THE USE OF CELLULOSE STRUCTURAL PARAMETERS TO EVALUATE PRETREATMENTS FOR ENZYMATIC HYDROLYSIS

by

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B.S., Kansas State University, 1977

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemical Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1979

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I. INTRODUCTION

The world depends extensively on fossil fuels for energy and chemicals. Consequently, the supply of petroleum, natural gas, and coal is dwindling rapidly, and many forecasters predict the depletion of these resources and our concurrent doom within the next 200 years. In the meantime, it would seem advantageous to consider alternative sources of energy and chemicals. The world community has realized the necessity for the development of these sources, and the scientific community has committed itself to the challenge. The current sources of energy and chemicals have resulted largely from the ancient deposit of plant materials in the earth and represent an accumulation of fixed solar energy. The future sources must result from the exploitation of recently generated plant materials that represent an efficient accumulation of fixed solar energy.

The traditional utilizations of plant materials, such as burning as fuel, papermaking, construction, and textiles must be augmented if the void left by the exhausted fossil fuels is to be filled. Innovative methods of plant material utilization are already technical realities and will eventually become economically feasible. Included in these methods are pyrolysis, gasification, anaerobic fermentation, and cellulose bioconversion. This work has focused on a specific aspect of the latter.

The biological conversion of cellulosic materials can follow different paths and result in different products. Acid hydrolysis of cellulosics to glucose has the advantage of a short reaction time. However, enzymatic hydrolysis appears to be more promising than acid hydrolysis due to larger yields, lack of formation of unwanted by-products, reaction specificity to

cellulose, low temperature and pressure of operation, and the noncorrosive nature of the reactants.

Several feasible enzymatic hydrolysis processes have been investigated. A process utilizing a thermophilic actinomyces has been developed to produce single cell protein (SCP) from cellulose (Armiger, et al., 1976). At least part of the enzymes used in this process are cell-wall-bound, necessitating contact of the cells and the substrate. Another process aims at producing glucose from its polymer, cellulose, through hydrolysis by extracellular enzymes obtained from the fungus Trichoderma reesei (formerly T. viride). This fungus can be induced to secrete large quantities of cellulolytic enzymes in a preliminary cultivation. These enzymes are completely extracellular and can be separated from the cell mass to produce an enzyme broth. This potent enzyme broth may be used to hydrolyze cellulose to glucose in the complete absence of microorganisms. The process using extracellular enzyme is versatile because the produced glucose may be used to generate either SCP, ethanol or fructose through subsequent processes. However, the structural complexity of cellulose makes it resistant to enzymatic hydrolysis.

Cellulosic plant materials are comprised mainly of cellulose, hemicellulose, and lignin. Cellulose is a polymer of the six-carbon reducing sugar, glucose, and appears in amorphous and crystalline forms. The crystalline form is highly resistant to hydrolysis due to its structural integrity. Hemicelluloses are polymers of five-carbon sugars, e.g., xylose, and are very reactive compared to cellulose. Lignin is a complex phenolic compound that acts as the "cement" in cellulosic materials. The intimate relationship of hemicellulose and lignin with cellulose increases the resistance of cellulosics to hydrolysis. In order for an enzymatic

hydrolysis process to be successful, the structural integrity of the cellulose must be destroyed and the negative effects of the associated materials, especially lignin, must be neutralized. This can be accomplished through a judicious choice of pretreatment methods.

The best sequence of pretreatment procedures must meet several requirements. The high degree of crystallinity that is typically exhibited in cellulose must be reduced substantially. Due to the heterogeneous character of the enzyme-cellulose reaction, the surface area of the cellulose available for reaction must be increased. In addition, lignin must be removed from its position in the complex structural matrix of natural cellulosics. All of these aspects must be a part of the cellulose pretreatment scheme to afford a substantial hydrolysis rate increase.

This section reviews all pertinent aspects of enzymatic hydrolysis; cellulase production, the hydrolysis reaction, cellulose structure, and pretreatments. The objectives of this thesis and their rationale are also presented.

A. Review

Cellulase production. Intensive work has been completed on the optimization of cellulase production by a Trichoderma reesei mutant strain, QM 9414 (Mandels, et al., 1975; Sternberg, 1976; Ghose, 1977). This organism was chosen for study because it produces large and highly active amounts of all components of the cellulase enzyme system. The optimal cultivation conditions have been established to a great degree. Optimums in pH, temperature, nutrient concentrations, substrate concentration, surfactant concentration, agitation, etc. for laboratory cultivation are known and employed. Large improvements in enzyme yields are now looked for through the discovery of further mutations of the organism and the discovery of suitable cellulase inducers.

Enzyme hydrolysis. The enzyme hydrolysis of cellulose is a complex reaction, and many factors affect its rate. Type of substrate, degree of pretreatment, the character of the enzyme preparation, temperature, pH, concentration of substrate, product inhibition, and type of reactor are all interdependent factors which contribute to the hydrolysis process. The hydrolysis procedure used in this work was similar to that used by Mandels, et al. (1974) to evaluate pretreatment methods. It represents the optimum hydrolysis conditions for a laboratory shaker flask hydrolysis.

On a larger scale, the enzymatic hydrolysis process presents a challenge to the plant designer. Process design schemes have been generated for large scale plants (Wilke, et al., 1976; Allen, 1976). The accurate design of the plants is difficult because little work has been done on modeling the kinetics of the hydrolysis reaction due to its inherent complexity. Consequently, data have been used to develop the process schemes. The results of the economic evaluations suggest two major areas whose costs sorely need to be reduced: enzyme production and substrate pretreatment.

Cellulose structure. Cellulose is a high molecular weight linear polymer composed of D-glucose residues joined by ß-1,4-glucosidic bonds (Sihtola and Neimo, 1975). In native cellulose, up to 10,000 ß-anhydroglucose residues are linked to form a long chain molecule. The length of the anhydroglucose unit is .515 nm; therefore, the total length of the native cellulose molecule is about 5 µm. The degree of polymerization of wood pulp and filter paper cellulose ranges from 500 to 2,100. The anhydroglucose units in cellulose adopt the chair configuration and every other chain unit is rotated 180° around the main axis. The glycosidic linkage and the hydroxyl groups mainly determine the chemical properties of cellulose.

Cellulose, hemicellulose, and lignin are the three principal components of the structural fibers of plants (Cowling and Kirk, 1976). The relative amounts of these materials vary with the plant source. Cotton contains up to 95% cellulose, 5% hemicellulose, and no lignin. The stems of grasses contain 25-40% cellulose, 25-50% hemicellulose, and 10-30% lignin. Wood fibers contain 40-55% cellulose, 25-40% hemicellulose, and 20-35% lignin. Wood fibers that have undergone a sulfite bleaching process are typically 99%+ cellulose.

A wood fiber is basically the remains of a wood cell wall and is typically 15 to 20 μm in diameter and about 150 μm long. The primary wall, composed largely of hemicelluloses, forms an encasing layer over the three secondary lamellae. The lumen, or central canal is filled with mostly proteinaceous material. The bulk of the cellulose fiber consists of the secondary lamellae which together are about 4 μm thick (the primary wall is about 0.1 μm thick). The cellulose and other constituents in the secondary layers are aggregated into long slender bundles called microfibrils. They are distinct entities with few, if any, cellulose molecules crossing over from one microfibril to the next. Within the microfibril, cellulose molecules are aligned in a parallel manner and great stability results from hydrogen bonding between hydroxyl groups on adjacent molecules.

The structure of the microfibril is still a matter of debate. The microfibril is made up of the most basic unit of a cellulosic material, the elementary fibril. The elementary fibril is pure cellulose and all of the cellulose molecules are arranged in a parallel fashion with hydrogen bonding responsible for their crystalline nature. Four or more elementary fibrils are held in a lignin and hemicellulose matrix that acts as the

"cement" of the microfibril. An elementary fibril is believed to be 40 Å wide, 30 Å thick, and 100 Å long and the microfibril is typically 50 Å wide and 100 Å thick. It is this complex microstructure that makes cellulosics the perfect structural material for plants and also makes them highly resistant to degradation.

The structural features of native cellulose that have been mentioned as significantly affecting hydrolytic enzyme attack include (1) degree of water swelling, (2) crystallinity, (3) molecular arrangement, (4) content of associated materials, such as lignin, and (5) capillary structure of fibers (Cowling and Kirk, 1976; Fan, et al., 1979). These features that constitute the capillary structure of cellulose are significant because the susceptibility of cellulose to hydrolysis is determined largely by the accessibility of the active cellulose surface to cellulolytic enzymes. Stone, et al. (1969) used the solute exclusion technique to show that the initial enzyme reaction rate is proportional to the surface area accessible to a molecule 40 Å in diameter. This diameter is approximately equal to that of cellulolytic enzymes. High crystallinity has repeatedly been shown to slow the hydrolysis reaction (Norkrans, 1950; Baker, et al., 1959; Caulfield and Moore, 1974). Caulfield and Moore (1974) suggested that the influence of each aspect of cellulose morphology could be more effectively studied by coupling crystallinity measurements with surface area measurements.

Pretreatments. Ball milling has been shown to be a highly effective method of cellulose pretreatment for enzymatic hydrolysis (Mandels, et al., 1974). The action of porcelain balls in a rotating jar mill produces a reduction of particle size and crystallinity with a concomitant increase

in external surface area and bulk density. After 7 days of milling with porcelain balls in a rotating jar mill, newsprint was totally digested in 48 hours by enzymes from a T. viride mutant, QM 9414.

High energy radiation has been utilized to enhance the digestibility of native cellulose (Kunz, et al., 1972). The increase in the presence of phenolic groups in irradiated wood fibers indicates lignin is affected by the treatment (Lawton, et al., 1951). Pentoses and hexoses are also produced and depolymerization is evident. The effects of different types of high energy radiation, e.g., high speed electrons or gamma radiation, are essentially the same. The total absorbed dosage is important while the dose rate has little effect. At extremely high dosages, above 500 Mrad, decomposition of the glucose to a form not usable by microorganisms begins. The presence of an oxygen atmosphere during irradiation enhances decomposition to a small extent over the presence of an inert atmosphere. Extremely low moisture content results in additional depolymerization occurring for up to 30 days after the initial radiation treatment (Glegg and Kertesz, 1957).

Pyrolysis has recently been examined as a process to increase the susceptibility of cellulosic materials to hydrolysis (Shafizadeh, 1977). Above 300°C, cellulose rapidly decomposes producing gaseous and tarry compounds which evaporate and leave a small amount of char residue. However, at intermediate temperatures the decomposition proceeds slowly and relatively few volatile products are formed. Pyrolysis of microcrystalline cellulose for 8 hours in a nitrogen atmosphere produces a three-fold increase in digestibility by commerical enzymes isolated from T. viride. It is speculated that rapid, higher temperature processing in a fluidized bed may yield similar results.

Sodium hydroxide has been repeatedly investigated as a reagent for chemical treatment to increase cellulose digestibility (Mandels, et al., 1974; Millet, et al., 1975). The process has been used to prepare cellulosics for enzyme hydrolysis as well as to increase the nutritive value of ruminant feeds. Various NaOH concentrations have been employed but Millet, et al. (1975) indicate that 5 to 6 grams of NaOH per 100 grams substrate gives maximum digestibility for several natural substrates. The mechanism of NaOH action involves the swelling of the cellulose structure. The heating of the sodium hydroxide-cellulose mixture enhances the swelling effect.

When cellulose is dissolved in a solvent, the major aspects that deter degradation, high crystallinity and lignin, are removed. In the case of the solvent CMCS, the addition of water to the cellulose-CMCS solution readily precipitates the cellulose in the form of a soft floc that is highly susceptible to enzymatic conversion to glucose (Tsao, 1978). CMCS is essentially a 5% sodium hydroxide solution that contains small amounts of sodium tartrate, ferric chloride, and sodium sulfite. Up to 4% cellulose can be dissolved in CMCS at room temperature. The solvent can be recovered almost completely after washing the cellulose with three volumes of wash water per volume CMCS. This solvent is less effective than other cellulose solvents but has the advantage of lacking toxicity.

Another treatment that can be considered as a cellulose solvent method involves the use of concentrated sulfuric acid (Sasaki, et al., 1977). Strong acid treatments have been avoided because much of the cellulose may be hydrolyzed to glucose which is subsequently lost in washing procedures. In addition, the strong acid produces secondary reaction products that may inhibit enzyme action if not completely removed. The method employed

by Sasaki, et al. (1977) utilized a very short reaction time and a novel precipitation procedure that circumvent the problems mentioned. The addition of acetone precipitates the cellulose and facilitates separation. Up to 95% of the acetone can be recovered by vacuum distillation. The process was applied to rice hulls, and the cellulose was 95% converted to glucose by commercial enzymes in 24 hours.

B. Objectives and Rationale

The objective of this thesis was to evaluate the effectiveness of various cellulose pretreatment methods for enzymatic hydrolysis. The pretreatments were judged by scrutinizing their effects on the structural parameters of cellulose as well as by establishing their effects on the enzyme hydrolysis rate. This approach determined the magnitude of the effectiveness of each pretreatment and the mechanism of each pretreatment. In addition, an attempt was made to relate the important structural parameters of cellulose to the rate of enzyme hydrolysis.

The evaluation of cellulose pretreatments had been conducted by other investigators. However, such a study had not been undertaken coupled with the examination of the fundamental structural changes wrought by the treatments. This knowledge could eventually lead to an effective treatment or combination of treatments in a practical hydrolysis process. The determination of the effects of two important structural parameters, crystallinity and surface area, simultaneously on hydrolysis had not yet been accomplished. This knowledge will establish their relative importance to hydrolysis as well as aid in understanding the mechanism of the enzyme reaction.

II. THEORETICAL

This section provides the fundamental theoretical basis for each of the techniques used in this thesis: the pretreatments, the solvent drying technique, the x-ray diffraction crystallinity methods, and the nitrogen adsorption specific surface area method. The enzyme hydrolysis reaction mechanism is also developed in this section.

A. Mechanism of Pretreatments

Ball milling. The action of porcelain balls in a rotating jar mill produces a reduction of particle size, degree of polymerization, and crystallinity and a simultaneous increase in external surface area and bulk density. All of these changes have a positive effect on the rate of enzymatic hydrolysis. The effects of increased surface area and reduced crystallinity on hydrolysis have already been mentioned while the reduction of particle size and degree of polymerization and increase of bulk density improve the hydrodynamics of an enzyme degradation. It has been noted that hammer milling as a means of pretreatment is ineffective compared to ball milling (Mandels, et al., 1974). This is due to the different action of the two types of mills. The ball mill imparts high shear to the cellulose in a crushing, grinding action, whereas the hammermill works with a clean, cutting action. In essence, the hammermill has little effect on crystallinity and increases only the external surface area, while ball milling has more prodigious effects.

Gamma irradiation. Gamma radiation produces cleavage of the β-1,4-glucosidic bond of the cellulose molecules which results in depolymerization. The cleavages are apparently random and glucose is produced during irradiation (Kunz, et al., 1972). The reaction is probably one of oxidative

degradation of the reducing type, but little is known of the chemical nature of the products (Blouin and Arthur, 1960). Crystalline and amorphous cellulose are affected equally by the radiation. The depolymerization may be accompanied by breakage of hydrogen bonds between parallel cellulose molecules. These two effects eliminate important factors that limit hydrolysis rate.

Pyrolysis. At temperatures between 150° and 200°C, the decomposition of cellulose proceeds slowly and relatively few volatile products are evolved (Shafizadeh, 1977). In the presence of oxygen, the pyrolytic reactions of depolymerization, oxidation and dehydration are accelerated. In an inert atmosphere, the depolymerization occurs more slowly, but the unwanted by-products of oxidation and dehydration are also formed more slowly. The by-products contain enolic double bonds, carbonyl and carboxyl groups, which destabilize the molecule. For pretreatment applications, depolymerization must be maximized while formation of enzyme-inhibiting compounds and the decomposition of glucose must be minimized.

Swelling in 1% NaOH. The mechanism of action of 1% NaOH on cellulose involves the swelling of the cellulose structure. In natural cellulosics, this swelling may be due to the saponification of intermolecular ester bonds, which contributes to the swelling of cellulosics beyond their water-swollen dimensions (Millet, et al., 1975). In pure cellulosics, sodium hydroxide take-up has been postulated by some to be a stoichiometric process and by others to be a non-stoichiometric process. It is agreed, however, that considerable intercrystalline swelling occurs.

Solvent dissolution and reprecipitation. Concentrated H_2SO_4 and CMCS are two effective cellulose solvents. During dissolution, intracrystalline

swelling occurs which involves a penetration of the crystalline and amorphous regions (Millet, et al., 1975). Dissolution results in unlimited swelling and essentially complete molecular dispersion. Upon precipitation, the cellulose forms a soft floc which is highly susceptible to hydrolysis.

B. Mechanism of Solvent-drying

The measurement of both the specific surface area and the CrI of cellulose are performed on dry samples. However, water has a profound effect on the structure of cellulose. It is known that water-soaking causes an increase in the crystallinity of cellulose (Howsmon and Marchessault, 1959). Also, the specific surface area of cellulose increases dramatically on wetting. The water-swollen surface area of cellulose fibers is reported to be about 200 m^2g^{-1} (Browning, 1963). When cellulose is air-dried from the water-swollen state, the capillary structure collapses and the physical parameters change drastically. This collapse occurs on drying because surface tension forces of the polar solvent, water, bring together neighboring surfaces which become hydrogen bonded. It has also been found that drying cellulose from polar liquids causes greater collapse than drying from nonpolar liquids (Merchant, 1957). The crystallinity of cellulose increases on air-drying and the specific surface area can decrease from 200 to 0.4 m^2g^{-1} (Weatherwax and Caulfield, 1971). Thus, the problem arises of how to measure the water-swollen properties of cellulose when it is in the dry state.

The water-swollen properties of cellulose may be partially preserved using special solvent-drying techniques. The properties of the solvent-dried cellulose may approximate those of water-swollen fibers. These approximations can not be used as quantitative results, but valuable qualitative

results are obtained and trends that appear for the approximations should reflect actual trends.

C. Theory of X-Ray Diffraction

The crystalline order of cellulose has been shown to influence the rate of enzymatic hydrolysis (Norkrans, 1950; Caulfield and Moore, 1974). Determination of the exact extent of crystalline order, or crystallinity, is difficult although many methods have been employed, e.g., acid hydrolysis, infrared absorption, and x-ray diffraction. An x-ray diffraction method was chosen for use in this work because of the considerable refinement and widespread use of the technique. The spectrometric powder technique of x-ray diffraction is the basis for the crystallinity analysis scheme.

Bragg explained x-ray diffraction in terms of "reflection" from a stack of parallel planes (Klug and Alexander, 1954). An incident beam of x-rays is not reflected from the crystal surface but actually penetrates several million layers before being appreciably absorbed. A small amount of the beam may be considered as reflecting from each of the successive layers in the stack of identical planes. For a beam to emerge from the crystal, it must not be absorbed by the atomic layers and the beams "reflected" from all of the layers must not interfere with each other. This occurs only when the path difference is a whole number of wavelengths and this is the essence of Bragg's Law.

In the random powder method a narrow beam of monochromatic x-rays strikes a crystalline powder composed of randomly oriented particles. For best results the powder should pass a 230 mesh sieve. Larger particles tend to become preferentially oriented when the sample is prepared. A

Geiger counter receiver directly measures the intensity of the diffracted x-ray beam. Only at angles that satisfy the Bragg Law for each crystal plane does the Geiger counter record a peak in the diffracted x-ray intensity. The result is a strip chart record of the relative intensity of the diffracted x-ray beam as the angle of incidence, θ, is varied. The recorded peaks correspond to the crystal planes of different interplanar spacings.

Native cellulose is not a purely crystalline material. It possesses various amounts of amorphous cellulose as well as crystalline cellulose. The relative amounts of amorphous and crystalline cellulose present, or the crystallinity, is dependent upon the origin and treatment history of the cellulose and can be determined using x-ray diffraction data. The crystallinity index proposed by Segal, et al. (1959) is based on the diffracted intensities which represent crystalline and amorphous cellulose. Highly crystalline cellulose exhibits a prominent peak at the angle $2\theta \doteq 22°$ which is a manifestation of the 002 crystal plane. Intensely ball milled cellulose, assumed to be amorphous, generates a flattened hump as an x-ray pattern which has its highest point at the angle $2\theta \doteq 18°$. Therefore, Segal, et al. (1959) proposed the following crystallinity index which is employed in this work:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

where I_{002} is the intensity of the 002 peak and I_{am} is the intensity at $2\theta = 18°$.

The effects of crystallinity on hydrolysis have been previously well established. However, the study of these effects has not been made in concert with a study of the effects of specific surface area on hydrolysis.

This combination leads to a better understanding of the mechanism of the enzyme-cellulose reaction.

D. Theory of the BET Method

Cellulose is porous and possesses considerable internal and external surface areas. The external surface area of typical wood fibers is reported to range from 0.6 to 1.6 m^2g^{-1} (Browning, 1963). Total specific surface areas of wood fibers have been reported to range from 0.7 to 300 m^2g^{-1}, depending on the fibers' treatment history (Merchant, 1957). The internal surface is composed of cracks, crevices, and pores of various sizes. The total surface area of cellulose can be measured by applying the BET equation to nitrogen adsorption data (Merchant, 1957; Browning, 1963). The results of hydrolyses of cellulose samples with differing surface areas should reveal any relationship between specific surface area and hydrolysis rate.

The BET equation, developed in 1938 by Brunauer, Emmett, and Teller, establishes a general theory of multi-layer physical adsorption (Brunauer, et al., 1938). Physical adsorption of gases onto a solid surface occurs under suitable conditions of temperature and pressure. The binding forces of physical adsorption are of the order of Van der Waals forces and the process resembles liquefaction. Adsorption occurs initially as a mono-layer, a layer of adsorbed gas one molecule thick, and subsequently, multiple layers of gas molecules adsorb.

The BET equation has been developed for the case of a free surface, where there is no limitation to the number of adsorbed gas layers (Carberry, 1976). The final equation is cast in the following form:

$$\frac{p}{V(p_0 - p)} = \frac{1}{V_m c} + \frac{c-1}{V_m c}\frac{p}{p_0}$$

where

p = partial pressure of adsorbate

p_0 = vapor pressure of adsorbate

V = volume of gas adsorbed

V_m = volume of gas in monolayer

c = constant

The constants V_m and c can be found from a plot of the left hand side of the equation versus p/p_0. The surface area accessible to the gas can be found from V_m and the cross sectional area of the adsorbate molecule. The plot is usually linear for nitrogen partial pressures between 0.05 and 0.30 (Browning, 1963). The highly convenient flow method used in this work is limited to low stream concentrations of adsorbate because high concentrations (greater than 0.30) can result in flow disturbances during adsorption.

The specific surface areas measured in this work are not the actual water-swollen surface areas of the cellulose samples, but the reported surface areas are valuable, nevertheless. In this work, the area measurements were performed on solvent-dried samples whose nitrogen adsorption areas were probably smaller than their actual water-swollen areas due to a small amount of structural collapse during the solvent-drying procedure. The areas measured were those accessible to nitrogen molecules, which are much smaller than the macromolecular enzymes. This factor would tend to make the measured areas larger than the actual area accessible to enzymes. The areas, as measured in this work, are useful because the solvent-drying procedures were constant and the measured areas should have a direct relationship with water-swollen areas accessible to enzymes.

E. Mechanism of Enzyme Hydrolysis

The mechanism of the enzyme hydrolysis reaction is complex because the reaction is heterogeneous in nature. In addition, the enzyme contains several components, and the cellulose substrate is structurally complex. Extensive research has revealed that three enzyme components are necessary for the complete degradation of native cellulose (Pettersson, 1975; Wood, 1975). The C_1 enzyme, or cellobiohydrolase, which attacks the unreactive crystalline portion of cellulose, acts by splitting a cellobiose unit from the end of a cellulose molecule. The C_x enzyme, or β-1,4-glucanase, is composed of two components, endo- and exo-β-1,4-glucan glucohydrolase. The endo-component attacks amorphous or soluble cellulose molecules in a random manner, hydrolyzing at any point along the cellulose molecule to produce two smaller cellulose molecules. The exo-component attacks only at the non-reducing end of a cellulose molecule to produce a single glucose unit. The exo-β-1,4-glucan glucohydrolase is of limited importance to the overall scheme. However, it is believed that the endo-β-1,4-glucan glucohydrolase acts synergistically with the C_1 enzyme when attacking native crystalline cellulose. While C_1 or C_x enzyme alone has little effect on crystalline cellulose, a combination of the two results in substantial degradation. The third type of enzyme is β-glucosidase which attacks mainly cellobiose to produce two glucose units. The molecular weights of the enzyme molecules range from 40,000 to 75,000. The aim of this work was to facilitate the accessibility of these enzymes to the cellulose through pretreatments.

III. EXPERIMENTAL

The objective of this thesis was to evaluate pretreatments by scrutinizing their effects on the structural parameters of cellulose and by establishing their effects on the enzyme hydrolysis rate. This was accomplished by first measuring the crystallinity and specific surface area of the untreated cellulose standard substrate. The pretreatments were then performed and the structural parameters of the treated cellulose were measured and compared to those of the standard. The treated cellulose was hydrolyzed by enzymes and the rates of hydrolysis were compared to the rate of hydrolysis of the untreated cellulose. The data was also used to determine relationships between the parameters and the hydrolysis rate.

A. Materials

Substrate. Solka Floc SW-40 (Brown Co.), a hammer milled sulfite wood pulp, was the principal substrate used in this work. The pulp is composed of crystalline and amorphous cellulose. It is pure enough for human consumption, containing 99.5% cellulose and less than 0.4% ash. Under microscopic observation the Solka Floc particles have a long, cylindrical shape with a diameter of about 20 μm and an average length of 100 to 140 μm. A screen analysis showed 60 to 75% passes through 100 mesh and 25 to 40% passes through 200 mesh.

The Solka Floc was apportioned into various particle size fractions using a Ro-Tap sieve shaker. The fraction passing 270 mesh and not passing 400 mesh was used as the basic substrate in this work. It is hence referred to as the standard substrate.

Wheat straw was used as an example of a natural substrate in this work. The wheat straw was collected from a field of stubble from a recently harvested area. To reduce its particle size, the straw was milled to pass a 1/16 inch screen in a Fitzmill. The mill did not grind but nearly broke the straw into pieces by high velocity impaction with the rotating blades. This left the actual structural characteristics of the wheat straw largely intact during size reduction. The wheat straw contained 30.4% hemicellulose, 9.9% lignin, and 41.7% cellulose by the Van Soest fraction method.

The wheat straw was sieved in the same manner as the Solka Floc, and the straw fibers which passed 270 mesh but did not pass 400 mesh were collected and will hence be referred to as the wheat straw standard substrate.

Enzyme. The source of the enzyme used was the culture filtrate from batch cultivations of the fungus Trichoderma reesei (viride) QM 9414 on Solka Floc. This strain is a mutant that produces high quantities of enzymes, and it was supplied by the U.S. Army Natick Research and Development Command. The culture was maintained on PDA (Potato Dextrose Agar) slants at 30°C. Slants 1 to 3 weeks old were used for inoculating shaker flask cultures. The medium composition for flask cultivation is listed in Table 1.

The substrate employed for the enzyme production cultures was Solka Floc SW-40 at 1% concentration. A 300 mℓ flask was filled with 100 mℓ of culture medium, plugged, and autoclaved. It was inoculated with 10 mℓ of distilled water containing the suspended spores from a slant. The fungus was cultivated for about 7 days at 30°C and 250 rpm. This small flask was then used to inoculate 2 liter flasks containing 500 mℓ of medium which were cultivated under the same conditions. The enzyme was harvested from the large flasks by filtration and stored in a refrigerator until used for

Table 1. Medium composition for cultivation of T. reesei.

Component	Amount
Distilled Water	1000 mℓ
$(NH_4)_2SO_4$	1.4 g
KH_2PO_4	2.0 g
Urea	0.3 g
$CaCl_2$	0.3 g
$MgSO_4 \cdot 7H_2O$	0.3 g
Trace Metal Stock	1.0 mℓ
Tween 80	2.0 g
Proteose Peptone	1/10 the concentration of the substrate

Trace Metal Stock

Distilled Water	495 mℓ
Conc. HCℓ	5.0 mℓ
$FeSO_4$	2.5 g
$MnSO_4 \cdot H_2O$	.98 g
$ZnSO_4 \cdot H_2O$	1.76 g
$Co(NO_3)_2 \cdot 6H_2O$	1.25 g

the hydrolysis. The soluble protein content of the enzyme broth, measured by the Lowry method using bovine serum albumin as a standard, ranged from 2.2 to 2.5 mg/mℓ. The enzyme activity was measured by the filter paper method developed by Mandels, et al. (1975) to be between 1.3 and 1.5 IU/mℓ.

B. Procedures

Pretreatments. The ball mill used in this work was a 5 liter porcelain jar mill with a 50 volume percent charge of 1 inch porcelain spheres. The jar was rotated at about 52 rpm for various time periods. The dry substrate was added in an amount that filled only the void volume of the balls. The milled samples were again dried at 80°C overnight as a precaution and stored in a desiccator.

Prior to the gamma radiation treatment, the cellulose samples were taken from a desiccator and sealed in glass jars in the presence of air. The jars were placed in a Co^{60} gamma irradiator with a dose rate of 800 rad/min until various dosages up to 50 Mrads were reached. For higher dosages the cellulose was tightly packed in polyethylene vials and dropped into the core of an operating 250 Kw Triga MARK II nuclear reactor where various dosages of gamma radiation were applied. Some neutron flux was also present at the core of the reactor. All treated samples were stored in a desiccator for at least one month before analysis.

For the first type of pyrolysis treatment, the substrate was placed in a glass container in air and heated in a 170°C oven for 24 hrs. The second treatment was the same except the air was evacuated and replaced with an inert gas, helium. The samples were stored in a desiccator after treatment.

The 1% NaOH soaking treatment was performed at a 5% substrate concentration. The cellulose was soaked in one case for 30 min. at room temperature and in a second procedure for 20 min. in an autoclave at 124°C and 17 psig. The substrates were washed with water until the wash water was neutral.

For the CMCS treatment, the solvent was made by dissolving 20 g sodium tartrate, 15.5 g ferric chloride, and 14.5 g sodium sulfite in 1000 g of a 5% NaOH solution. Cellulose at a 4% concentration was dissolved in the solvent and the mixture was allowed to stand for 6 hrs. The dissolved cellulose was reprecipitated by the addition of water and allowed to settle. The excess solvent was then decanted and the residue was washed with water until the wash water was neutral. The treatment imparted an orange tint to the cellulose that persisted even after washing and solvent drying.

For the acid solvent treatment, sulfuric acid was prepared at 60% concentration and allowed to cool at room temperature. Substrate cellulose was dissolved at 5% and immediately precipitated by the addition of acetone. The precipitate was washed with acetone and then water until the wash water was neutral.

All chemically treated samples were solvent-dried before parameter measurement and hydrolysis. All physically treated samples were solvent-dried before parameter measurement but were hydrolyzed without previous solvent-drying.

Solvent-drying. The solvent-drying procedure was adopted to partially preserve the water-swollen characteristics of cellulose during drying. The cellulose was soaked in water for at least 30 min. to insure that the sample

was completely wetted. The water-swollen cellulose was then placed in an extraction thimble and mounted in a Soxhlet extraction apparatus. First, 400 mℓ of methanol was used to extract the residual water. The Soxhlet extractor was allowed to cycle for 3 to 4 hours. Then, the methanol was replaced by 400 mℓ of fresh methanol and the extractor was again cycled for 3 to 4 hours. The methanol was next replaced by 400 mℓ of benzene which was cycled for 3 to 4 hours. A final charge of fresh benzene was cycled to complete the water exchange procedure. The benzene was removed from the sample by air-drying at 80°C overnight. The solvent-dried samples were stored in a desiccator.

Crystallinity determination. The crystallinity was measured by employing the powder method of x-ray diffraction using a Norelco Diffractometer. The substrate sample was dried overnight at 80°C and stored in a desiccator. Care was exercised in handling the samples to minimize exposure to the atmosphere because adsorption of moisture from the air tends to increase the crystallinity. The samples were prepared by the powder mounting method of McCreery (Klug and Alexander, 1954). The specimen was mounted horizontally while the Geiger counter moved in a vertical arc. The samples were scanned over a range of 2θ from 10° to 30°. A complete list of instrument settings and conditions is provided in Table 2. The calculation of the crystallinity index of Segal, et al. (1959) requires the measurement of the intensity of the 002 peak and the amorphous intensity at $2\theta = 18°$. Figure 1 shows that the intensities were measured above an approximate baseline representing background intensity.

Specific surface area determination. The specific surface area of each sample was determined by applying the BET equation to the nitrogen adsorption data obtained with a Perkin-Elmer Shell Model 212D Flow Sorptometer.

Table 2. X-Ray diffractometer conditions and setting.

Chart Speed	30"/hr
Scan Speed	1°/min
Target	CuKα
Voltage	35 KV
Current	18 Ma
Scale Factor	1 K
Time Constant	2 sec
Detector Voltage	1.65 KV
Baseline	3.5 V
Window	3.5 V
Gain	512 V
Mode	linear
Filter	nickel

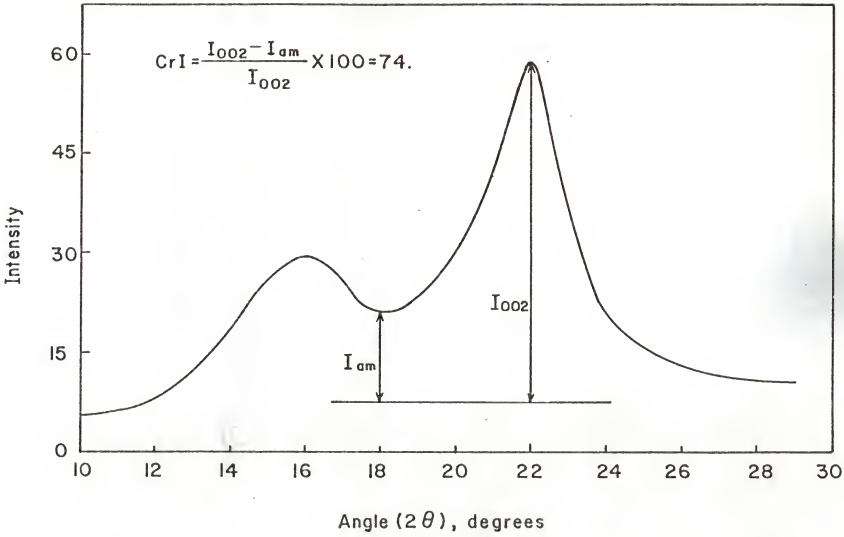


Fig. 1. X-ray diffraction pattern of Solka Floc.

Each sample was degassed overnight to prepare the cellulose surface for adsorption measurements. This was effected by passing helium at 80°C slowly through the U-shaped sample tube which contained 0.2 to 0.7 g of sample. An enlarged area at the bottom of the U-tube held the sample, but enough room was always left to allow the gas to pass through without entraining the powder. In addition, a small amount of glass wool was placed in the tube downstream from the sample to prevent any entrained powder from fouling the detector during measurement. Nitrogen was used as the adsorbate gas and helium was used as the carrier gas. A strip chart recorder was connected to the sorptometer to record the peaks caused by adsorption and desorption of nitrogen. The tube containing the sample was attached and the flow rates of the nitrogen and helium were determined with a bubble flowmeter. The baseline was zeroed and liquid nitrogen was applied to the sample tube to effect adsorption. The adsorption, which was carried out for 4 minutes, was typically recorded as a peak with a moderate amount of tailing. The polarity of the recorder was then reversed and the liquid nitrogen was removed to effect desorption. A room temperature water bath was used to speed up the desorption process and prevent tailing of the desorption peak. Consequently, the desorption peak was typically sharper than the adsorption peak. The release of a known amount of nitrogen from a calibration loop produced a reference peak. The area of the desorption peaks were compared to the area of the calibration peak to determine the volume of nitrogen adsorbed on the sample. A planimeter was used to measure the peak areas. Adsorptions and desorptions at three different nitrogen flow rates (partial pressures) were performed in order to plot a three point BET graph. The specific surface area was calculated from the slope and intercept of the plot. The details of the calculations can be found in Appendix A.

Enzyme hydrolysis. The substrate samples were dried in an oven overnight at 80°C and stored in a desiccator to insure that they contained no moisture. Two 5 g samples of the substrate were placed into two 300 mℓ flasks. Then, 40 mℓ of distilled water, 5 mℓ of 1.0 M citrate buffer (pH 4.8), and 50 mℓ of the enzyme solution were added to each flask to obtain a 5% substrate solution. All of the components of the solution were brought to 50°C and the enzyme, also at 50°C, was added at time zero. This insured that the reaction started immediately and that there was no lag time from warming up. The flasks were plugged with rubber stoppers and placed in a 50°C incubator set at 250 rpm (rotary motion). Samples were taken at various times by pipette. Each sample was comprised of about 2 mℓ from each of the two flasks containing a particular substrate. This 4 mℓ sample was immediately boiled for 5 min. to denature the enzyme and, hence, stop its action. The samples were then centrifuged and the supernatent was refrigerated until the DNS (dinitrosalicylic acid) reducing sugar assay was performed. A detailed procedure of the DNS method, a colorimetric assay, can be found in Appendix B.

IV. RESULTS

A list of the specific surface area, the CrI, and the relative hydrolysis rate for the standard substrate and the various treated celluloses is presented in Table 3. Both non-solvent-dried and solvent-dried values are listed for CrI and specific surface area of several of the cellulose samples. The first column under Relative Hydrolysis Rate lists the rate which was calculated including any sugar produced during pretreatment and the second column lists the rate due to sugar production by enzyme hydrolysis alone.

The ball milling treatment data have been plotted in graphical form. The effect of ball milling on the crystallinity of the solvent-dried and non-solvent dried samples is shown in Fig. 2. The influence of milling on the cellulose specific surface area is shown in Fig. 3. Figure 4 reveals the relationship between ball milling time and the relative hydrolysis rate.

The gamma irradiation data have also been graphically illustrated. It should be noted that the dosages from 2 to 50 Mrad were performed in a gamma-cell and the 100 and 500 Mrad dosages were performed in the core of a nuclear reactor. Figure 5 shows the amount of reducing sugar produced during the pretreatment as a function of dosage. The effect of radiation dosage on the CrI is presented in Fig. 6. The specific surface area as a function of dosage is plotted in Fig. 7. Figure 8 represents the relationship between gamma radiation dosage and the cellulose relative hydrolysis rate.

The influence of the cellulose structural parameters on the relative hydrolysis rate has been illustrated in the next two figures. Figure 9 is

a plot of specific surface area versus relative hydrolysis rate for all of the treated samples plus the Solka Floc standard and microcrystalline cellulose. The solid circles represent samples with a CrI between 70 and 80, the triangular points represent samples with a CrI below 66, and the square point represents microcrystalline cellulose with a CrI of 88.8. Figure 10 is the plot of CrI versus relative hydrolysis rate for all of the treated samples plus the Solka Floc standard and microcrystalline cellulose. The solid circles represent cellulose samples with specific surface areas between 1.1 and 8.2 m^2g^{-1}. The triangular points represent cellulose samples with specific surface areas greater than 12.0 m^2g^{-1}.

Sample	Specific Surface Area, $m^2 g^{-1}$ not solvent dried	solvent dried	Crystallinity Index, CrI not solvent dried	solvent dried	Relative Hydrolysis Rate $mg \cdot ml^{-1}$	
Standard Substrate	1.71	8.18	74.2	77.4 (2)	1.00	1.00
Ball Milled 12 hours	2.09	1.54	66.3	65.1	1.16	
Ball Milled 24 hours	2.26	1.59	30.8	59.4	1.30	
Ball Milled 48 hours	2.36	1.59	18.7	58.1	1.61	
Ball Milled 96 hours	1.91	1.15	4.9	36.5	2.12	
γ-radiation 2 Mrad		5.37	68.1	74.1	.699	.683
γ-radiation 5 Mrad		4.25	75.3	77.5	.643	.616
γ-radiation 10 Mrad		5.50	71.3	76.0	.747	.662
γ-radiation 20 Mrad		7.94	72.1	75.9	.856	.733
γ-radiation 50 Mrad		12.8	71.1	73.4	1.25	.988
γ-radiation 100 Mrad		4.07	72.1	75.9	1.74	1.47
γ-radiation 500 Mrad		106.2	64.2	70.2	4.10	2.55
Pyrolysis in Air, 170°C		2.07	75.9	74.9	1.25	.748
Pyrolysis in He, 170°C		3.61	71.0	74.6	2.18	1.81
NaOH, 1% Room Temp.		12.2		75.5	1.37	
NaOH, 1% Autoclaved		23.2		75.9	1.45	
CMCS, Room Temp.		27.4		79.3	1.44	
Conc. Acid, H_2SO_4		29.2		74.3	1.41	
Wheat Straw Std.		25.5	46.2	45.1	1.00	
Wheat Straw NaOH-Autocl.		89.1		62.9	3.64	
Wheat Straw 96 hour ball-m.		47.2		0	—	

Fig. 2. Effect of ball milling on cellulose crystallinity

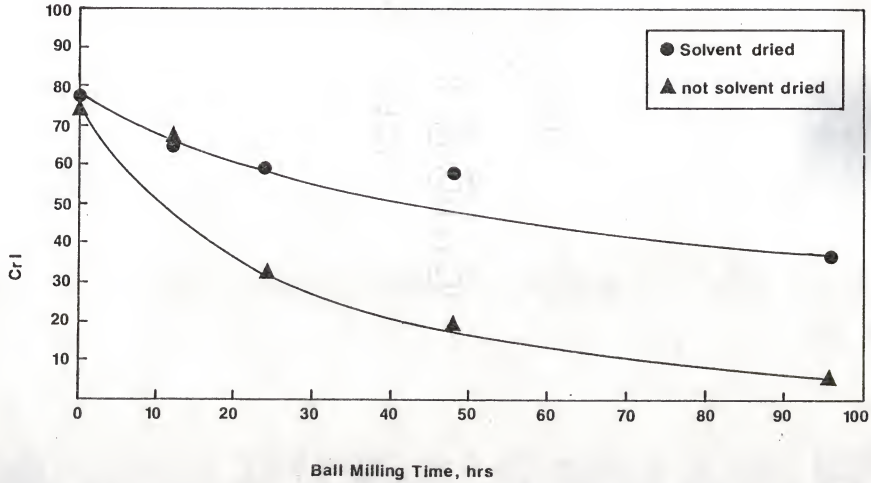


Fig. 3. Effect of ball milling on the specific surface area of cellulose

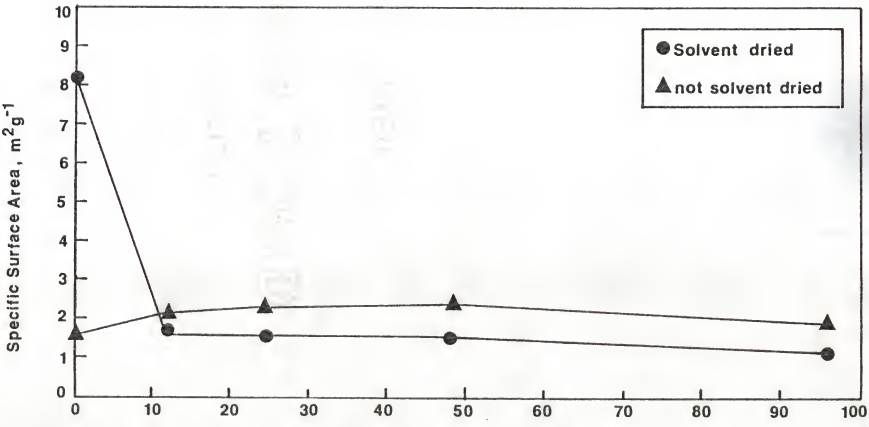


Fig. 4. Effect of ball milling on the relative hydrolysis rate of cellulose

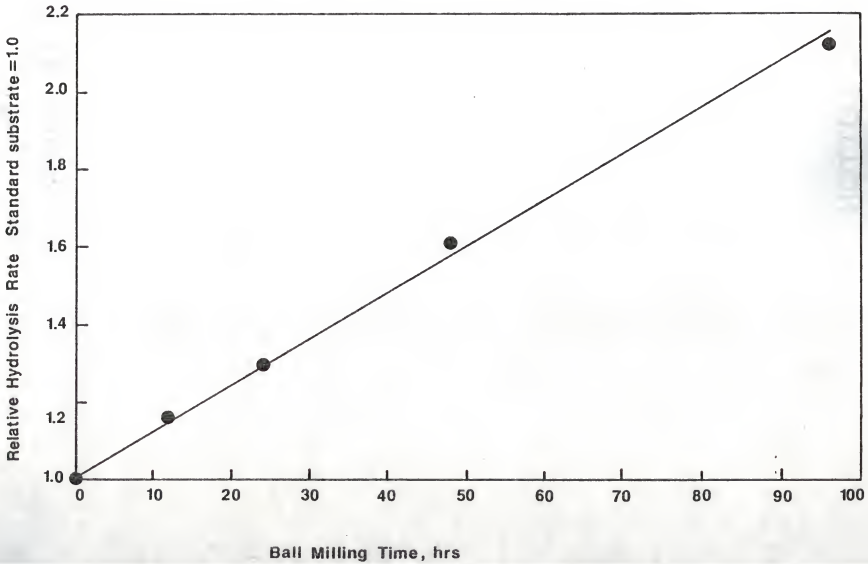


Fig. 5. Reducing sugar production during gamma irradiation

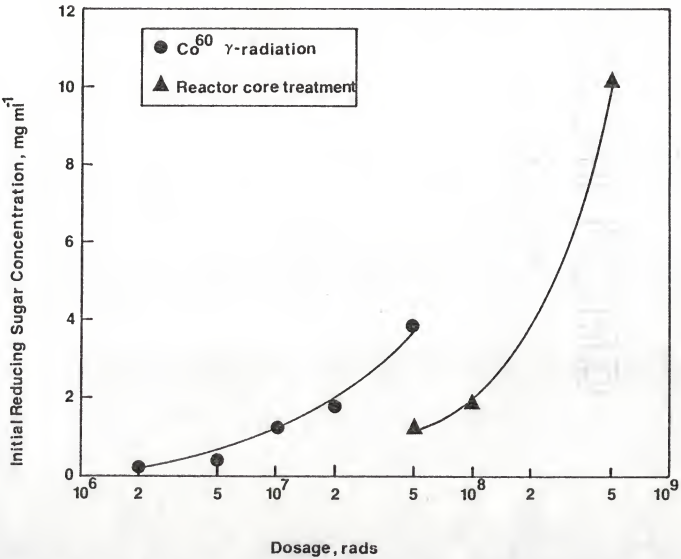


Fig. 6. Effect of gamma radiation dosage on cellulose crystallinity

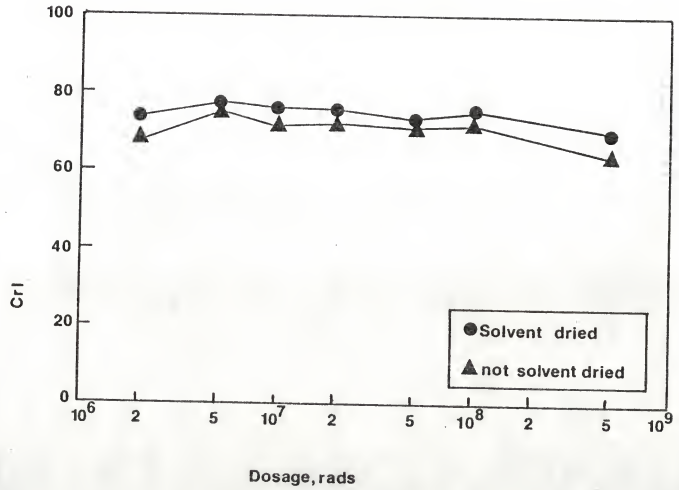


Fig. 7. Effect of gamma radiation dosage on the specific surface area of cellulose

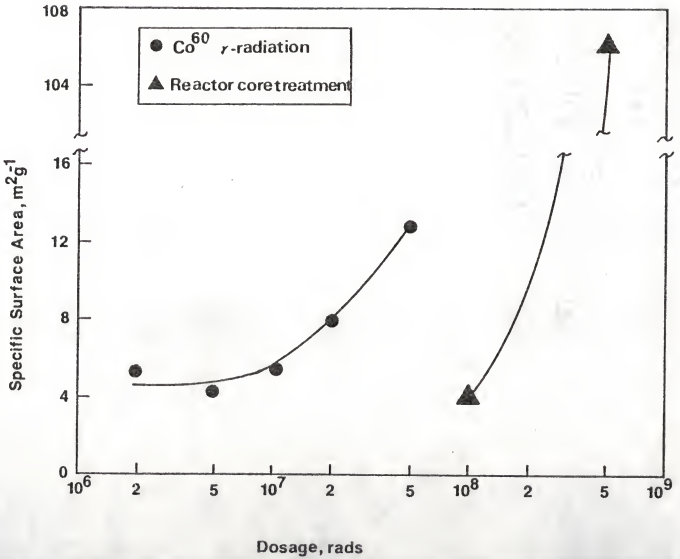


Fig. 8. Effect of gamma radiation dosage on the relative hydrolysis rate of cellulose

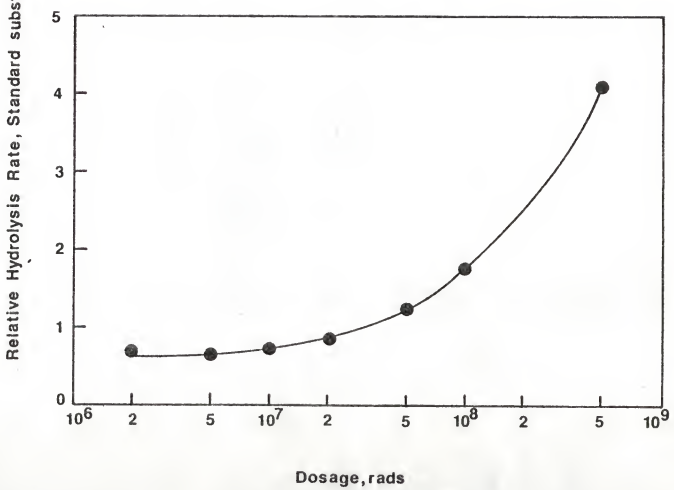


Fig. 9. Effect of the specific surface area of cellulose on the relative hydrolysis rate

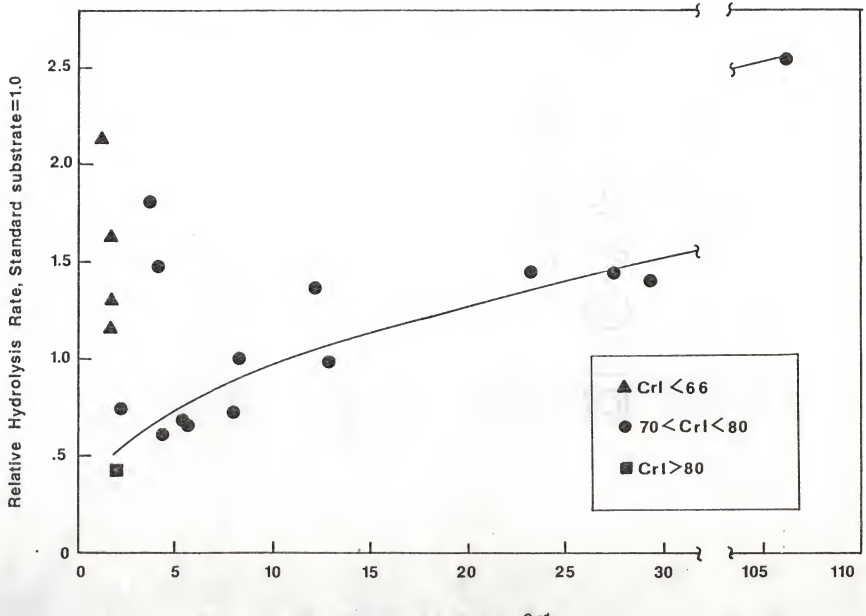
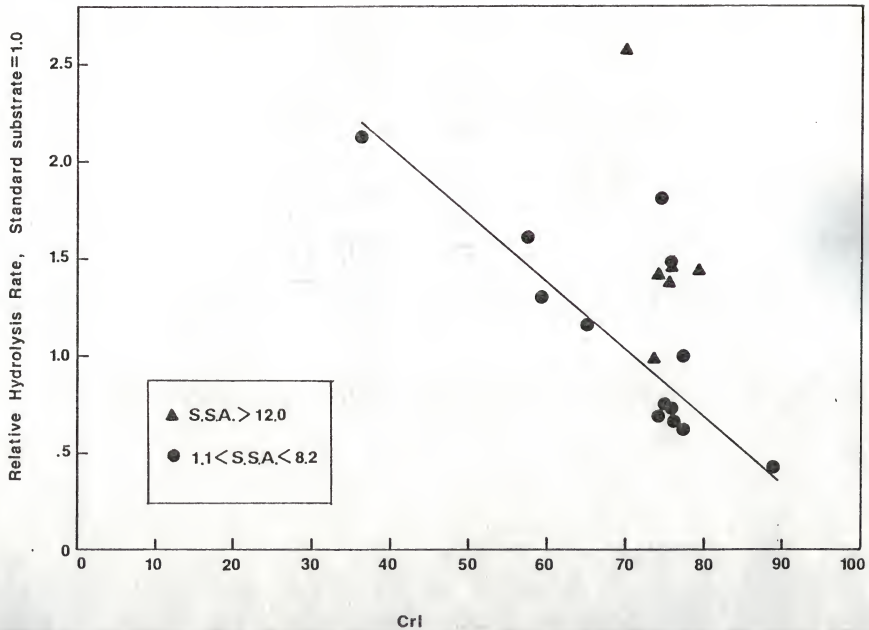


Fig. 10. Effect of the cellulose crystallinity on the relative hydrolysis rate



V. DISCUSSION

The physical parameters of the Solka Floc standard substrate were measured so that comparisons could be made between this "untreated" cellulose and the standard substrate which had been subjected to the various pretreatments. The specific surface area of the non-solvent-dried standard substrate was 1.71 m^2g^{-1} (see Table 3). This Solka Floc had been sieved as received from the Brown Co., dried overnight in an 80°C oven, and stored in a desiccator. This specific surface area, 1.71 m^2g^{-1}, is probably no more than 2 or 3 times the external area of the fibers. It is most likely so small because at the end of the sulfite bleaching process the fibers were probably dried from water, which causes a great deal of structural collapse. The standard substrate after solvent-drying possessed 8.18 m^2g^{-1} specific surface area (see Table 3). This value does not match the 200 m^2g^{-1} often reported in the literature for the solvent-dried area of never-dried wood fibers (Browning, 1963). However, fibers that were at one point dried from water, resoaked in water, and then solvent-dried exhibited 60% less surface area, from about 50 to 90 m^2g^{-1} (Merchant, 1957). The solvent-dried specific surface area obtained in this work is lower because of two probable reasons. During the solvent-drying procedure, all of the water may not have been removed. On drying, its presence in even small amounts may have contributed to considerable collapse. Also, the surface tension of benzene may have been different than the solvents used in the literature, contributing to further collapse. The final drying temperature of a solvent-drying procedure also affects the final surface area, but this work used a similar temperature to those found in the literature.

The crystallinity index of the non-solvent-dried standard substrate, as measured by x-ray diffraction, was 74.2 (see Table 3). This agrees with literature values for a similar type of cellulose fiber (Caulfield and Moore, 1974). When solvent-dried, the CrI of the standard substrate was 77.4. The higher CrI after solvent drying may be interpreted as evidence of the recrystallization phenomenon.

Recrystallization occurs when partially or totally amorphous cellulose is exposed to water, either by wetting or exposure to atmospheric moisture (Howsmon and Marchessault, 1959; Caulfield and Steffes, 1969). For example, when brought to equilibrium with air saturated with water vapor at 24°C, the standard substrate possessed a CrI of 81.8. It has been postulated that the moisture helps plasticize longer segments of amorphous cellulose chains (Wadehra and Manley, 1965). This enables them to become better aligned in an orderly array through hydrogen bonding and results in an increase in the CrI. The evidence leads to the rather puzzling conclusion that the action of water on cellulose produces a great degree of swelling causing a dramatic increase in surface area and at the same time produces an increase in the crystalline order due to alignment of the amorphous portions.

The reliability of the crystallinity index measurements is good. There is a 3% maximum possible human error that could occur while measuring the intensities which are needed to calculate the CrI. To check the reproducibility of the values, the crystallinity indices of Solka Floc standard substrate and Sigmacell 50 microcrystalline cellulose were measured twice; 77.1 and 77.6 for the Solka Floc and 82.9 and 83.5 for the Sigmacell 50. These results indicate that the error may be substantially less than 3%.

The reliability of the BET specific surface area measurements was checked for both the solvent-dried and non-solvent-dried Solka Floc standard substrate. The non-solvent-dried values were 1.59, 1.77, and 1.78 m^2g^{-1} for three identical samples. The solvent-dried Solka Floc resulted in specific surface areas of 7.92 and 8.44 m^2g^{-1}. These and other results indicate that at low surface areas (1-2 m^2g^{-1}) the values for identical samples may vary as much as 10%. As the surface areas increase, the values are found to vary less.

A. Effects of Pretreatments on Physical Parameters and Extent of Hydrolysis

Ball milling. The ball milled Solka Floc standard substrate showed similar characteristics to the wood pulp ball milled by Howsmon and Marchessault (1959). The samples became much finer powders with higher bulk densities and acquired a slightly greyish color. Microscopic observation revealed considerable particle size reduction, but there was also some evidence of agglomeration. The original fiber structure becomes almost entirely destroyed after 96 hours of milling. It is evident in Fig. 2 that ball milling has a dramatic effect on the crystalline structure of cellulose. However, the crystalline structure, which is almost entirely lost after 96 hours of milling, is partially regained on solvent-drying. This is evidence of the recrystallization phenomenon described earlier. It is clear that recrystallization on wetting is more pronounced for the highly amorphous samples. The data suggest that the CrI of the cellulose is a simple function of ball milling time and, hence, energy input.

The non-solvent-dried data show very little change in specific surface area with ball milling time (see Fig. 3). This was not expected because

newly created surface area should result from the energy input. There is a possible explanation for the results, however. It is difficult to perform the milling operation while excluding atmospheric moisture from the mill. Any moisture entering the mill may have contributed to the collapse of the fine pore structure of the cellulose and to agglomeration of the particles.

The solvent-dried data show a similar trend (see Fig. 3). The specific surface area drops after ball milling and levels off at a low value. It was expected that the ball milled samples would swell extensively in water and that the solvent-dried data would reflect the swelling. This was not the case and the results may have been due to collapse caused by an imperfect solvent-drying technique. The expected and, perhaps, actual trends were not reflected in the data, possibly due to the mentioned handling problems.

The enzyme hydrolysis of the ball milled cellulose well illustrated the effect that ball milling has as a pretreatment. Figure 4 shows how ball milling affects the hydrolysis rate as represented by the extent of hydrolysis after 8 hours. This time period was chosen because it represents the hydrolysis before substantial product inhibition becomes important and because a practical commercial process will have to deal with short reaction times in order to prove economical. The relative hydrolysis rate is the ratio of the extent of hydrolysis after 8 hours of the treated sample to the extent of hydrolysis after 8 hours of the standard substrate. The extents of hydrolysis were determined from reducing sugar production data for each sample. An equation of the following form:

$$\frac{AT^C}{B+T}$$

was fit to the data by a regression technique and the value of the equation at 8 hours was recorded as the extent of hydrolysis. Any reducing sugar

present in the initial unhydrolyzed sample was included in the sugar production values. The increasing hydrolysis rate is at least partially the result of decreased crystallinity caused by ball milling. The data do not show that ball milling increases available surface area, but it is suspected that ball milling does cause such an increase which would contribute to the enhanced hydrolysis rates. The 96 hour sample resulted in the largest relative hydrolysis rate, 2.12. Although ball milling requires a great deal of energy, it is a simple, clean, and effective method of pretreatment.

Gamma irradiation. The effects of gamma irradiation on cellulose were, in part, visually perceived. Higher dosages resulted in a yellowish color in the samples. In addition, a sweet, sugary smell was present and more obvious as the dosages increased. These observations were due to sugar production by depolymerization during treatment (see Fig. 5). Under microscopic observation, the irradiated fibers appear unchanged.

There is no significant effect on the crystalline nature of cellulose by gamma irradiation until the dosage approaches 5×10^8 rad (see Fig. 6). This implies that a moderate degree of depolymerization, as evidenced by the sugar production, can occur without a great disruption of the crystal lattice structure.

The five samples with dosages between 2 and 50 Mrad show increasing surface area with increasing dosage in Fig. 7. The 100 and 500 Mrad samples differ slightly due to the different method of irradiation. These samples had only a small amount of air present in the tightly packed polyvials. It is known that the presence of oxygen enhances depolymerization slightly (Blouin and Arthur, 1960). The radiation treatment appears

to cause a substantial increase of specific surface area, and the increase becomes larger as the dose becomes larger. The specific surface area of the 500 Mrad sample, 106.2 m^2g^{-1}, is the largest area recorded in this work.

The effects of the radiation pretreatment on the enzyme hydrolysis rate do not become substantially positive until high dosages are reached (see Fig. 8). The low dosage samples are actually less susceptible to hydrolysis than the standard substrate cellulose. This may be due to the generation of an enzyme inhibiting by-product. If this is the case, the inhibiting power of the by-product is overcome at higher dosages by the increase in availability caused by depolymerization, surface area increase, and, in the 500 Mrad sample, crystallinity decrease. If high dose rates of gamma radiation are readily available, this pretreatment shows great promise. The 500 Mrad sample surpasses the 96 hour ball milled sample in digestibility with a relative hydrolysis rate of 4.10.

Pyrolysis treatment. The colors of the two pyrolyzed samples were dark brown and light brown for the air- and helium-pyrolyzed cellulose, respectively. The air-pyrolyzed sample smelled sweet to a greater degree than the helium-pyrolyzed sample. Microscopic examination of the samples revealed no major changes in the fibers, except that the air-pyrolyzed fibers possessed a brownish tint.

The specific surface areas of the solvent-dried air- and helium-pyrolyzed samples were 2.07 and 3.61 m^2g^{-1}, respectively (see Table 3). These values are small compared to the specific surface area of the solvent-dried standard substrate, 8.18 m^2g^{-1}. The heating of the cellulose may have caused this, and the presence of oxygen apparently enhanced the effect. One possible explanation for the low areas is that tarry products

were formed which, when evaporated, have the same effect as evaporating water. The structural collapse should be more pronounced in the sample with the most tarry products, and the air-pyrolyzed sample does exhibit a smaller specific surface area. Preferred destruction of the amorphous regions during heating may also contribute to the decreased specific surface area.

The pyrolysis treatments had little effect on crystallinity. The solvent-dried crystallinity indices were 74.9 for air-pyrolysis and 74.6 for the inert atmosphere technique compared to 77.4 for the standard substrate. The decrease is significant but not major.

Despite the negative effect on the specific surface area, the pyrolysis procedures had substantial positive effects on the enzymatic hydrolysis rate. The relative hydrolysis rates were 1.25 and 2.18 for the air- and helium-pyrolyzed samples, respectively. The air-pyrolyzed sample was obviously more highly degraded by the pretreatment as evidenced by a higher initial sugar concentration, 1.45 as compared to 1.05 mg ml^{-1}. However, it was less easily hydrolyzed by enzymes than the helium-pyrolyzed cellulose. Two explanations for this are possible. First, a large amount of the most highly accessible cellulose may be destroyed in the presence of oxygen, whereas, in the helium atmosphere, the cellulose is merely depolymerized. Secondly, the enzyme reaction may be inhibited by pretreatment reaction by-products, and the air-pyrolyzed sample obviously had a greater amount and variety of these by-products. The inert atmosphere pyrolysis procedure is approximately as effective as 96 hours of ball milling as a hydrolysis pretreatment. Not a great deal of work has been done on the heat treatment of cellulose for enzymatic hydrolysis, so further research could lead to an even more promising technique.

Swelling in 1% NaOH. The two 1% NaOH treatments effected a profound change on the measured specific surface area of the cellulose fibers. The surface areas of the solvent-dried celluloses were 12.2 and 23.2 m^2g^{-1} for the room temperature and autoclaved samples, respectively (see Table 3). This is evidence of the swelling effect of sodium hydroxide, which is apparently enhanced under autoclave conditions. Microscopic observation has revealed no obvious change in either of the two cellulose fiber samples. Table 3 shows that the crystallinity indices for the solvent-dried room temperature and autoclaved samples, 75.5 and 75.9, respectively, were significantly but only slightly lower than that of the solvent dried standard substrate, 77.4.

The relative hydrolysis rates of the 1% NaOH treatments were 1.37 for the room temperature method and 1.45 for the autoclaved sample. Both of these hydrolyses were performed on cellulose samples that had been treated and then solvent-dried. If the solvent-drying procedure was imperfect in its water removal role, then a slight amount of irreversible structural collapse may have occurred during drying. If this is the case, then the relative hydrolysis rates of the 1% NaOH treatments may be slightly larger for an actual process that does not include solvent-drying. It should be remembered that the solvent-dried surface areas and CrI's may not portray the characteristics of the actual water-swollen cellulose, but because the solvent-drying procedure should be constant, qualitative conclusions can be drawn from relative evaluations. The dilute sodium hydroxide treatments are not as effective in improving hydrolysis rates as are the physical treatments already discussed. The sodium hydroxide treatments could become important if the economics of these treatments is favored over the more effective treatments or if the 1% NaOH is especially effective on natural substrates containing extraneous materials.

CMCS solvent treatment. The CMCS treatment imparted an orange color to the cellulose that persisted even after solvent-drying. Under the microscope, the fibers appeared less frayed than untreated fibers. During the treatment, the cellulose fibers could still be seen and it appeared that the cellulose did not actually dissolve. The fibers did, however, appear to be different than fibers in, for instance, the 1% NaOH solution. The fibers did not appear to be white but looked translucent, as if totally wetted.

The specific surface area of the solvent-dried CMCS cellulose was 27.4 m^2g^{-1} (see Table 3). This is substantially greater than the specific surface area of room temperature 1% NaOH treated cellulose. This and the difference in appearance during treatment suggest that the CMCS solvent causes structural changes, such as intracrystalline swelling, that are more prodigious than the intercrystalline swelling mechanism of the low concentration NaOH. The CrI of the CMCS treated sample was inexplicably high, 79.3 (see Table 3). This is slightly higher than the CrI of the standard substrate, 77.4.

The relative hydrolysis rate of the CMCS treated cellulose was 1.44, higher than that of the room temperature 1% NaOH treatment, 1.37. Again, this may be due to the solvent's penetrating capacity. The CMCS sample was solvent-dried before the hydrolysis was performed and this, as was the case for the dilute NaOH treatment, may be responsible for a slight irreversible structural change. The high CrI value may be an indication of this. The CMCS treatment was not as effective in increasing the hydrolysis rate of cellulose as were the physical treatments, but the large effect on specific surface area indicates that this method warrants further study.

Sulfuric acid solvent treatment. The sulfuric acid treatment was similar to the CMCS treatment in that it seemed that the fibers did not actually dissolve but did appear translucent, rather than white. Due to the short reaction time, little cellulose was lost to acid hydrolysis and other yield-reducing reactions. The specific surface area of the solvent-dried acid treated cellulose was the largest of all of the chemically treated samples, 29.2 $m^2 g^{-1}$ (see Table 3). As mentioned before, it seems that the "dissolution" process opens up substantial surface area. The CrI of the solvent-dried acid treated sample, 74.3, was not a great deal lower than the standard substrate's CrI, 77.4. In both the CMCS solvent and the acid solvent techniques, the major effect was to increase the specific surface area while the CrI's changed only slightly.

The relative hydrolysis rate of the acid treated sample was 1.41, which compares closely with that of the CMCS treatment, 1.44. These two "solvent" methods are not as effective in increasing digestibility as the physical methods mentioned before. The relative hydrolysis rates of the two solvent methods would probably increase slightly if the cellulose was not solvent dried before hydrolysis. A process economics study may indicate which of the two procedures is the most economically favorable.

Examining all of the treatment methods and their influence on cellulose structural parameters gives insight to the relative effectiveness of the methods. The ball milling method was by far the most effective method of destroying the crystal lattice structure of the cellulose fibers. This was probably due to the crushing, shearing action of the ball mill. The nonmechanical treatments were not able to effect the same magnitude of change in the CrI. The three chemical treatments, CMCS, sulfuric acid, and 1% NaOH-autoclaved, all generated new surface area to a greater extent

than any of the physical treatments, except the 500 Mrad gamma radiation sample. This was probably due to the fact that the swelling action of water was enhanced by the chemical bond-breaking capacity of the reagents used. The large surface area of the 500 Mrad sample might be due to extensive depolymerization. The pretreatments that were most effective in increasing hydrolysis rates were the 96 hour ball milling, the helium pyrolysis, and the 500 Mrad gamma irradiation techniques. Interestingly, all three were physical techniques that involved depolymerization, though the mechanisms of attack were different.

Wheat straw. Wheat straw was briefly examined as a substrate for enzymatic hydrolysis. Table 3 indicates that non-solvent-dried wheat straw standard possesses a specific surface area of 1.92 m^2g^{-1}, which compares closely with the 1.71 m^2g^{-1} for the Solka Floc standard. However, the solvent-dried specific surface area, 25.5 m^2g^{-1}, is much greater than the corresponding value for the Solka Floc. This may be due to the presence of extraneous materials in the wheat straw. Another possibility is that because the wheat straw had never been dried from water, as had the Solka Floc, its capillary structure was never irreversibly collapsed by drying. The CrI of the wheat straw was 46.2 for non-solvent-dried and 45.1 for solvent-dried substrate. The slight decrease in CrI is contrary to the expected increase due to recrystallization and to removal of amorphous solubles during solvent exchange.

The 1% NaOH soaking treatment under autoclave conditions was applied to the wheat straw substrate. The wheat straw turned darker brown during the treatment and a great deal of the substrate was apparently solubilized. This was evidenced by the color of the wash water and the solvent exchange fluids. After solvent-drying the substrate was extremely fluffy with a

much lower bulk density. The specific surface area of the wheat straw increased more than three-fold to 89.1 m^2g^{-1}. The removal of some of the extraneous materials from the cellulose structural matrix by the NaOH apparently exposed a great deal of the capillary surface of the wheat straw. The CrI actually increased to 62.9, but this might have been due to the removal of a large amount of the extraneous materials. When the hemicellulose and lignin were removed, newly freed amorphous cellulose might have recrystallized when wetted because physical deterrents no longer intervened. The treated wheat straw was hydrolyzed 3.64 times faster than the untreated wheat straw substrate. This emphasizes the importance of another structural parameter of cellulosics; namely, the amount of extraneous materials present.

The wheat straw was also ball milled for 96 hours. However, the data obtained were questionable because of a large amount of ceramic dust that was produced and mixed with the cellulose. Nevertheless, it appears that the method was very effective because there was a sweet smell after milling and the x-ray crystallinity index was essentially 0. The hydrolysis rate of the milled cellulose leveled off after only 4 to 8 hours, suggesting that all available cellulose had been digested.

B. The Effect of Structural Parameters on the Rate of Enzymatic Hydrolysis.

Specific surface area. As described earlier, the specific surface areas were measured by nitrogen adsorption after solvent-drying. It should be remembered that these areas are not the actual surface areas accessible to cellulase in solution. However, the surface area of solvent-dried cellulose better reflects the properties of the water-swollen state than does the surface area of water-dried cellulose. These measurements

were carried out not to determine the exact water-swollen surface area of cellulose but to make a semiquantitative or qualitative comparison of the effects of the pretreatments on the structural parameters of cellulose. It was expected that a general trend would appear regardless of treatment that would show an increasing hydrolysis rate with increasing specific surface area. The heterogeneous nature of the enzyme hydrolysis reaction dictates such a relationship. Figure 9 supports this contention to a large degree.

It was expected that both crystallinity and surface area would affect the hydrolysis rate. If this is the case, a plot of the specific surface area against the relative hydrolysis rate would not indicate a dependence relationship between them because the crystallinity would also be varying. Only if the CrI were constant would such a curve be produced. This type of curve is drawn in Fig. 9. The solid circles represent cellulose samples that possess CrI's within a fairly narrow range, betwen 70 and 79.3. All of these points except two fall close to the curve, indicating the existance of the expected relationship between specific surface area and relative hydrolysis rate. The two nonconforming points represent the 100 Mrad gamma irradiation sample and the helium-pyrolysis sample. The triangular points represent the ball milled samples, all of which had crystallinity indices below 66. These data points were expected to lie above the curve because their substantially lower crystallinity would tend to increase their relative hydrolysis rates. The square point, representing microcrystalline cellulose, lies just slightly below the line; it had a higher CrI, 88.8, than the round points, making its relative hydrolysis rate lower.

Crystallinity index. This section examines the effect of crystallinity, as represented by the CrI, on the relative hydrolysis rate. There would not be a direct correspondence between the CrI and the relative hydrolysis

rate unless the specific surface area of a group of samples was constant. In Fig. 10, the solid circles represent the data for samples with low surface areas, between 1.15 and 8.18 m^2g^{-1}. All solid circles lie within a narrow band along the smooth curve except for the 100 Mrad gamma irradiation and the helium-pyrolysis sample points. This indicates that, in general, the crystallinity does have the expected effect on hydrolysis rate. The triangular points, which represent samples with specific surface areas above 12.18 m^2g^{-1}, all fall above the line. This was expected because a substantially greater surface area should logically raise the relative hydrolysis rate. The 500 Mrad gamma irradiation sample point was expected to be and is located farthest from the line because its specific surface area was the largest.

The previous two sections present convincing evidence that crystallinity and surface area are two factors that influence the enzymatic hydrolysis rate of cellulose. If the two graphs, Figs. 9 and 10, were combined in a three-dimensional plot of CrI, specific surface area, and relative hydrolysis rate, a rough surface would be described by the data points. This implies that crystallinity and specific surface area are the principal two parameters needed to predict the hydrolysis rate of pure cellulose.

Only two points on Figs. 9 and 10 were blatantly inexplicable, the 100 Mrad gamma irradiation and the helium pyrolysis sample points. These two points do not conform for two possible reasons. The solvent-drying of these two samples may have been imperfect, and the resulting structural collapse would explain their positions on the graphs. Another plausible explanation is that there is another structural parameter that can become important in some instances. Because both of these samples were subjected

to harsh depolymerization reactions, the degree of polymerization is a logical possibility for a third important structural parameter.

VI. CONCLUSION

The relative effectiveness of several promising cellulose pretreatment techniques was established. Until recently, evaluation of pretreatments had been limited to studies on their effects on hydrolysis rates. This work used the same approach augmented by the determination of structural changes during pretreatment. The understanding of each pretreatment's mechanism of action could lead to the development of actual or a multiple pretreatment scheme. In synthesizing such a process, a combination of two or more pretreatments that affect different aspects of cellulose structure would be utilized. This would insure that a total structural deterioration of the substrate would be accomplished.

The results indicate that both the CrI and the specific surface area influence the enzyme hydrolysis rate. It is difficult to conclude that one parameter is more important than the other. It is essential that further research be done on this subject. This research should include the perfection of a solvent-drying technique that would generate a more accurate and reproducible representation of water-swollen cellulose. The development of a different surface area determination method, such as a solute exclusion technique, may also be beneficial. The task of ascertaining the dependence of the relative hydrolysis rate on the CrI and the specific surface area would still be problematical. For instance, there is evidence (Betrabet and Paralikar, 1978) that different cellulose crystal surfaces are hydrolyzed at differing rates.

The next step in expanding research in the field should also involve the characterization of the hydrolysis rate of native cellulosics. Here,

the amount of extraneous substances becomes as significant as the two other structural parameters investigated in this work. When all of these challenges are met, a practical, economical process of the enzymatic conversion of cellulose to glucose will be a reality.

ACKNOWLEDGMENTS

Financial support was supplied by the Agricultural Experiment Station. Appreciation for guidance is extended to Dr. L. T. Fan and Yong-Hyun Lee.

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APPENDICES

Appendix A

Specific Surface Area Calculations

(Instructions, The Perkin-Elmer Shell Model 212D Sorptometer, Perkin-Elmer, Norwalk, Connecticut, Revised Feb., 1967)

The calculation of the specific surface area (S_s) is based on the BET equation, and the plot of this equation which yields the desired data. The following formulae indicate the pre-calculations necessary for plotting.

Flow Rates

The flow rates of the gases have to be calculated from the measured transit time of a film between the two calibration marks in the bubble flowmeter.

$$F_c = \frac{60}{t_c} v_{meter} \quad (1)$$

$$F_t = \frac{60}{t_t} v_{meter} \quad (2)$$

$$F_a = F_t - F_c \quad (3)$$

where

F_c = carrier gas flow rate (ml/min.)

F_t = total gas flow rate (ml/min.)

F_a = adsorption gas flow rate (ml/min.)

t_c = soap film transit time for the carrier gas flow (sec.)

t_t = soap film transit time for the total (carrier and adsorption) gas flow (sec.)

v_{meter} = soap bubble flowmeter volume between the calibration marks (ml)

Partial Pressure of Nitrogen (p)

$$p = \frac{F_a}{F_t} p_T \tag{4}$$

Volume of Adsorbed Nitrogen

$$v_{ads\ (R)} = \frac{A_{des}}{A_{cal}} v_{cal} \tag{5}$$

where:

A_{des} = area under the desorption peak (counts or surface units)

A_{cal} = area under the calibration peak (counts or surface units)

$v_{ads\ (R)}$ = volume of nitrogen adsorbed by the sample at room temperature and barometric pressure (ml)

v_{cal} = volume of nitrogen in the injection tube at T_R temperature and P_T pressure (ml)

Now, v_{ads} has to be corrected to standard temperature and pressure

$$V_{ads} = v_{ads} \frac{P_T \cdot 273}{760 \cdot T_R} = 0.3595 \frac{P_T}{T_R} v_{ads} \tag{6}$$

where:

V_{ads} = the volume of nitrogen adsorbed by the sample, corrected to 760 mmHg pressure and 0° C temperature (ml).

The symbol "f" is used for the correction factor:

$$f = \frac{P_T \cdot 273}{760 \cdot T_R} = 0.3595 \frac{P_T}{T_R} \tag{7}$$

The BET Plot

With the help of the formulae given above, the values necessary for the BET plot can be calculated. The BET plot is the graphical representation of the BET equation:

$$\frac{p}{V_{ads}\,(p_o - p)} = \frac{c-1}{V_m c}\,\frac{p}{p_o} + \frac{1}{V_m c} \tag{8}$$

where:

p = partial pressure of the adsorption gas

p_o = saturation pressure of the adsorption gas over the solid sample at the temperature of the coolant

V_{ads} = total volume (STP) of adsorbed gas on the surface of the adsorbant

c = constant expressing the net adsorption energy

V_m = volume (STP) of adsorbed gas when the entire adsorbent surface is covered with a monomolecular layer.

With V_m and c constant for a given system, the BET equation assumes the form of a linear equation where the slope is given by:

$$\alpha = \frac{c-1}{V_m c} \tag{9}$$

and the intercept is given by

$$\beta = \frac{1}{V_m c} \tag{10}$$

and equation (8) can be written in the following form:

$$y = \alpha x + \beta \tag{11}$$

where:

$$x = \frac{p}{p_o} \tag{12}$$

and

$$y = \frac{p}{V_{ads}\,(p_o - p)} \tag{13}$$

From the plot of y vs. x, the slope and intercept can be evaluated graphically. From equations (9) and (10), the following expression can be obtained for V_m:

$$V_m = \frac{1}{\alpha + \beta} . \qquad (14$$

Once V_m is known, the total area of the sample can be calculated with the help of the value of the area covered by one ml (STP) of adsorbate molecules:

$$S = V_m s_o \qquad (15)$$

where:

S = the total area of the sample (m^2)

V_m = the volume (STP) of adsorbed gas when the entire adsorbent is covered with a monomolecular layer (ml)

s_o = area covered by one ml (STP) monolayer of nitrogen (m^2).

Values for s_o and p_o for nitrogen as adsorbate have been tabulated. Now, the specific surface area of the sample can be calculated using equation (16):

$$S_s = \frac{S}{w} = \frac{V_m s_o}{w} \qquad (16)$$

where:

S_s = the specific surface area of the sample (m^2/g)

w = the weight of the sample (g).

Appendix B

Glucose Assay - DNS Method

(Miller, G. L., Analytical Chem., 31, 426 (1959).

I. Preparation of DNS Reagent

Combine until dissolved:

Distilled water	1416 ml
Dinitrosalicylic acid	10.6 g
NaOH	19.8 g

Then add:

Rochelle Salts (NaK Tartrate)	306 g
Phenol	7.6 ml
Na meta bisulfite	8.3 g

Titrate a 3 ml sample of the DNS reagent with .1 N Hcl. This should take 5-6 ml of HCl. Add NaOH if required (2g NaOH = 1 ml 0.1 N HCl). Store in a refrigerator and keep away from light.

II. Glucose Determination

Dilute the sample until it contains 0.2 to 1.0 mg/ml glucose. Place a 0.5 ml sample in a test tube and add 3 ml of DNS reagent. Boil tube for 5 min. in a water bath and then cool to room temperature. Glucose standards containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml glucose should be included in the assay. The % transmittance at 550 nm should be read with a blank prepared identically using citrate buffer (.05 M, pH 4.8) for 100% transmittance. Convert % transmittance to absorbance and plot the standards. They should give a straight line intersecting the glucose axis at .04 mg glucose. The value of glucose concentration in the sample can be determined by interpolation of the standard line. The method is non-specific and measures any reducing compound. If glucose is used as a standard, values for cellobiose will be 15% low and values for xylose will be 15% high on a weight basis.

THE USE OF CELLULOSE STRUCTURAL PARAMETERS TO EVALUATE PRETREATMENTS FOR ENZYMATIC HYDROLYSIS

by

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B.S., Kansas State University, 1977

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

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1979

ABSTRACT

This thesis examined various pretreatments for cellulose to be used as a substrate for enzymatic hydrolysis to glucose. The effectiveness of these pretreatment schemes was evaluated in two ways. The extent of hydrolysis of the treated cellulose was determined by measuring reducing sugar production during the enzyme reaction. Also, the changes in two structural parameters of pure cellulose, crystallinity and specific surface area, were established for each pretreatment method. These two structural parameters may be the two most important structural factors affecting the hydrolysis rate of pure cellulose and are measured using x-ray diffraction and nitrogen adsorption techniques, respectively. The treatment methods studied were ball milling, gamma irradiation, pyrolysis, soaking in 1% NaOH, dissolution in CMCS solvent, and dissolution in 60% H_2SO_4. It was found that the most effective treatments to enhance sugar production were gamma irradiation at 500 Mrad dosage, ball milling for 96 hours, and pyrolysis in helium at 170°C for 24 hours. These treatments improved the sugar production to, respectively, 4.10, 2.18, and 2.12 times the sugar production of untreated cellulose. The most effective treatment for reduction of crystallinity was the ball milling treatment, which reduced the crystallinity index from 77.4 to 36.5 after 96 hours of milling. The most effective treatment for increasing the specific surface area of cellulose was gamma irradiation at 500 Mrad dosage which increased the specific surface area of pure cellulose from 8.18 to 106.2 m^2g^{-1}. Three other treatments that significantly increased the specific surface area were dissolution in 60% H_2SO_4, dissolution in CMCS solvent, and soaking in 1% NaOH under autoclave conditions. These pretreatments increased the cellulose surface area from 8.18 to 29.2, 27.4 and 23.2 m^2g^{-1}, respectively. For a natural substrate, wheat straw, swelling in 1% NaOH under autoclave

conditions was an effective treatment. Sugar production occurred at 3.64 times faster than sugar production for untreated wheat straw, and the specific surface area increased from 25.5 to 89.1 m^2g^{-1} after treatment.

This thesis also investigated the relationship between the two structural parameters of cellulose, crystallinity and specific surface area, and the enzyme hydrolysis rate. In general, it was found that, regardless of treatment, an increasing crystallinity index resulted in lower hydrolysis rates and an increasing specific surface area resulted in higher hydrolysis rates. Both of the parameters have a major effect on the hydrolysis reaction, and they act in a coupled manner.