Kinetics of Cellobiase has been investigated and its kinetics constant accurately determined. Immobilization of this enzyme on porous glass was successful.
- (3) Absorption of SO_2 at atmospheric pressure increased digestibility of delignated cellulose but not the natural materials such as corn stalk. A pressurizable absorption unit is being built.
- (4) Kinetics models for purified C_1 and C_x are postulated and equations derived. Experimental tests will be made when sufficient quantities of purified C_1 and C_x enzyme are prepared.
- (5) A lignin digesting, white-rot fungus, Pleurotus ostreatus has cultivated in our laboratory. It has grown well and heavy. Attempts will be made to extract and identify lignin digesting "enzymes".
- (6) A graduate student has been trained for using electro-microscopes. A number of micrographs were made with untreated cellulosic materials, which showed that the method of drying the specimens for electromicroscopy is important. Ordinary drying procedures will alter their physical structures. A technique, Critical point drying, is being practiced, which will not change the structure.

SCOPE OF INVESTIGATION

A two-stage process involving a chemical pretreatment followed by an enzymatic hydrolysis is speculated as an efficient way of converting cellulose into glucose and fermentable sugars. Cellulose hydrolysis is hindered by two major factors: (1) lignin interference and (2) cellulose crystalline structures. Another difficulty that one is often faced with in designing a process of cellulose hydrolysis is the lack of information regarding the kinetics parameters of the cellulolytic enzymes. Furthermore, these factors are not independent but interact with one another. For instance, the enzymatic reaction rate is strongly affected by the cellulose crystalline structures.

Since the initiation of this project on June 1, 1975, we have been concerned with research on the following fundamental problems:

(1) Isolation and purification of cellulase components

In order to elucidate the mechanism and the kinetics of cellulase reactions, we believe we should first deal with pure enzyme components. Essentially homogeneous C_1 , C_x and Cellobiase enzymes have been isolated in our laboratory. From the kinetics parameters of individual components, we will then study the joint action of enzyme mixtures on cellulosic materials.

(2) Acidic Pretreatment

Experiments have been conducted to absorb acidic gases such as SO_2 and HCl into cellulosic particles under various controlled conditions. The acids formed in situ will hopefully rupture lignin and disintegrate some of the cellulose bonds.

(3) Electronmicroscope Observation

To examine the effect of acid treatment and enzymatic attack on cellulose, we have been attempting to use electronmicroscope

for visual observation. Currently, we are testing techniques to distinguish cellulose from lignin on electronmicrographs. In addition to the above research plans as originally proposed, another area of research has been added to our program.

(4) Microbial degradation of lignin

Recent publications have shown evidence of coupled degradation of cellulose and lignin in wood rotting fungus. Cellulose has been found to enhance lignin degradation. A white rot, lignin digesting, fungus, Pleurotus Ostreatus has been cultivated in our laboratory. This organism can grow reasonable fast (in fact very fast comparing with other wood rotting fungi). We plan to extract from the organism and identify lignin digesting "enzymes".

Results of Project Research

(1) Isolation and Purification of Cellulase Components

Essentially homogeneous C_1 , C_x , and Cellobiase enzymes have been successfully isolated from a commercial cellulase preparation, Cellulase-Onozuka. Figure 1 shows the electrophoresed bands on SDS-polyacrylamide gels of the purified fractions.

Before reporting the details of the purification procedure, it is useful to describe how the verions C_1 , C_x and Cellobiase enzymes are identified and assayed.

(1-1) Enzyme Identification

A. Cellobiase or beta-glucosidase activity was detected by its ability to hydrolyze p-nitrophenyl-glucoside to form glucose and p-nitrophenol. Confirmation tests were then carried out using pure cellobiose as a substrate.

B. C_x cellulase or endo-1,4 glucanase was identified by its ability to hydrolyze high-polymerized cellulose (methyl cellulose, water soluble) and by its inability to hydrolyze microcrystalline cellulose (Avicel of FMC Corp.) in a short time incubation.

C. C_1 cellulase or exo-1,4 glucanase was identified by its high capacity to attack microcrystalline cellulose, Avicel, and to release cellobiose and glucose which were confirmed and identified by silica gel thin-layer chromatography. The enzyme activity assay were done according to procedures given in Appendix 1.

(1-2) Isolation Procedure

A crude cellulase enzyme (lot no. WR 1432) was obtained from Enzeco which retails the Japanese enzyme product called Cellulase-Onozuka derived

Figure 1. Homogeneous Cellulase Components Electrophoresed on SDS - Polyacrylamide Gels.

a. C_1 , b. beta-glucosidase
c. low molecular weight C_x , d. C_x .

from Trocoderma viride by surface culturing. Eleven grams of crude cellulase powder were suspended in 150 ml 0.01 M sodium phosphate buffer at pH 6.0 and then centrifuged to pellet down the insoluble materials. The supernatants were brought to 80% saturation of ammonium sulfate to precipitate proteins which was collected by centrifugation and redissolved in 25 ml of the same buffer. Five ml portions of the protein solution were passed through a Sephadex G-25 column (1.5 x 42 cm) to separate salts from proteins. The resulting solution was then concentrated by pressing through Dalflo ultrafiltration with non-cellulosic membranes UM 10 and PM 10 (Amicon Corp.).

About 50 mg crude cellulases from above were fractionated on a DEAE-Sephadex (A-25) column (2X25 cm). Three enzyme components were resolved by elution with a linearly increased concentration of sodium phosphate buffer of pH 6.0 from 0.01 M to 0.5M. The elution results are shown in Figure 2. Enzyme activity assay of the fractions showed that some cross-contamination of cellulase components still exist.

The cellulase enzyme fractions from the DEAE-Sephadex column were pooled, then concentrated by 80% ammonium sulfate, followed by Sephadex G-25 gel filtration for de-salting and then concentrated by Diaflo ultrafiltration. The concentrated enzymes were then added individually to a hydroxylapatite gel (Bio-rad) column for further purification. The 2x20cm column was eluted with a linearly increased concentration of the sodium phosphate buffer. Results given in Figure 3 indicate that after the hydroxylapatite column separation, cross contamination of cellulase components no longer exist. The measurement of optical density (OD) at A_{540nm} gives the C_1 activity with Avicel as the substrate. The OD measurement at A_{280nm} indicates the various protein fractions. The protein fractions

Figure 2. DEAE-Sephades (A-25) column chromatography of crude cellulases.

Figure 3 Hydroxylapatite column chromatography of cellulases

from Fraction 15 to Fraction 22 contain essentially C_1 activity only.

(1-3) Further Identification of the Individual Components

The enzyme fractions from hydroxylapatite column were then pooled and concentrated by Diaflo ultrafiltration. The concentrated enzymes were applied to a Bio-gel P-150 column (1.5 x 42 cm) which was eluted with 0.01 M sodium phosphate buffer. The OD measurement at A_{420nm} given in Figure 4 indicates the cellobiase activity by the p-nitrophenyl-glucoside assay. The peak activities (assay results not shown in Fig. 4) of other cellulases and the peak fractions ($OD A_{280nm}$) of marker proteins (of known molecular weight) are also indentified in Fig. 4. There seems to have an cellulase component which shows C_x activity having a molecular weight of about 10,000.

The peak enzyme fractions from Bio-gel P-150 gel filtration were precipitated by 5% trichloroacetic acid (TCA), dissolved in 0.1% sodium dodecyl sulfate (SDS) sodium phosphate buffer and dialyzed against the same buffer overnight. The dialyzed proteins were further denatured in boiling water for 10 minutes in the presence of 5 mM beta-mercaptoethanol. The denatured protein were then electrophoresed individually on SDS-polyacrylamide gels. The results are shown in Fig. 1. The cellobiase showed a major protein band at a molecular weight of 53,000. C_x appeared to be a single protein band at a molecular weight of 40,000; while C_1 showed a major component with a molecular weight of 48,000 and a minor band. The molecular weight estimates were done with marker proteins of known molecular weight as shown in Fig. 5.

(1-4) Summary of Enzyme Purification

The above described procedures are diagrammed in Fig. 6. The degrees of purification achieved at various steps are summarized in Table 1. The specific activity of beta-glucosidase increased from 17 OD/mg protein to

357 OD/mg protein. The specific activity of C_x showed little change throughout the procedure; while C_1 activity actually reduced from 123 OD/mg protein to 15 OD/mg protein. This reduction in specific activity is likely due to synergistic effects of cellulase components when present together in crude preparations.

Figure 4. Gel filtration of cellulases on Bio-gel P-150.

solid line: protein concentration

dotted line: cellobiase activity

arrows also showing marker proteins of known molecular weights

FIGURE 5. ESTIMATION OF MOLECULAR WEIGHT OF MAJOR PROTEIN BANDS PRESENT IN THE PURIFIED ENZYME FRACTIONS FROM BIO-GEL P-150 GEL FILTRATION

Fig. 6. A schematic representation of the procedures used for the purification of cellulases from commercial crude cellulases preparation of Trichoderma viride

TABLE 1

The partial purification of cellulases from crude cellulases of *I. viride*

Fractions	step ^a	Specific ^b activity (OD/mg protein)	Total protein (mg)	Total activity	Fold Purifi- cation	Recovery ^b (%)
<u>B-Glucosidase</u>						
Soluble protein	2	17	2,310	39270	1	100
Sephadex G-25	4	29.7	1,483	44045	1.75	112
DEAE-Sephadex	6b	85.2	344.4	29343	5	74.7
Hydroxylapatite	8b	207	130.5	27013	12	68.8
Bio-gel P-150	9b	357	62	22134	21	56.4
<u>C_x cellulase</u>						
Soluble protein	2	4.8	2,310	11088	1	100
Sephadex G-25	4	6.36	1,483	9432	1.3	85
DEAE-sephadex	6a	5	114.5	572	1.04	5.2
Hydroxylapatite	8a	5.23	34	177.8	1.09	1.6
Bio-gel P-150	9a	5.7	20	114.	1.19	1
<u>C₁ Cellulase</u>						
Soluble protein	2	123	2,310	284130	1	100
Sephadex G-25	4	78	1,483	115674	0.634	40.7
DEAE-Sephadex	6c	55	342.5	11837	0.45	4.2
Hydroxylapatite	8c	9.4	120	1128	0.076	0.4
2nd DEAE-Sephadex	9c	12.9	80	1032	0.105	0.3
Bio-gel P-150	10c	15	56.3	844	0.122	0.3

a. These numbers refer to the steps in Fig. 6

b. See Methods section in Appendix for the definition of specific activity of each enzyme.

(1-5) Low Molecular Weight Cellulose Components

Two additional low molecular weight cellulase components have also been purified from crude cellulases. One which has a molecular weight of about 10,000 was obtained after gel-filtration of beta-glucosidase (step 8b, Fig. 6) on Bio-gel P-150. This protein component exhibited a high ability to hydrolyze methyl cellulose (produce 2.65 OD A_{540nm} reducing sugar per mg protein) but has no apparent capacity to attack microcrystalline cellulose. The other protein component was separated from C_7 cellulase (step 8c, Fig. 6) on a DEAE-sephadex column with a linear salt elution (step 9c, Fig. 6). This protein has a molecular weight of 23,000 when analyzed on SDS-gel electrophoresis and exhibited a high ability to attack microcrystalline cellulose (produced 15 OD A_{540nm} reducing sugar per mg protein). Whether these two enzymes are part of the cellulases enzyme system produced by the fungus I. viride, or if they are instead an artifact due to the proteases action on the original cellulases remains to be seen. The protease activity of the fractions throughout the purification process (Table 2) was appreciable in each and every fraction (up to step 6; Fig. 6) during the early stage of purification. The control experiments yet to be done which will include a protease inhibitor in the buffer during the purification processes to determine the origin and the significance of these low molecular weight cellulase components which might be involved in the hydrolysis of native cellulose. If these low molecular weight proteins are true cellulase components, we will also examine their possible role in "de-crystallization" of native cellulose.

Table 3 gives the various fractions of cellulase component from the present work as a summary. The success of our project in quickly achieving a high degree of purification of these cellulase components, which often have taken several years in some foreign laboratories, should be largely

Table 2. Protease Activity of Some of the Protein Fractions

Fractions	Step ^a	Relative Activity (A _{520nm} /mg protein)	Total Activity	Amount of protease ^b (ug protease/mg ptotein)
Soluble protein	2	1.528	3529.7	12
Sephadex G-25	4	1.976	2930.4	15.8
DE-1	6a	2.824	972.6	22.6
DE-2	6b	2.432	278.5	19.5
DE-3	6c	0.624	213.7	5

a. These numbers refer to the steps in Fig. 6.

b. Approximately eight μ g of protease (Calbiochem) is capable of releasing one OD A_{520nm} of dye from Azocoll when incubated the reaction mixture at 37 C for 15 min.

Table 3. Summary of the Activities and Molecular Weights of Cellulases

Enzyme	Amount (mg)	Specific activity (OD/mg protein)	Molecular weight
Glucosidase	62	357	53,000
C _x cellulase	20	5.7	40,000
C ₁ cellulase	56.3	15	48,000
Low M.W. cellulase (C _x)	5.6	2.7	10,000
Low M.W. cellulase (C ₁)	10	15.2	23,000

credited to the contribution of Dr. C.S. Gong who has worked as a Research Associate for our project since Oct. 1, 1975 and who has had a great deal of experience in enzyme purification.

2. Enzyme Kinetics

2-1 Cellobiase kinetics

A purified cellobiase preparation was obtained from Step 8b of Figure 6. It contains 4.85 mg/ml protein. Cellobiase hydrolyzes cellobiose, the dimer, to monomeric glucose. Cellobiose reportedly has a product inhibition effect on C_1 . Thus, removal of cellobiose by converting it into glucose would be encouraged in order to enhance the overall hydrolysis process.

The method used to study the kinetics was to combine a 0.5 ml each of substrate (cellobiose) and buffer (0.1 M citrate, pH 4.8), heat to 30°C, add 0.2 ml cold (0°C) enzyme, incubate in a water bath at 30°C, and then measure glucose formed as a function of time using a glucose analyzer.

Results thus obtained are described below:

Extent of Reaction: As shown in Table 4, the reaction goes to completion given sufficient time.

Time Course: The extended time course of the reaction is shown in Figure 7 at various substrate levels. The initial cellobiase activity is shown in Figure 8.

Kinetic Constants: A Lineweaver-Burk plot of data taken from Figure 8 is shown in Figure 9. From a least squares fit, estimates of the kinetic constants are:

$$k_m = 2.51 \text{ mM (cellobiose)}$$

$$v_{\max} = 1.0 \frac{\text{nmole}}{\text{min}} \text{ (as glucose)}$$

Product Inhibition: Using reaction velocities obtained by estimating tangents to curves in Figure 7, an attempt was made to verify the existence of possible product inhibition. The data points obtained, plotted in Figure 9, are too scattered to draw any conclusion. This experiment will be repeated using a more sensitive glucose detection method.

Table 4. Extent of Reaction @ 210 Minutes

Initial cellobiose Concentration		Glucose Equivalent ^b	Glucose formed @ 210 minutes	% of Maximum Glucose Possible
mM	Total ^a			
15	18 moles	36 moles	26.3	73
7.5	9	18	16.5	91.7
3.75	4.5	9	9.67 2%*	107.
1.5	1.8	3.6	3.93 6%*	109.

* Error in measurement due to insensitivity of glucose analyzer at low glucose levels.

^a Based on 1.2 ml. total incubation volume

^b Glucose formed if all cellobiose $\rightarrow$ glucose

Assay Conditions:

T = 30°C

pH = 4.8 w/41.8 mM citrate buffer enzyme: 0.81 mg protein/ml

(prep. G1)

FIGURE 7 GLUCOSE FORMED AS A FUNCTION OF TIME

FIGURE 8 INITIAL CELLOBIASE ACTIVITY

FIG 9 Lineweaver-Burk Plot
For Cellobiase

Comparison of Specific Activity:

Another purified cellobiase preparation was obtained from Step 8b of Figure 6 containing 7.2 mg protein/ml. The specific activity of this preparation was found to be 5.5 EU/mg protein. This is more than 3 times more active than the purified cellobiase reported by Shikata and Nisizawa (in Ref. 1 with 1.67 EU/mg protein).

Immobilization of Cellobiase and Reaction of Immobilized Cellobiase

As mentioned before, it might be desirable to add excess cellobiase in a cellulose hydrolysis process in order to remove cellobiose-inhibition on C_1 enzyme. For economical reasons, addition of cellobiase immobilized on a solid support can aid in recycling of the enzyme.

By following the procedure of Weetall (2), cellobiase from Step 8b of Fig. 6 was successfully immobilized on Corning porous glass coated with CrO_2 in the alkylamine form. The detailed chemical procedure is now more or less standard and will not be reported here. The results in Fig. 10 A and B give a preliminary indication of the advantage of immobilized enzymes. The soluble cellobiase tests (Fig. 10A) were conducted in a flask, while the immobilized cellobiase on glass particles was packed in a small column. The productivity of the packed column reactor with cellobiose solution flowing through the column is about 75% of that of free enzyme. But, at the end of the reaction, the immobilized enzyme was still intact and could be used over and over again by simply pumping more cellobiose solution through the column reactor; while in the case of free enzyme, it would be very difficult and expensive, if at all successful, in recycling the enzyme.

(2-2) C_1 kinetics

In investigating cellulose hydrolysis, there are currently three types

of chemical analyses available for us to follow the reaction: (1) glucose determination, (2) reducing power determination and (3) product distribution of up to G_{12} (i.e. glucose polymers of a degree of polymerization (DP) of 12). Glucose determination is done by a glucose analyzer which is specific for the monomer. Reducing power can be done by Fehling solution titration. Since each molecule of glucose polymers has only one reducing group (the chain terminal, number one carbonyl group), the reducing power measurement reflects the molar concentration of total glucose polymers. The product distribution can be determined by a high pressure, liquid chromatography (LC), a fairly expensive instrument, which has been recently purchased and installed for our project. In developing kinetics models, one is to relate kinetics constants to some quantities that can be measured experimentally. Actual experiments will then test the "correctness" of the models and determine the kinetics constants. If we had unlimited analytical capability, philosophically speaking, kinetics would never be a problem.

In dealing with C_1 kinetics, we have adapted an approach that is based upon product distribution, glucose and reducing power measurements.

First the following reactions are assumed

For the above reactions, one can write, according to Michaelis-Menten kinetics

$$\begin{aligned}\frac{d(G_{n-2})}{dt} &= \frac{k_3(C_1)_0(G_n)}{K_m + S} \\ \frac{d(G)}{dt} &= \frac{2 k_3(C_1)_0(G_n)}{K_m + S} \\ \frac{d(G_n)}{dt} &= k_{-1} \frac{(C_1)_0(G_n)}{K_m + S} - k_{+1}(G_n)(C_1)\end{aligned}\quad (1)$$

where (G_n) is the concentration of polymer of a DP of N, $(C_1)_0$ is the total C_1 enzyme concentration, (C_1) is the concentration of C_1 enzyme in free uncoupled form. The term S has the following meaning

$$S = \sum_{N=3}^{\infty} (G_n) = \sum_{N=1}^{\infty} (G_n) - (G_2) - (G)$$

Since we have assumed excess cellobiase, (G_2) should be zero. Therefore

$$S = \sum_{N=1}^{\infty} (G_n) - (G)$$

The 1st term on the right side of the above equation is the total reducing sugar which can be measured by Fehling solution titration. The 2nd term is the glucose concentration which can be measured by the glucose analyzer. Therefore

$$S = \text{Reducing power expressed in glucose equivalent, DE} - (G)$$

S is thus a measurable quantity but it varies as hydrolysis proceeds.

When a mixture of glucose polymers of a distribution of different DP is the substrate, we will write the following summed expressions

$$\begin{aligned}\frac{d(G_n)}{dt} &= \frac{k_3(C_1)_0(G_{n+2})}{K_m + S} - \frac{k_3(C_1)_0(G_n)}{K_m + S} \\ \frac{d(G_{n-1})}{dt} &= \frac{k_3(C_1)_0(G_{n+1})}{K_m + S} - \frac{k_3(C_1)_0(G_{n-1})}{K_m + S}\end{aligned}\quad (2)$$

$$\frac{dG}{dt} = \frac{k_3(C_1)_0}{K_m + S} [2S + (G_4) + 1/2 (G_3)]$$

Equations (2) can be numerically integrated if given the initial product distribution and can be tested against actual experimental data.

Equations (2) do have the following analytical solutions by applying Laplace transform for some extreme cases.

$$\begin{aligned}(G_{n-2r}) &= \sum_{q=0}^r \frac{A^q}{q!} G_{(n-2r)+2q}^{\circ} e^{-at_t q} \\ (G_{n-2r-1}) &= \sum_{q=0}^r \frac{A^q}{q!} G_{n-2r-1+2q}^{\circ} e^{-at_t q}\end{aligned}\quad (3)$$

$$\begin{aligned}G_1 &= a \int_0^t e^{-at} \left[\sum_{r=0}^{\frac{n}{2}-3} \sum_{q=0}^r (G_{n-2r+2q}^{\circ} + G_{n-2r-1+2q}^{\circ}) \frac{A^q}{q!} t^q \right. \\ &\quad \left. + \sum_{q=0}^{\frac{n}{2}-2} \frac{t^q a^q}{q!} (4G_{4+2q}^{\circ} + 3G_{3+2q}^{\circ}) \right] dt + G^{\circ}\end{aligned}$$

where G_n° is the initial concentration of G_n with n being the largest initial DP and a is equal to $k_3(C_1)_0/K_m$. For Equations (3) to hold, we need

$K_m \gg S'$, a condition likely to exist in the cases of natural, wood rotting microorganisms and in fermentation on cellulose substrates where as soon as solid cellulose is solubilized, the soluble carbohydrates will be quickly assimilated by the microbial cells and thus S is at all times very small.

As soon as sufficient quantities of purified C_1 and cellobiase are prepared, the above equations can be tested against actual experimental results.

(2-3) C_x kinetics

C_x enzyme supposedly exerts random cleavage of glucosidic bonds of cellulose polymers. The following reaction model is assumed

For the above reactions, one can write

$$\frac{d(G_i)}{dt} = k_2 (G_j C_x) - k_1 C_x (i-1) (G_i) + 2k_3 \sum_{n=i+1}^{\infty} \frac{(G_n C_x)}{n-1}$$

the factor $(i-1)$ in the 2nd term on the right hand side of the above equation is a weight factor for the $(i-1)$ - bonds, each of which may break.

The factor $2/(n-1)$ in the 3rd term is to reflect the fact that for a n -mer, a cleavage has to take place at two of the $(n-1)$ bonds in order to form an i -mer, i.e. counting from one end, the i th and the $(k-i)^{th}$ bond. Assuming that the concentration of $(G_i C_x)$ complex is at a quasi steady state, we can derive

$$d(G_i) = - \frac{k_3 (C_1) (i-1) (C_x)_0}{\sum_{n=2}^{\infty} (n-1) G_n + K_m} + \frac{2k_3 (C_x)_0 \sum_{j=i+1}^{\infty} (G_j)}{\sum_{n=2}^{\infty} (n-1) G_n + K_m}$$

The above equation can be solved numerically if given the initial product distribution.

(3) Pretreatment by Acidic Gases

Preliminary screening tests have been carried out in ordinary vacuum desiccators. Tests have been so far limited to gases at atmospheric pressures or less. A continuous pressurizable column reactor is being designed for tests at higher pressures. The present simple experimental set-up is shown in Figure 11.

In our experiments, cellulosic raw materials were subjected to acidic gases at constant temperature and moisture for a controlled length of time. After the exposure, the solid samples were submerged in a standard cellulase solution (one gram of cellulase-onozuka per 100 ml). Glucose contents of the solution before and after the enzyme treatment were determined with a glucose analyzer. Large increase in glucose content by the enzyme treatment is judged as a criterion of a good acid pretreatment. Even though reducing power measurement rather than glucose alone will be more indicative of the achievement of the enzyme hydrolysis, the use of glucose analyzer in glucose analysis is fast (takes about one minute) and is suitable for screening purpose.

Results up to now indicate that exposure to SO_2 gas does increase the digestability of cellulose (filter paper and solka floc, a commercial de-ligninized cellulose). But, atmospheric SO_2 treatment of natural materials such as sawdust and corn crop waste produced no appreciable increase in glucose formation. It seems that atmospheric SO_2 treatment has not been able to rupture lignin to the extent that would aid cellulose digestion by enzymes. Literature information suggests that probably SO_2 at higher pressures would be needed.

(4) Electronmicroscopic Observation

To prepare electronmicroscopic pictures is, itself, a technique that

FIG.11 GAS ABSORTION APPARATUS

one has to master before one can interpret the results on micrographs. Since the initiation of our project, a graduate student, Miss R. Amick, has taken special trainings to prepare specimens and to take electron-microscopic pictures.

Shown in Fig. 12 are pictures of dried but otherwise untreated corn stalk specimens at 800 magnification (800x) and 10,000 x. Unless we have means to distinguish lignin from cellulose, it will be difficult to visually observe the effects of acids and enzymes. All specimens need to be dried before they can be prepared for picture taking. The method of drying is very important. The physical structure of the cellulosic material will be altered by ordinary drying method. For instance, Figure 13 shows results of (a) air drying, (b) replacing water with alcohol and then air drying off alcohol and (c) replacing water with acetone and then air drying off acetone. In the case of acetone, there seems to have considerable structure collapse.

Presently, we are practicing with the technique of critical point drying in which water is replaced by liquid CO_2 which is then removed by evaporation at its critical temperature. This technique supposedly will leave the physical structures of the specimens essentially unaltered.

(5) Cultivation of Lignin Digesting Microorganism

Pleurotus ostreatus is a white-rot (lignin digesting leaving white colored cellulose) wood rotting fungus. It grows on dead trees and its fruiting body is commonly known as "oyster mushroom" which is edible and delicious. The organism has been cultivated in our laboratory on wet sawdust and also in submerged culture. It can grow quite fast and heavily.

For quantitative studies, some of the difficulties in dealing

800x

10,000x

Figure 12. Electronmicrographs of cross-sections of corn stalks

a

b

c

Figure 13. Micrographs of corn stalk sections (300x)

- a. air drying
- b. alcohol replacing water and air drying off alcohol
- c. acetone replacing water and air drying off acetone

with microbial growth on solid substrate are reproducible methods of making uniform inoculation and reliable methods of measuring growth. To solve the first problem, we have cultivated a large batch of this organism in shaker culturing, harvested cells, dispersed the mycelium type of cells with a Waring Blender into homogeneous suspensions, frozen and stored the suspensions in freezers in small vials. When needed, they are used as inoculants. For quantifying growth, we plan to measuring either nucleic acid or protein content of the cells after rupturing with ultrasonic treatment and extracting with a buffer. The testing procedures are being worked out.

Compliance with Contract Requirements

Our work as reported in the last chapter complies with the contract requirements except on two accounts:

(1) We have added the work on cultivation of a lignin digesting micro-organism, Pleurotus ostreatus. The growth has been heavy and we plan to extract and identify lignin digesting "enzymes" from the culture.

(2) Original plan involves the use of a electromicrobalance in measuring HCl absorption into cellulosic materials. The corrosiveness of the gas makes it difficult with the highly precise microbalance. We have changed plans to use a standard enzyme solution to examine the success of acid absorption on rupturing cellulosic structures and increasing its digestibility (by the enzyme).

Time and Effort of Investigators

(1) Principle Investigator: Dr. George T. Tsao, spends about 10% of his time on this project. He is concerned with the general direction of the project, coordination of project research, literature, discussion at weekly group seminar on cellulose hydrolysis and preparation of reports and publications.

(2) Research Associate: Dr. C.S. Gong spends 100% of his time on this project. His main concern has been the purification of cellulase components and their characterization.

(3) Research Assistants: Mr. Mike Ladisch, a senior graduate student, is concerned with purification of larger quantities of cellulase enzymes and kinetics and immobilization of cellobiase. Miss R. Amick, Mr. A. Heubner, Mr. C. Hong and Mr. F. Taylor are all first year new graduate students. They have been concerned with kinetics models, electronmicroscope observation, and the lignin digesting microorganism, Pleurotus ostreatus. Mr. Ladisch is a research fellow and spends about 50% of his time on research. Miss Amick and Mr. Hong are teaching assistants and they each spend currently about 25% time on cellulose project. Mr. Heubner and Mr. Taylor are research assistants and each spends 50% time on the project.

The level of effort is expected to remain about the same for the rest of the project year. The 1st year graduate students, after completing more course work, will become more involved in research on cellulose.

Technical Reports

Since the project has been only 8 months old, no publication has yet been in print. A paper on purification of cellulase component is being prepared. A second paper on kinetics and immobilization of highly purified cellobiase is also in preparation. A paper for oral presentation on utilization of cellulosic waste is on the program of the Annual Conference of Industrial Microbiology to be held in August, 1976. Copies of these papers will be submitted to ERDA as soon as they become available.

APPENDIX(I.) Procedures of Enzyme Activity Assay

- (1) Cellobiase (beta-glucosidase): The reaction mixture contained 0.05 ml of 0.0136M p-nitrophenyl beta-glucoside, 0.1 ml of 0.1M sodium acetate (pH 4.5) and 0.05 ml of enzyme solution. After 3 minutes of incubation at 30 C, 5 ml of 0.1 M sodium carbonate was added to stop the reaction. The liberated p-nitrophenol was measured at $A_{420\text{nm}}$. The enzyme activity was expressed in terms of optical density OD at 420 nm per milligram of proteins. In the control experiments, p-nitrophenyl-alpha-glucoside was used to replace p-nitrophenyl-beta-glucoside.
- (2) Microcrystalline cellulose (Avicel) hydrolysis assay: The reaction mixture which was placed in corex tubes each containing 50 mg of Avicel powder, 2 ml of 0.1 M sodium citrate (pH 4.8) and 2 ml of enzyme solution is 0.01M Na-phosphate. The tubes were incubated for 20 minutes at 48 C with continuous stirring using a stirring bar and then were centrifuged at 5000 rpm for 5 minutes. The reducing sugar which was released into solution was measured by the methods of Somogyi (3) and Nelson (4). The activity was expressed as the amount of absorption at $A_{540\text{nm}}$ per milligram of proteins per assay tube.
- (3) Methyl cellulose hydrolysis assay: The reaction mixture contained 0.5 ml of 1% methyl cellulose in 0.1 M sodium citrate (pH 4.8) 0.5 ml enzyme solution in 0.01 M Na Phosphate. After 4 hours of incubation at 48 C, the reducing sugar produced was determined as described above.

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