

THE EFFECT OF PRETREATMENTS ON THE RATE OF ENZYMATIC  
HYDROLYSIS OF WHEAT STRAW AND ITS STRUCTURAL FEATURES

by

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## 1. INTRODUCTION

The conversion of agricultural residues into fuel has been receiving increasing attention in recent years. The upward spiral in the price of fossil fuels, coupled with impending shortages, has made agricultural residues an attractive candidate as a supplemental source of energy. Wheat straw represents a renewable and low cost energy resource and is available in significant amounts in select areas of this country.

Enzymatic hydrolysis of native lignocellulosics is prohibitively slow for developing an economically feasible process at this time. The resistance of biomass to enzymatic attack can be attributed to the following three major factors (13,15,48);

- i. Cellulose in lignocellulosic biomass possesses highly resistant crystalline structure.
- ii. Lignin surrounding cellulose forms a physical barrier.
- iii. Sites available for enzymatic attack are limited.

Cellulose in lignocellulosics is composed of crystalline and amorphous components. The amorphous component is more readily digested by enzymatic attack than the crystalline component, and any means that will increase the amorphous content will enhance the hydrolysis rate. The presence of lignin forms a physical barrier for enzymatic attack, and hence, pretreatments, causing disruption of the lignin seal, increase the accessibility of cellulose and eventually its hydrolysis rate. The limitation of available sites for enzymatic attack stems from the fact that the average size of the capillaries in biomass is too small to allow the entry of large enzyme molecules, and enzymatic attack is confined, therefore, to the external surface. Thus, pretreatment is a prerequisite to enhance the susceptibility of lignocellulosic

residues to enzyme action. An ideal pretreatment would accomplish reduction in crystallinity, concomitant with a reduction in lignin content, and an increase in surface area. Many different pretreatments have been attempted and the literature on this subject is voluminous (12,14,33). The pretreatments can be classified into physical and chemical depending on their modes of action on the substrate.

Physical pretreatments can be grouped into mechanical and non-mechanical pretreatments. The mechanical pretreatments include ball-milling (12,28,34), two-roll milling (45), hammer-milling (28), colloid-milling (20), vibro-energy milling (17,32,40), and extrusion (19). These pretreatments utilize shearing and impacting forces to yield a fine substrate possessing a low crystallinity index, and hence its susceptibility to enzyme action is enhanced. The use of this fine substrate allows a higher slurry concentration, thus reducing the reactor volume. The non-mechanical pretreatments cause decomposition of lignocellulosics by exposing them to harsh external forces other than shearing and impacting forces. These include  $\gamma$ -irradiation (10, 12,23,24,41), pyrolysis (43), and steaming (3). In irradiation, cellulose undergoes extensive depolymerization. In pyrolysis depolymerization, oxidation, or dehydration takes place in addition. Although considered to be effective, physical pretreatments have received less attention, as evident from fewer publications appearing in the literature compared to chemical pretreatments.

Chemical pretreatments have been used extensively as a means of lignin removal and structural modification of lignocellulosics. Conventional pulping processes, such as kraft, sulfite, and soda processes, are suited for delignification, but these processes have been designed for removal of lignin so that the quality of cellulose is preserved rather than for maximizing the

yield of sugars upon enzymatic hydrolysis. Furthermore, these processes are too costly as bioconversion pretreatments. The possibility of using modified pulping processes and other innovative procedures has been suggested (9). The most common chemical pretreatment has been caustic swelling (10,16,19,34, 35). Pretreatment with caustic soda leads to an increased surface area due to swelling and disruption of the lignin structure. In acid pretreatment, acids, such as HCl (19),  $\text{H}_2\text{SO}_4$  (19,23), and  $\text{H}_3\text{PO}_4$  (50), have been used, which remove hemicellulose by hydrolysis. Another technique is chemical oxidation of lignin by an oxidizing agent, e.g., peracetic acid. This technique liberates cellulose for enzymatic digestion (46,47). Recently, gases, such as  $\text{SO}_2$  (10) and  $\text{NO}_2$  (51), have been used for delignification. Pretreatment with gases is a clean and effective process. Considerable attention also has been paid to delignification using solvents, such as, ethanol, butanol, and acetone along with a suitable catalyst; the possibility of solvent recovery makes this an attractive proposition (36,38). Although most of the chemical pretreatments are effective, waste chemicals are often difficult to recycle or dispose.

It appears that most of the research efforts on pretreatments for enzymatic hydrolysis have been focused on pure cellulose, whereas pretreatments for lignocellulosics have not received sufficient attention. In addition, the enhancement of the hydrolysis rate has hardly been studied from the standpoint of structural modification. Furthermore, efforts so far have been primarily directed to single pretreatments, even though the possibility of multiple pretreatment schemes has been proposed (26). A combination of two or more pretreatments would possibly alter different structural features of lignocellulosics, resulting in total structural

deterioration of the substrate.

The objective of this work, is therefore, twofold: i) to evaluate a wide variety of pretreatments for lignocellulosics in terms of enhancement in the hydrolysis rate and cost of treatment, and ii) to conduct an overall structural feature study for the substrate subjected to single as well as multiple pretreatments to understand the mechanisms of various pretreatments in terms of enzymatic hydrolysis.

The substrate chosen for this purpose was wheat straw. In the first part of this work, various physical and chemical pretreatments were applied to the substrate. The structural features were determined experimentally, and their effects on the hydrolysis rate were studied. This will help identify promising pretreatments and the relative importance of individual structural features on the rate of enzymatic hydrolysis. In the second part of this work, the promising pretreatments identified earlier were combined to provide multiple pretreatments. In multiple pretreatments, two pretreatments were combined and the order of treatment was physical pretreatment followed by chemical pretreatment. This was followed by an overall structural feature determination for the pretreated substrate. This will help, first to identify the effects of various pretreatments on the structural features of the substrate, and second to examine the relationship between structural features and the hydrolysis rate. This relationship, together with the measured values of the structural features, will facilitate understanding of the mechanisms of various pretreatments.

## 2. LITERATURE REVIEW

### 2.1 Availability, Structure, and Composition of Wheat Straw

Wheat straw is abundant in the United States, with its availability ranging from 54 - 108 million tons annually. For every ton of harvested wheat grain, about 2.5 tons of wheat straw is produced. This vastly available resource is used to some extent for pulp manufacture. In spite of its vast availability, only a small fraction of wheat straw is used in pulp manufacture. The disadvantages involved are to some extent technical, but mainly economic. The bulky material is costly to handle, collect, and transport; it is produced at scattered and small areas in typical seasonal variations with the sharp peak in the late summer weeks, and it tends to deteriorate rapidly on storing without special precautions. The material usually has short fibers which do not give a very strong paper, and the presence of an outer cuticula, rich in silica, presents special technical problems. As to the advantages, wheat straw has a chemical composition comparatively rich in carbohydrates and a fairly low content of easily accessible lignin, which makes it easy to pulp to good yields when properly cooked. The rate of growth of the material is, as a rule, as high or higher than in the case of wood (7).

The chemical composition of wheat straw resembles that of hardwoods with a slightly lower lignin and cellulose content but a somewhat higher hemicellulose content (see Table 1).

Wheat straw is composed of the following tissues as shown in Fig. 1: (1) the epidermis, (2) the hypoderm or zone of mechanical tissue beneath the epidermis, (3) green assimilating parenchyma, (4) colorless ground parenchyma, and (5) the vascular bundles (39).

The epidermis, which is about 25-30 $\mu$  thick, is formed of narrow

Table 1. Composition of wheat straw (7)

Moisture	Ash	Lignin	Pentosans	Cellulose
6.6	6.6	16.7	28.2	39.9

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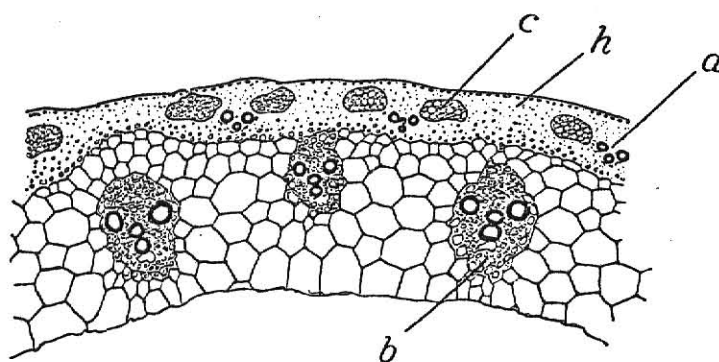


Fig. 1. Transverse section of wheat straw culm (x70)  
a. small vascular bundle in the hypoderm (h); b.  
large vascular bundle; c. band of chlorophyllous  
tissue (39)

elongated cells 150 - 250 $\mu$  long, with short square cells 9 - 13 $\mu$  across intercalated among them at intervals.

The hypoderm is a strong elastic cylinder of mechanical tissue, consisting of lignified fibers with narrow lumina and strong walls about 4 $\mu$  thick. A transverse section of straw appears as a continuous zone of cells immediately within the epidermis, and is of variable thickness being more or less wavy in outline on its inner side. The assimilating tissue of the stem resembles that of the leaf, consisting of delicate parenchymatous cells.

The colorless ground parenchyma extends from the hypoderm to the center or to the hollow pith-cavity found in most wheats. It is composed of thin-walled finely-pitted cells polygonal or rounded in section. In the lower internodes, the walls of the parenchyma become thickened and lignified; the tissue then materially assists the hypodermal stereome in strengthening the base of the straw.

In a transverse section, vascular bundles are found distributed symmetrically in the wall of the straw. Imbedded in the hypoderm is a ring of very small bundles separated from each other by somewhat wide intervals. Nearer the center, in the soft parenchyma, are the large bundles of the stem, arranged more or less regularly in a ring with an occasional bundle of intermediate size close to the inner edge of the hypoderm (39).

## 2.2. Pretreatments for Lignocellulosics

Numerous pretreatments have been attempted on both pure cellulose and lignocellulosics to enhance their digestibility. These pretreatments can be classified into physical, chemical, and biological depending upon their mode of action.

Ball-milling is an effective pretreatment for promoting enzymatic hydrolysis of lignocellulosics. Ball-milling accomplishes a reduction in crystallinity, a decrease in the mean degree of polymerization, an increase in bulk density, and a marked decrease in particle size through its compressive and shearing forces. Several investigators (12,28,34) studied the effect of ball-milling on the digestibility of cellulose or lignocellulosics. Wilke and Yang (52) reported a conversion of 72.9% for 325 mesh ball-milled newsprint upon 48 hours of hydrolysis. Millett et al. (33) noted that ball-milling is species sensitive, with softwoods showing the least response; this severely limits its applicability. Fan et al. (12) ball-milled Solka Floc for a period up to 96 hours. They found a marked decrease in the crystallinity; however, an expected increase in the surface area was not observed. They also reported that the extent of hydrolysis after 8 hours was proportional to the ball-milling time.

Fitz-milling has not been used previously for lignocellulosic pretreatment. The cutting action of the Fitz-mill provides a substantial size reduction in a short period of time.

Recently, roller milling has been applied to lignocellulosics to increase their digestibility. Tassinari and Macy (45) tested two-roll milling on a wide variety of cellulosic substrates. Their findings indicated that two-roll milled Maple chips yielded 17 times more reducing sugar than the untreated Maple. On the other hand, two-roll milled newspaper showed 2.5 fold increase over untreated newspaper and a significant increase over ball milled newspaper. Factors that control the susceptibility to enzymatic attack are the clearance between the mill rolls and the processing time. According to Spano et al. (44) as the clearance between the rolls decreases

and the processing time increases, the susceptibility to hydrolysis increases.

Extrusion, as a means of pretreatment, has been investigated recently. Brenner (55) carried out extrusion of newspaper concomitant with acid hydrolysis, and reported remarkable hydrolysis results. The high shearing action along with heating may make it a successful pretreatment.

The digestibilities of cellulose and lignocellulosics are enhanced by the use of high energy radiation (10,12,23,24,41). The radiation of pure cellulose results in oxidative degradation of the molecules, dehydrogenation, destruction of anhydroglucose units to yield carbon dioxide, and cellulosic chain cleavage. Fan et al. (12) showed that gamma irradiation of Solka Floc was very effective in increasing the specific surface area, but not effective in decreasing the crystallinity index. They also noted that the hydrolysis rate increased only after the dosage exceeded a certain level. The digestibility can be further increased by milling the substrate before irradiation or by adding nitrate and nitrite salts prior to irradiation (10,41).

Caustic soda pretreatment has been mainly used to enhance the digestibility of lignocellulosic materials for ruminants. Caustic soda pretreatment results in structural swelling leading to an increased surface area and a decreased crystallinity index. In addition, it causes separation of structural linkages between lignin and carbohydrates and disruption of the lignin seal. This pretreatment is reported to be species sensitive, with hardwoods undergoing more drastic enhancement in digestibility (16). The optimum quantity of caustic soda required to enhance the digestibility of aspen wood is 5 - 6 g NaOH per 100 g of wood, whereas that for wheat straw is 12 g NaOH per 100 g of wheat straw (10,53).

So far, sodium sulfite has not been used for lignocellulosic pretreatment. It is used commonly for pulping of straw, typically wheat straw (7). Since this treatment results in substantial delignification of straw, it may become a promising pretreatment method.

Sodium hypochlorite has not been used for pretreatment of lignocellulosics. Hypochlorite is commonly used in bleaching of pulp. Hypochlorite bleaching is mainly an oxidation which decolorizes and solubilizes the lignin, natural dyes, and other impurities in the fiber (7).

Toyama and Ogawa (46,47) used 20% peracetic acid for delignifying corn stalks, sawdust from broad leaved trees, and sawdust from coniferous trees. A significant increase in the rate of enzymatic hydrolysis was observed by them. This pretreatment, due to its potential to achieve high degree of delignification, deserves consideration.

Recently, considerable attention has been paid to organosolv delignification. April et al. (1) investigated the use of n-butanol for delignification of sweet gum. A 92% delignification was reported upon two hours of autoclaving at 200°C. This pretreatment may turn out to be economical on a large scale due to the possibility of solvent recovery.

Selvam and Ghose (38) used ethylene glycol to extract lignin from rice husks. Their results have indicated that an optimum treatment involves heating the rice husks at 170°C with a substrate to ethylene glycol ratio of 8 g per 100 ml and an acid concentration (conc. HCl) 0.9% for 1 hour. After 48 hours of enzymatic hydrolysis, 46 g/l of reducing sugar was obtained.

Dilute sulfuric acid has been commonly used for hydrolysis of pentoses. Tsao et al. (48) used this pretreatment to separate the pentose fraction. The remaining residue was dried and mixed with concentrated sulfuric acid

(70%-80%) to dissolve the cellulose, which was precipitated out of solution by addition of methanol. The resultant cellulose was saccharified easily by acid or enzymatic hydrolysis. Knappert *et al.* (22) studied partial acid hydrolysis using sulfuric acid as a pretreatment for enhancing enzymatic hydrolysis of oak, corn stover, newsprint, and Solka Floc. They employed a continuous flow reactor, with temperatures varying from 160° to 220°C and acid concentrations from 0 - 1.2% at a fixed treatment time of 0.22 minutes. For all substrates, except Solka Floc, increased glucose yields were obtained. In several cases, the pretreatment resulted in 100% conversion of the potential glucose content of the substrate after 24 hours of enzymatic hydrolysis. Dunning and Lathrop (11) carried out a study to establish an integrated process for utilization of agricultural residues. They pretreated a variety of agricultural residues varying the acid concentration, the temperature of pretreatment, and pretreatment time. It was found that pretreatment with 4.4% sulfuric acid at 100°C for 55 minutes gave significantly improved results upon acid hydrolysis. This pretreatment is interesting on account of yield of furfural as a by-product.

## 2.3 Structural Parameter Measurement

### 2.3.1. Crystallinity index

The orderly crystalline structure of cellulose has been shown to influence the rate of enzymatic hydrolysis (8,37). The precise determination of the extent of crystallinity is tedious; however, a variety of methods have been employed, e.g., acid hydrolysis, infrared absorption, and x-ray diffraction. X-ray diffraction method has been selected on account of its versatility. The spectrometric powder technique of x-ray diffraction forms the basis for the crystallinity analysis scheme.

Bragg explained x-ray diffraction in terms of "reflection" from a stack of parallel planes (21). An incident x-ray beam penetrates several million layers before being appreciably absorbed. A small fraction of the beam may be regarded as reflecting from successive layers stacked up together. The beams reflected from various layers do not undergo interference if and only if the path difference is a whole number of wavelengths.

In the random powder method, a narrow beam of monochromatic x-rays strikes the powder which is composed of randomly oriented particles. Finer powder is preferable for better results since larger particles have a tendency to orient themselves in a particular manner. A Geiger counter receiver directly measures the intensity of the diffracted x-ray beam; it records a peak in diffracted x-ray intensity only at angles specified by Bragg's law for each crystal plane (21). The variation in the relative intensity of the diffracted beam as a function of angle of incidence is recorded on a strip chart recorder. The peaks recorded represent planes with different interplanar spacings.

Native cellulose is composed of amorphous as well as crystalline fractions. The relative amounts of amorphous and crystalline cellulose present, or the crystallinity, is dependent upon the origin and treatment history of the cellulose. The crystallinity index proposed by Segal et al. (42) is based on diffracted intensities which represent crystalline and amorphous cellulose. Highly crystalline cellulose exhibits a prominent peak at the angle  $2\theta = 22^\circ$ , which represents the 002 plane, whereas the amorphous fraction is represented by the intensity at  $2\theta = 18^\circ$ . Hence, Segal et al. (42) proposed the following crystallinity index, which was also employed in this work,

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

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

### 2.3.2. Specific surface area

Lignocellulosics are porous in nature and possess considerable internal and external surface areas. The external surface area of typical wood fibers ranges from 0.6 to  $1.6 \text{ m}^2 \text{ g}^{-1}$ , depending upon the extent of fiber treatment (30). The surface of cracks, crevices, and pores of various sizes contribute to the internal surface area. Nitrogen adsorption data together with the BET equation can be employed for measurement of the surface area (4,30). The measurement of surface areas of substrates, subjected to various pretreatments and their subsequent hydrolysis rates, will reveal the 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 (5). Physical adsorption of gases onto a solid surface takes place under suitable conditions of temperature and pressure. The active forces in physical adsorption are of the order of Van der Waals forces. Adsorption occurs initially as a monolayer, a layer of adsorbed gas one molecule thick, followed by adsorption of multiple layers of gas molecules.

The BET equation is applicable in the case of a free surface where there is no limitation to the number of adsorbed gas layers (6). The final equation has the form:

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



where

$p$  = partial pressure of adsorbate

$p_o$  = vapor pressure of adsorbate

$V$  = volume of gas adsorbed

$V_m$  = volume of gas in adsorbed monolayer

$c$  = constant

The constants  $V_m$  and  $c$  can be found from a plot of  $p/V(p_o - p)$  vs.  $p/p_o$ . 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 (4).

### 2.3.3 Lignin content

The presence of a physical barrier in the form of lignin, has been shown to influence the rate of enzymatic hydrolysis of lignocellulosics (2,33,49). A variety of methods are available for the determination of lignin, most of which utilize strong mineral acids to hydrolyze the polysaccharides, leaving a residue which upon washing and drying is weighed as lignin. Some methods employ rapid reaction between lignin and oxidizing agents. Goering and Van Soest (18) have proposed permanganate oxidation method for lignin determination. This method has several advantages over other methods; such as, a shorter procedure, less corrosive reagents, no need for filter aids for the residue, and lignin values obtained are less sensitive to heat damage (18).

The lignin in unbleached pulps is readily oxidized by potassium permanganate, whereas the cellulose remains primarily unaffected. The consumption of permanganate under standardized conditions provides an estimate of residual lignin in unbleached pulps. In a simple control method,

the time required for decolorization of a permanganate solution by pulp is observed and expressed as "Roschier number"; however, most of the commonly used methods use an excess of potassium permanganate. The reaction is terminated after a specific period by addition of potassium iodide, after which the amount of potassium permanganate consumed is determined by titration.

The permanganate number of a pulp is usually expressed as the number of ml of 0.1 N potassium permanganate solution consumed by 1 g of dry pulp.. The permanganate number of a pulp is greatly influenced by the amount and concentration of permanganate. The temperature and reaction time have a significant effect too, but standardized conditions are easily fixed. The physical condition of the pulp and the manner of stirring have apparently no effect (4).

#### 2.4. Enzymatic Hydrolysis and Its Mechanism

The enzymatic hydrolysis of cellulose is a complex reaction, and several factors influence its rate. The type of substrate, extent of pretreatment, the conditions of enzyme preparation, temperature, pH, slurry concentration, product inhibition, and type of reactor employed are some of the factors which contribute to the hydrolysis reaction. The method of hydrolysis used in this work was similar to that used by Mandels et al. (28), since it represents optimal conditions for a laboratory shaker flask hydrolysis.

The enzyme system of Trichoderma sp. has been examined extensively. Based on this study several components of cellulase have been identified. Their functions have been described below (25).

Endo- $\beta$ -1, 4 glucanase: This contains several components with varying degrees of randomness. One of these may be the enzyme that acts on

crystalline cellulose; however, it acts randomly. This component does not act on cellobiose. The main products are cellobiose and cellotriose.

Exo- $\beta$ -1, 4 glucanases: This is present in several forms.  $\beta$ -1,4 Glucan glucohydrolase removes a single glucose unit from the non-reducing end of the chain.  $\beta$ -1,4-Glucan cellobiohydrolase removes a cellobiose unit from the non reducing ends of the chain.

$\beta$ -Glucosidase: This hydrolyzes cellobiose and shortchain cellooligosaccharides to glucose, but has no effect on cellulose. While it rapidly hydrolyzes cellobiose and cellotriose, its rate of attack decreases markedly with an increasing degree of polymerization.

### 3. MATERIALS AND METHODS

#### 3.1 Lignocellulosic Substrate

The winter crop wheat straw was hammer-milled and sieved. The fraction of wheat straw passing 10 mesh but retained by 45 mesh was designated as the standard substrate. This standard represented 77% of the total wheat straw.

For some experiments designated "fines", the finer fraction of substrate passing 45 mesh was used.

#### 3.2 Cellulase

The source of enzyme used was the culture filtrate from batch cultivations of the fungus Trichoderma reesei (viride) QM9414 on Solka Floc. This strain is a mutant that produces high quantities of enzyme; 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 2.

The substrate employed for the enzyme production was Solka Floc SW-40 at 1% concentration. A 300 ml flask was filled with 50 ml of culture medium and autoclaved. It was inoculated with 10 ml of sterile 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 and the cultivation was carried out under the same conditions. Enzyme was harvested from the large flasks by filtration through glass wool and stored in a refrigerator until used for the hydrolysis. The soluble protein content of the enzyme broth, measured by the Lowry method (27), using bovine serum albumin as the standard,

ranged from 2.2 to 2.5 mg/ml. The enzyme activity measured by the filter paper method (29) was between 1.3 and 1.5 IU/ml.

### 3.3 Pretreatments

The milling pretreatments include ball-milling, Fitz-milling, and roller-milling. The ball-milling method employed a 5ℓ porcelain jar mill with a 50 volume percent charge of 1 inch diameter porcelain spheres. The jar was rotated at about 52 rpm for various periods of time. Wheat straw was added in an amount that filled only the void volume between the balls. Fitz-milling was carried out using a Fitz mill, Model D, comminutor (Fitzpatrick Company, Elmhurst, Illinois). The extent of size reduction was controlled by varying the screen sizes. In roller-milling, the extent of size reduction was varied by changing the milling time. Two milling times, 15 and 30 minutes, were used.

In extrusion pretreatment, two experiments were performed using a Wenger Model X-20 extruder. One experiment was carried out with an outlet die to exert pressure and the other was carried out without an outlet die.

In gamma radiation pretreatment, wheat straw was sealed in sample jars in the presence of air. The jars were placed in a  $\text{CO}^{60}$  gamma cell and irradiated at the rate of 500 rad/min. (1 rad = 0.01 J/kg) until total dosages were reached.

The caustic pretreatment was performed using 1% (0.25 N) NaOH solution. One hundred grams of wheat straw were mixed with 1000 ml of the NaOH solution. One half of the mixture was allowed to stand for 2 hours at room temperature, and the other half was autoclaved for 2 hours at 129°C and 2.57 atm. (23 psig). In both cases, the treated wheat straw was washed until the wash water was neutral.

In the sodium sulfite pretreatment, 13.7% sodium sulfite solution was used. One hundred grams of wheat straw were mixed with 700 ml of the  $\text{Na}_2\text{SO}_3$  solution. One half of the mixture was allowed to stand for 2 hours at room temperature, and the other half was autoclaved for 2 hours at 129°C and 2.57 atm. (23 psig). In both cases, the treated wheat straw was washed until the wash water was neutral.

Dilute sodium hypochlorite solution (4-6%  $\text{NaOCl}$ ) was used in the hypochlorite pretreatment. One hundred grams of wheat straw were mixed with 1000 ml of hypochlorite solution. One half of the mixture was allowed to stand for 2 hours at room temperature, and the other half was autoclaved for 2 hours at 129°C and 2.57 atm. (23 psig). In both cases, the treated samples were washed until the distinct odor of hypochlorite was thoroughly removed.

In peracetic acid pretreatment (1:1 by volume of acetic anhydride and 35% hydrogen peroxide), 100 g of wheat straw were boiled with 1000 ml of peracetic acid at 100°C for 30 minutes. The substrate was washed until the wash water was neutral.

In butanol pretreatment, 40 g of wheat straw were autoclaved (1l autoclave by Autoclave Engineers Inc.) with 200 ml of butanol and 200 ml of water in the presence of a 0.005% aluminum chloride catalyst. The pretreatment was carried out at 175°C for 1.5 hours. The treated wheat straw was washed until the wash water was neutral.

In ethylene glycol pretreatments, 48 g of wheat were mixed with 600 ml of ethylene glycol and 13 ml of conc.  $\text{HCl}$ . One half of this mixture was boiled at 170°C for 30 minutes, and the other half was autoclaved at 129°C and 2.57 atm. (23 psig) for 1 hr. The treated wheat straw was washed until

the wash water was neutral.

In dilute sulfuric acid pretreatment, 21.6 g of wheat straw were mixed with 360 ml of 4.4%  $H_2SO_4$ . This was boiled at 98°C for 1 hour, after which the substrate was washed until the wash water was neutral.

### 3.4 Structural Parameter Determination

#### 3.4.1 Crystallinity index determination

The crystallinity index was measured by employing the powder method of x-ray diffraction using a General Electric Diffractometer. The substrate samples were dried overnight at 80°C. The samples were prepared by the powder mounting method of McCreery (21). The specimen was mounted vertically while a Geiger counter moved in a horizontal arc. The samples were scanned over a range of  $2\theta$  from 10° to 30°. The calculation of the crystallinity index of Segal *et al.* (42) requires the measurement of the intensity of the 002 peak and the amorphous intensity at  $2\theta = 18^\circ$ . Figure 2 shows that the intensities were measured above an approximate baseline.

#### 3.4.2 Specific surface area determination

The specific surface area of each sample was determined by applying the BET equation to the nitrogen adsorption data obtained using a Perkin-Elmer Model 212D flow sorptometer. Each sample was degassed overnight to prepare the lignocellulosic surface for adsorption measurements. This was achieved by passing helium at 80°C slowly through the U-shaped sample tube which contained 0.2 - 0.7 g of sample. An enlarged bulb at the bottom of the U-tube held the sample. Precaution was taken to provide sufficient room for the passage of gas through the sample without entrainment; in addition, a small amount of glass wool was placed in the tube to prevent any powder from fouling the detector during measurement. Nitrogen was used as

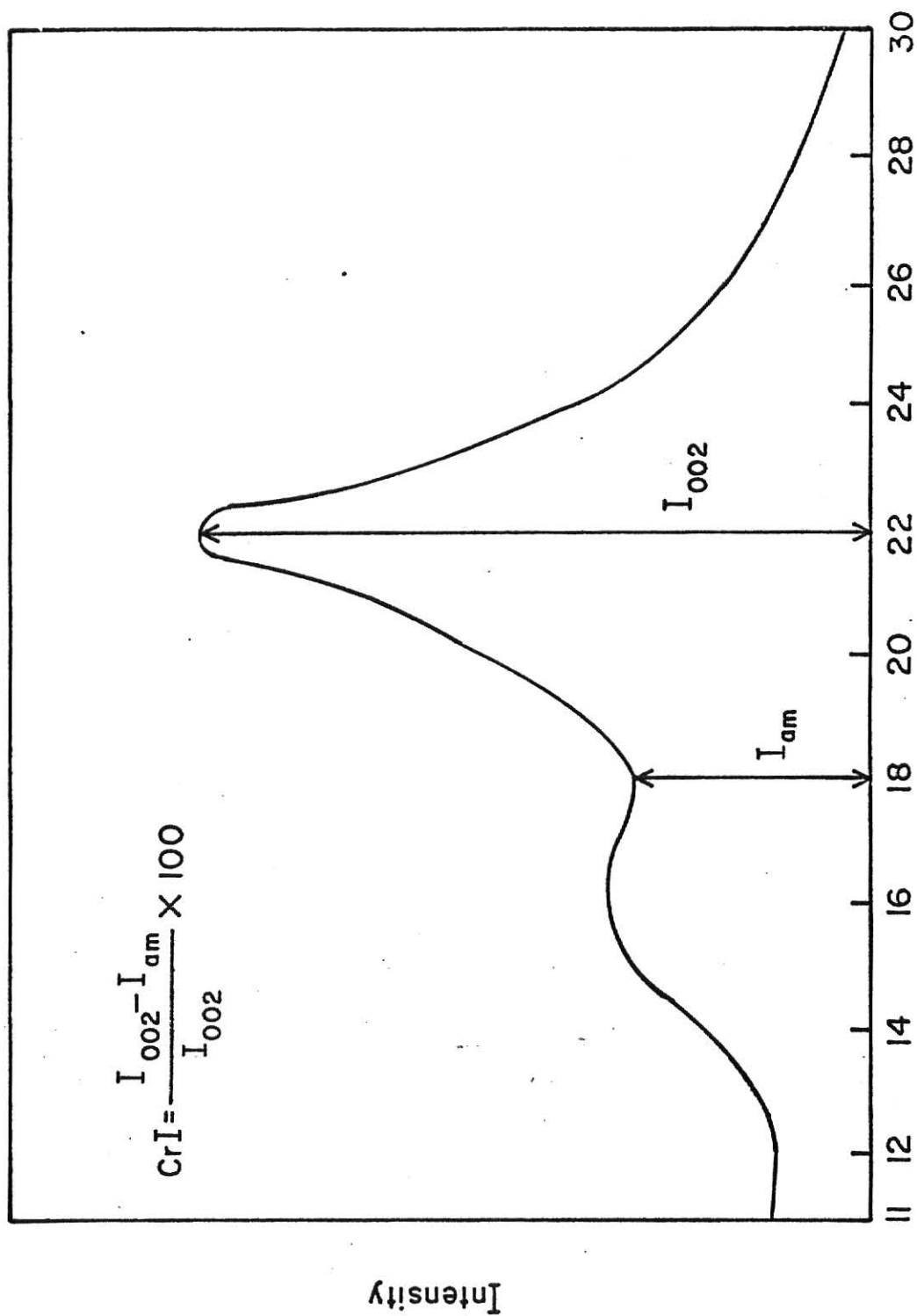


Fig. 2. X-ray diffraction pattern of wheat straw



the adsorbate gas, while helium was used as the carrier gas. A strip chart recorder was connected to the sorptometer to record the adsorption and desorption peaks. The sample tube was attached and flow rates of nitrogen and helium were determined with a soap bubble flowmeter. After baseline adjustment, liquid nitrogen was applied to the sample tube to effect adsorption. The adsorption was carried out for approximately four minutes. The polarity of the detector was then reversed, and liquid nitrogen was removed to effect desorption. A room temperature water bath was used to speed up the desorption. The desorption peak was typically sharper than the adsorption peak. The release of a known amount of nitrogen from a calibration loop produced a calibration peak. The areas of the desorption peaks were compared to the area of the calibration peak to determine the volume of nitrogen adsorbed by the sample. A planimeter was used to determine the areas of peaks. Adsorption and desorption at three different flow rates (partial pressures) were carried out to plot a three point BET graph. The specific surface area was calculated from the slope and intercept of the plot.

#### 3.4.3 Lignin content determination

Lignin determination was carried out by means of the permanganate oxidation method proposed by Goering and Van Soest (18).

#### 3.4.4 Electron microscopic observation

For electron microscopic observation, the wheat straw stems were placed perpendicular to aluminum stabs with colloidal carbon glue. The mounted straw was coated with Gold-Palladium to a thickness of about 15 nm, using a Kenny Vacuum Co. Model KSE-2-A-M evaporator. After this, the specimens were observed and photographed with an Etec Auto Scan Scanning Electron Microscope (SEM).

### 3.5 Enzyme Hydrolysis

The substrate was dried overnight at 80°C, and 5 g were weighed into a 250 ml flask, after which 45 ml of 0.05M citric acid buffer (pH 4.8), and 50 ml of the culture filtrate (enzyme solution) were added to the flask to obtain a 5% substrate suspension. The flask was placed in a 50°C incubator set at 250 rpm. Samples were withdrawn at different time periods, centrifuged, and the supernatant was refrigerated. The samples were analyzed for reducing sugar content by the dinitrosalicylic acid (DNS) method (31) after appropriate dilution.

Table 2. Medium Composition for Cultivation of T. reesei

<u>Component</u>	<u>Amount</u>
Distilled Water	1000 ml
$(\text{NH}_4)_2 \text{SO}_4$	1.4 g
$\text{KH}_2\text{PO}_4$	2.0 g
Urea	0.3 g
$\text{CaCl}_2$	0.3 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.3 g
Trace Metal Stock	1.0 ml
Tween 80	2.0 ml
Proteose Peptone	1/10 the concentration of the substrate

Trace Metal Stock

Distilled Water	495 ml
Conc. HCl	5.0 ml
$\text{FeSO}_4$	2.5 g
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.98 g
$\text{ZnSO}_4 \cdot \text{H}_2\text{O}$	1.76 g
$\text{Co}(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$	1.25 g

#### 4. RESULTS AND DISCUSSION

##### 4.1 Evaluation of Pretreatments for Enzymatic Conversion of Agricultural Residues

###### 4.1.1 Effect of chemical pretreatments on structure of substrate and its hydrolysis rate

The results obtained from the hydrolysis of the chemically pretreated wheat straw are presented in Table 3. It includes the extent of hydrolysis after 8 hours, relative extent of hydrolysis after 8 hours, and maximum conversion. The extent of hydrolysis after 8 hours is the reducing sugar produced during the first 8 hours of hydrolysis. It can be considered as an indicator of the hydrolysis rate. The relative extent of hydrolysis is the ratio of the extent of hydrolysis of the pretreated wheat straw to that of the untreated standard substrate; the magnitude of this parameter is a convenient measure of the degree of enhancement of the hydrolysis rate. The maximum conversion is the fraction of pretreated wheat straw that has been converted into sugars upon 96 hours of hydrolysis; the hydrolysis essentially ceases at this time. The yield from pretreatment, lignin content, and percent delignification are also listed in Table 3. The yield from pretreatment is the fraction of wheat straw that is recovered after the pretreatment.

In general, chemical pretreatments appear to be effective in enhancing the hydrolysis rate. Such pretreatments have given rise to large values of the relative extent of hydrolysis. This is particularly true for caustic soda, peracetic acid, and ethylene glycol pretreatments.

Caustic Soda. Pretreatment with caustic soda led to a significant increase in the hydrolysis rate, both at room temperature and under autoclave condition. The relative extent of hydrolysis was 4.3 for the former and 9.6 for the latter. The hydrolysis rate would be enhanced by two factors.

Table 3. Effect of chemical pretreatments on hydrolysis rate and lignin content

Type of pretreatment	Extent of hydrolysis after 8 hrs, g/l	Relative extent of hydrolysis after 8 hrs	Maximum conversion, %	Lignin content %	Delignification, %	Yield from pretreatment, %
Caustic-RT	9.1	4.3	36.0	10.6	8.6	45.7
Caustic-AC	20.5	9.6	62.0	6.5	43.5	50.3
Sulfite-RT	2.1	1.0	10.6	9.6	16.9	49.8
Sulfite-AC	16.0	7.6	38.0	6.4	45.5	60.0
Hypochlorite-RT	12.0	5.7	36.4	6.8	40.9	44.0
Hypochlorite-AC	13.3	6.3	55.9	9.7	16.6	39.1
Peracetic acid	20.9	9.9	54.5	2.8	76.2	46.7
Butanol	4.7	2.2	10.6	10.9	5.7	61.8
Ethylene glycol-RT	15.4	7.3	40.0	-	-	26.5
Ethylene glycol-AC	18.2	8.6	54.5	1.9	83.8	35.6
Dil. Sulfuric acid	9.4	4.5	25.4	17.0	-	50.3
Standard	2.1	1.0	5.5	11.6	0	100.0

One was removal of the lignin seal; lignin removal was 8.6% after pretreatment at room temperature, and 43.5% after autoclaving. The higher hydrolysis rate for the autoclaved substrate might be due to the higher extent of delignification achieved. The other was structural swelling of the substrate in alkaline medium; this might increase accessibility of the substrate to enzymatic attack. The substrate appeared light yellow after pretreatment at room temperature and light brown and fluffy after autoclaving. Thus, autoclaving might have caused more structural modification than the pretreatment at room temperature. The low yield of wheat straw from caustic pretreatment was probably due to the loss of components such as lignin, hemicellulose, and extractives.

Sodium Sulfite. Wheat straw autoclaved with sodium sulfite solution (13.7%) gave a value of the relative extent of hydrolysis of 7.6. The hydrolysis rate of the autoclaved substrate was enhanced presumably due to the lignin removal; Table 3 shows substantial delignification after autoclaving. Surprisingly, the hydrolysis rate was not enhanced by the pretreatment at room temperature. Relatively low extent of lignin removal was observed for the pretreatment at room temperature. This might be a reason for the relatively low hydrolysis rate for the substrate pretreated at room temperature. Both conditions rendered the wheat straw light yellow in color, but the autoclaved wheat straw was lighter and fluffier than the sample from pretreatment at room temperature, showing additional structural modification. The loss of lignocellulosic components, such as lignin, hemicellulose, and extractives resulted in a low yield of wheat straw from pretreatment.

Sodium Hypochlorite. Impressive hydrolysis results were obtained following pretreatment of wheat straw with hypochlorite solution both at room temperature and under the autoclave condition; the lignin contents of pretreated substrate were 6.8 for the former and 9.7% for the latter. Sodium

hypochlorite is a very strong oxidizing agent that reacts with lignin and forms water soluble compounds. Hypochlorite, however, also reacts with cellulose and other components of lignocellulosics upon prolonged treatment, and these reactions are further enhanced under high temperature and pressure. This dissolution of cellulose and other lignocellulosic components might possibly be the reason for higher lignin content in the substrate after pretreatment under the autoclave condition. The substrate appeared pale white, and it was very light in weight and fibrous after pretreatment at room temperature. The autoclaved substrate had a dark brown appearance and was light in weight. Thus it appeared that the substrate was structurally modified by the pretreatment. The yield from pretreatment under the autoclave condition was 39%; this low yield indicates the severity of the pretreatment.

Peracetic acid. Pretreatment of wheat straw with peracetic acid resulted in a substrate that was highly susceptible to the enzymatic hydrolysis. Approximately a ten-fold increase in the hydrolysis rate over the standard substrate was observed. The drastic increase in the hydrolysis rate might be due to the high degree of delignification achieved, as can be seen in Table 3. A similar delignification was reported by Toyama et al. (46,47) after pretreatment of sawdust with peracetic acid. The substrate appeared white, fibrous and light, possibly indicating extensive structural modification. This pretreatment can remove lignin extensively in a short period of time.

Butanol. Wheat straw subjected to butanol extraction under high temperature and pressure showed only a slight increase in the hydrolysis rate, as can be seen in Table 3. This might be due to the low extent of delignification attained by this pretreatment. Pretreated wheat straw appeared practically unchanged.

Ethylene Glycol. The data in Table 3 appear to indicate that ethylene

glycol is an excellent solvent for modifying the structure of wheat straw; the relative extent of hydrolysis upon pretreatment with boiling was 7.3 and that with autoclaving was 8.6. Pannir Selvam et al. (38) also reported a significant increase in the hydrolysis rate of rice straw and bagasse upon ethylene glycol pretreatment. Extraction of a large quantity of lignin by ethylene glycol might be responsible for substantial enhancement in the hydrolysis rate (see Table 3). The substrate was rendered dark brown in color and light in weight. The low yield of pretreated wheat straw reflects the possible loss of major lignocellulosic components.

Sulfuric acid. Pretreatment of wheat straw with dilute sulfuric acid gave approximately a four fold increase in the hydrolysis rate over the standard substrate. Preferential extraction of hemicellulose upon sulfuric acid treatment has possibly led to an increase in the lignin content, as seen in Table 3. Modification of the lignin-carbohydrate linkage might be a plausible reason for improving the hydrolysis rate. The substrate was light in weight and appeared brown in color after the pretreatment.

#### 4.1.2 Relationship between the extent of delignification and hydrolysis rate

Wheat straw, subjected to a variety of chemical pretreatments, yielded a wide spectrum of lignin content. It ranged from 11.5% for the standard substrate to 1.9% for the ethylene glycol treated substrate. Figure 3 shows the relative extent of hydrolysis plotted against the extent of delignification. The hydrolysis rate increased substantially with an increase in the extent of delignification upto about 50% delignification; however, beyond this the hydrolysis rate increased only slightly. A similar observation was reported by Millett et al. (33) for in vitro digestibility of paper birch and red oak. There are three plausible reasons for this phenomenon; (a) Delignification beyond 50% leads to a collapse of the lignocellulosic structure, thus, shrinking the available surface area for enzymatic attack, which is generated by



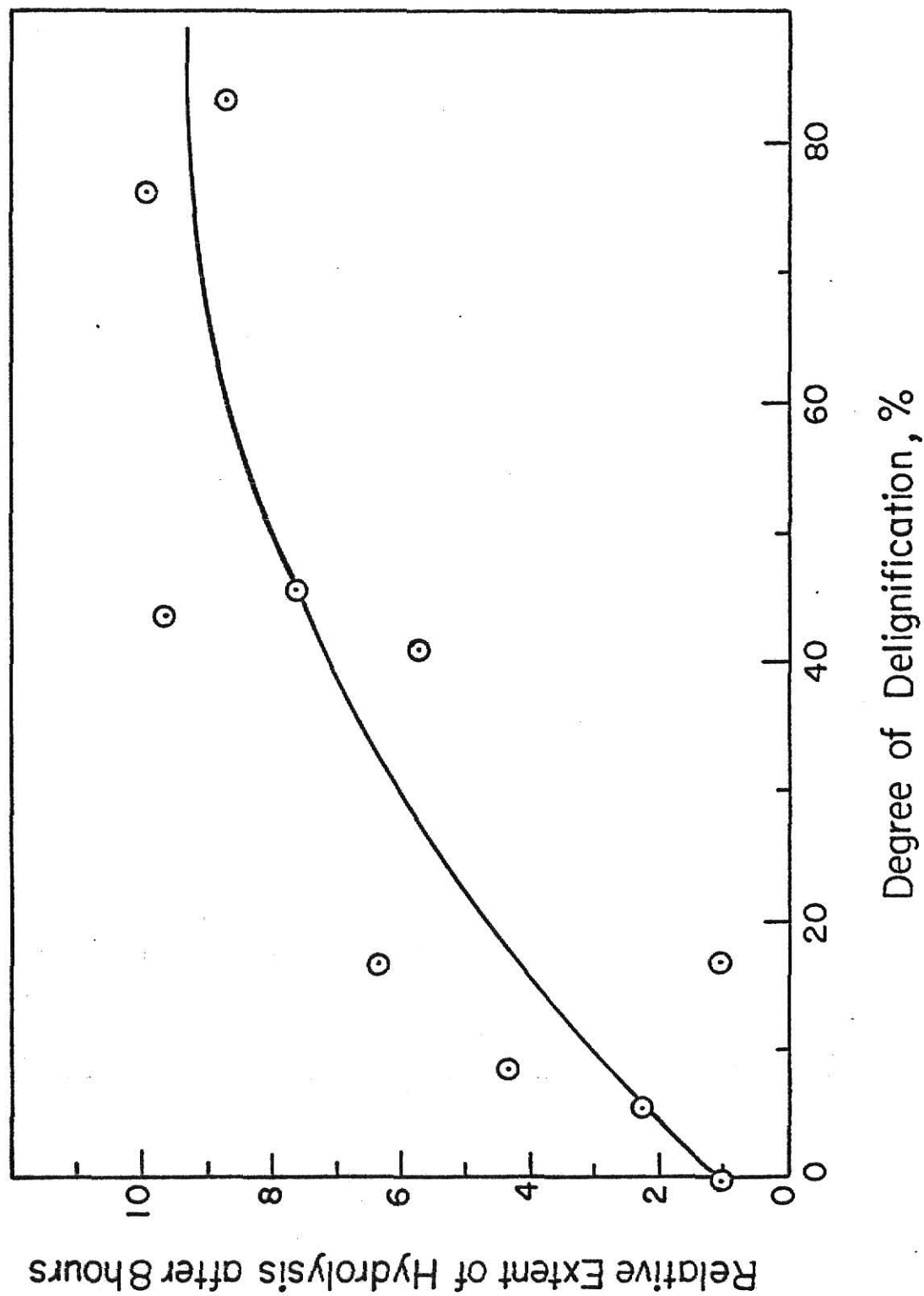


Fig. 3. The relationship between the relative extent of hydrolysis and degree of delignification

removal of the lignin seal. (b) Two kinds of pores, macropores and micropores, are present in the lignocellulosic structure. Exposure of the macropore surface by lignin removal increases the hydrolysis rate, but that of the micropore surface does not, because of their geometrical hindrance to large enzyme molecules. (c) The cellulose in lignocellulosics possibly undergoes structural rearrangement upon extensive delignification, e.g., recrystallization of cellulose.

#### 4.1.3 Effect of physical pretreatment on structure of substrate and its hydrolysis rate

The results obtained from the hydrolysis of the physically pretreated wheat straw are presented in Table 4. Crystallinity indices of these substrates are also listed in the table. In general, the physical pretreatments appear to give a substantial increase in the hydrolysis rate, but to be less effective than the chemical pretreatments. Ball-milling and roller-milling seem to be the most promising among the physical pretreatments.

Ball-milling. Extensive ball-milling of wheat straw enhanced substantially the hydrolysis rate, as seen in Table 4; for example, 8 hours of ball-milling gave approximately a four-fold increase in the hydrolysis rate over the standard substrate. Such an increase might be attributable to the decrease in the crystallinity index (see Table 4) and extensive size reduction caused by the shearing and compressive forces of ball-milling.

Fitz-milling. The cutting action of Fitz-milling gave minor improvement in the hydrolysis rate. The relative extent of hydrolysis was 1.3 for coarse milling and 1.4 for fine milling. Fine Fitz-milling resulted in substantial size reduction but only a slight reduction in crystallinity index; this low reduction in crystallinity might be responsible for the low hydrolysis rate.

Roller-milling. The roller-milling of wheat straw led to a considerable amplification of the hydrolysis rate. The relative extent of hydrolysis was

Table 4. Effect of physical pretreatments on hydrolysis rate and crystallinity index

Type of pretreatment	Extent of hydrolysis after 8 hrs g/%	Relative extent of hydrolysis after 8 hrs	Maximum conversion, %	Crystallinity index
Ball-milling				
4 hr	7.2	4.0	23.6	-
8 hr	9.1	4.8	23.3	54.5
16 hr	7.2	3.6	19.1	-
24 hr	9.8	4.4	19.1	-
Fitz-milling				
coarse	2.7	1.3	8.2	-
fine	3.0	1.6	9.1	65.6
Roller-milling				
¼ hr	5.3	2.5	12.4	-
½ hr	6.5	3.3	14.5	57.6
Extrusion				
under pressure	2.7	1.3	5.8	-
without pressure	2.5	1.2	5.8	-
γ-Irradiation				
10 Mrads	1.4	0.7	5.5	-
30 Mrads	4.2	2.0	13.9	-
50 Mrads	5.6	2.7	18.8	-
Standard	2.1	1.0	5.5	69.6

2.5 for 15 minutes roller-milling and 3.1 for 30 minute roller-milling. The high shearing action of the roller-mill caused fragmentation and a decrease in the crystallinity index of wheat straw. The roller-milling as a pretreatment is promising because it is capable of substantially enhancing the hydrolysis rate in a short period of pretreatment time.

Extrusion. Compared to the standard substrate, the hydrolysis rates were 1.3 and 1.2 times for extrusion with and without pressure, respectively (see Table 4). The substrate appeared fibrous but did not undergo any size reduction.

$\gamma$ -Irradiation. A dosage of 10 Mrad gave a slight reduction in the hydrolysis rate; this might have occurred due to formation of an inhibitory product which decomposed on extended irradiation. The hydrolysis rate increased only when the dosage of radiation reached a certain level; e.g., the dosage of 50 Mrad gave relative extent of hydrolysis of 2.7. The increase might be due to the depolymerization of cellulose caused by an intensive input of energy.

#### 4.1.4 Relationship between the crystallinity index and hydrolysis rate

A limited number of data on the crystallinity index are plotted against the relative extent of hydrolysis in Figure 4. It can be seen that the range of the crystallinity index observed was relatively narrow; it varied from 69.6 for the standard substrate to 54.5 for the 8 hr ball-milled substrate. The hydrolysis rate increased with decreasing crystallinity. Notice that the hydrolysis rate increased drastically, even though the crystallinity index underwent a small change.

#### 4.1.5 Comparison between physical and chemical pretreatments

It is clear that, in general, chemical pretreatments were more effective than physical pretreatments. Ball-milling and caustic pretreatments were found

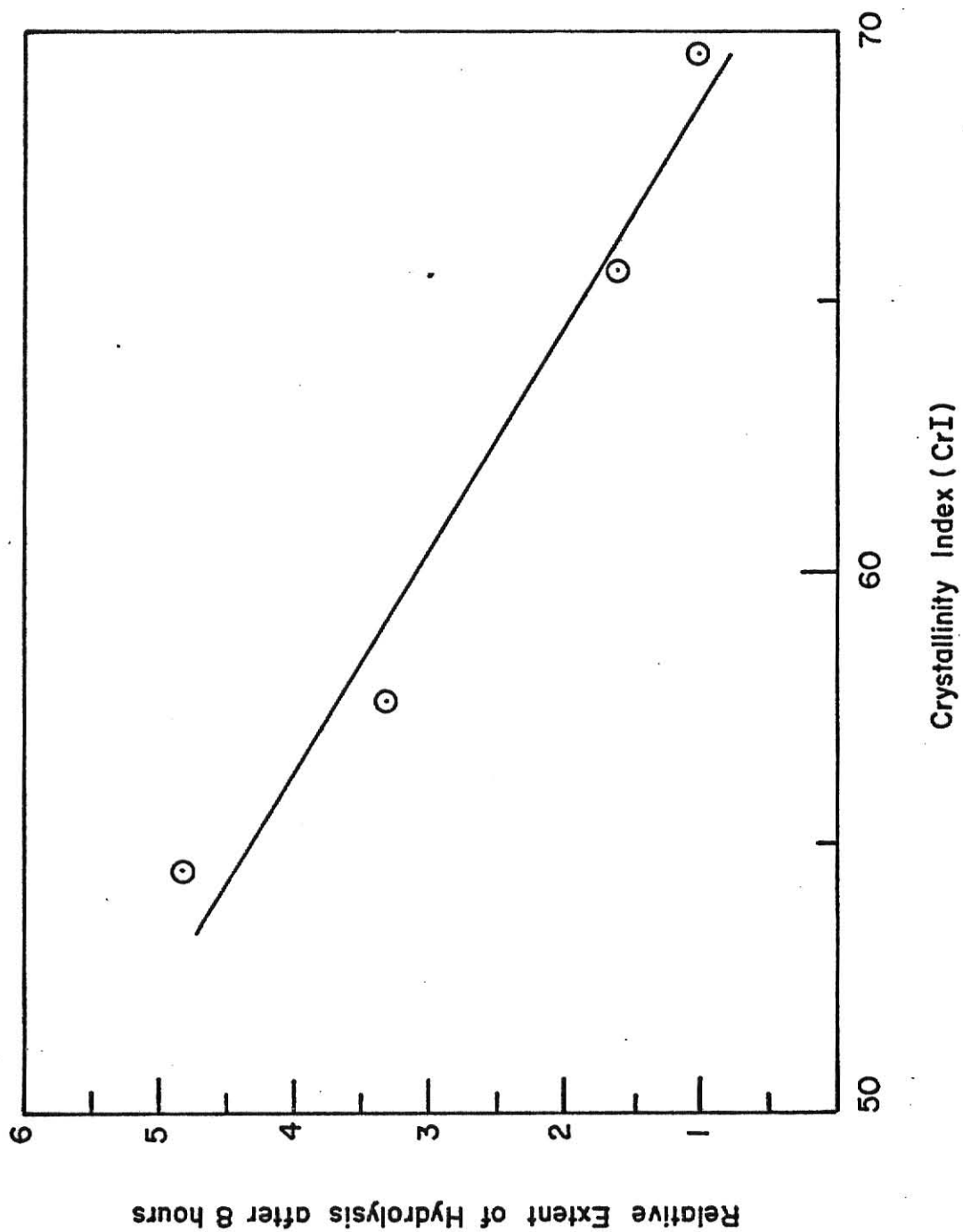


Fig. 4. The relationship between the relative extent of hydrolysis and crystallinity index

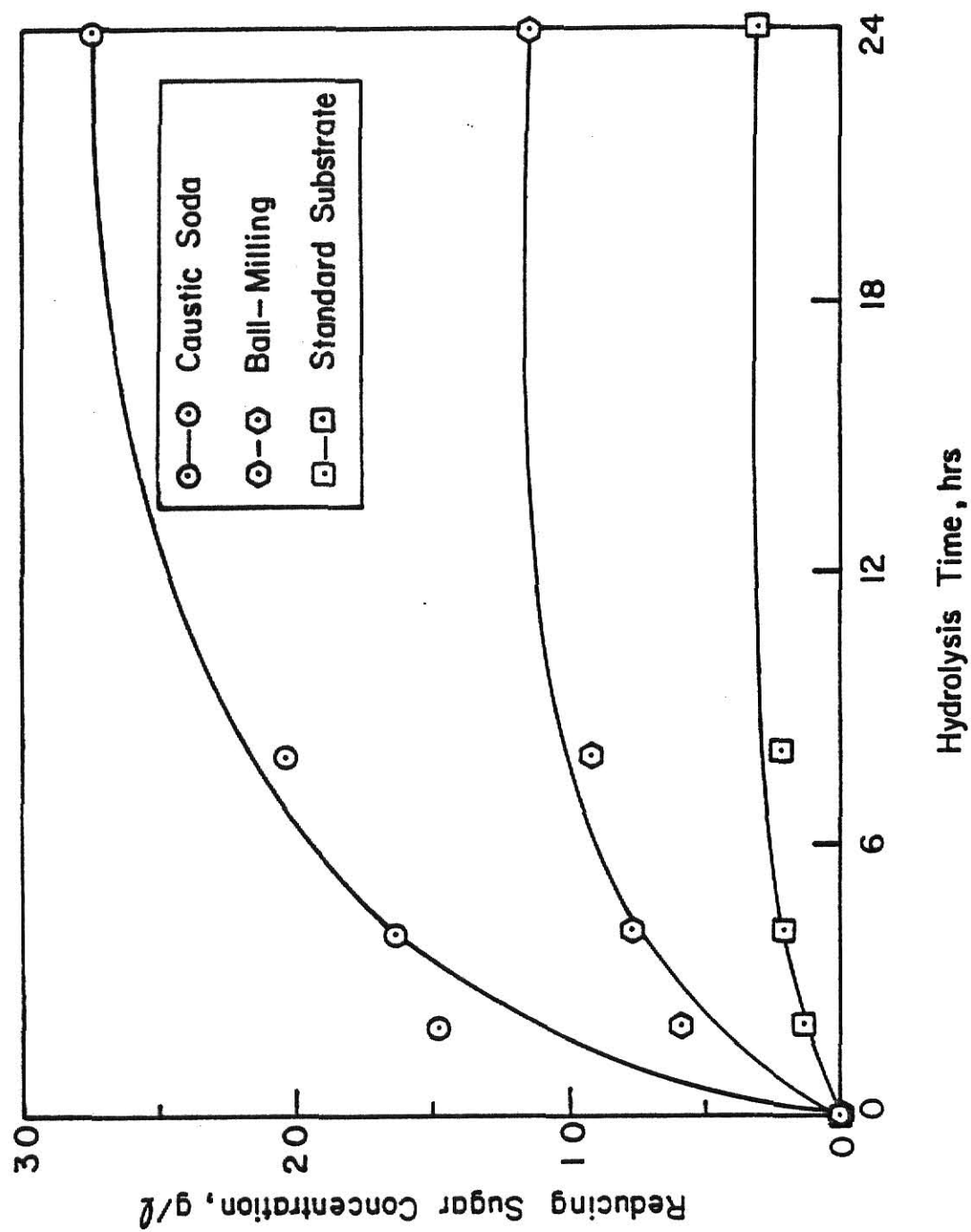


Fig. 5. The progress of hydrolysis for caustic treated, ball milled, and untreated wheat straw

to be the most effective among the physical and chemical pretreatments, respectively. Figure 5 represents the progress of hydrolysis for ball-milled wheat straw, caustic treated wheat straw, and untreated standard wheat straw.

Both physical and chemical pretreatments increased the hydrolysis rate; however, enhancement of the rate by the chemical pretreatments far exceeded that of the physical pretreatments. The chemical pretreatments caused delignification without any size reduction, whereas the physical pretreatments caused drastic size reduction and reduction in crystallinity index without any delignification. This has led to the following observations: (a) The presence of lignin predominantly controls the heterogeneous hydrolysis reaction. (b) Size and crystallinity reduction only moderately increase the hydrolysis rate.

#### 4.1.6 Scanning electron microscopic observations

Figure 6 shows a SEM photomicrograph (400 magnification) of the cross-section of an untreated mature wheat stem, with arrows indicating specific structures, such as cuticle (A), small vascular bundle (B), hypoderm (C), and large vascular bundle (D). Figure 7 shows the cross-section of caustic pretreated wheat stem. The structural swelling is obvious, along with the distortion of small vascular bundles and cell wall rupture. Sulfite pretreatment led to structural swelling, distortion of small vascular bundles, and cell wall rupture as illustrated in Fig. 8. Distortion and loosening of cells that resulted from peracetic acid pretreatment can be seen in Fig. 9. In addition, a large fraction of the middle lamellae was lost. Figure 10 shows drastic structural modification as a result of ethylene glycol pretreatment. It induced total rupture and separation of cells, in addition to separation of cuticle and hypoderm. These observations are in agreement with our earlier discussion on structural modification due to various pretreatments.

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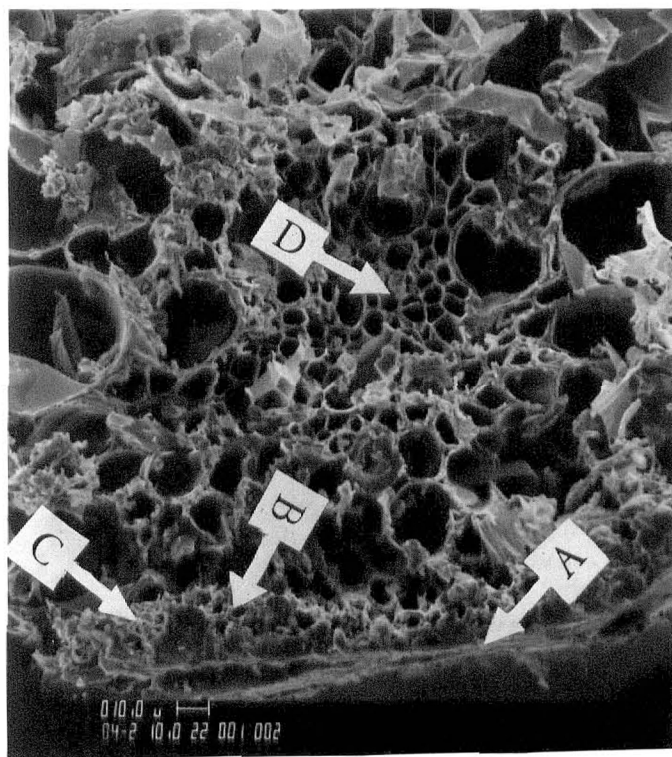


Fig. 6. SEM of untreated wheat straw stem cross-section (x 400). Arrows indicate cuticle (A), small vascular bundle (B), hypoderm (C), and large vascular bundle (D).

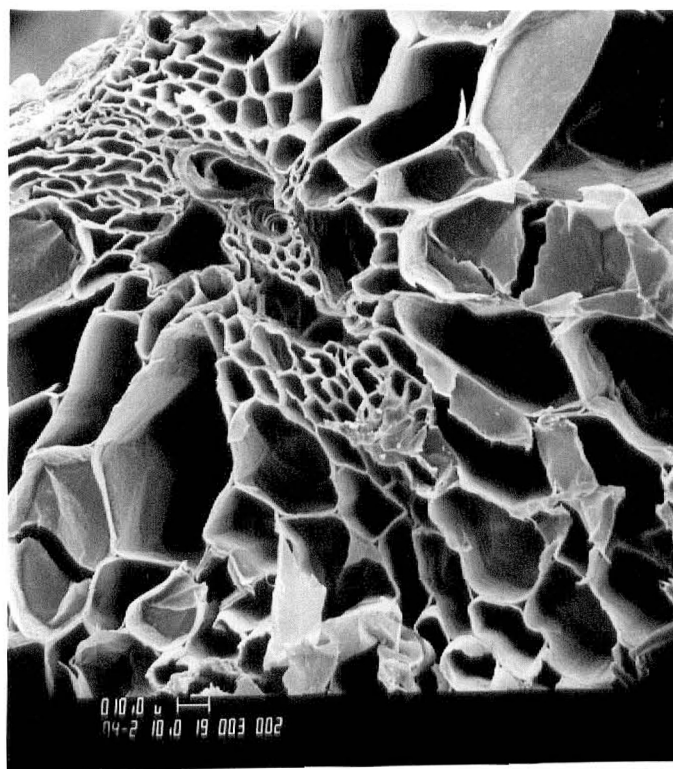


Fig. 7. SEM of caustic (autoclave) treated wheat straw stem cross-section (x 400)

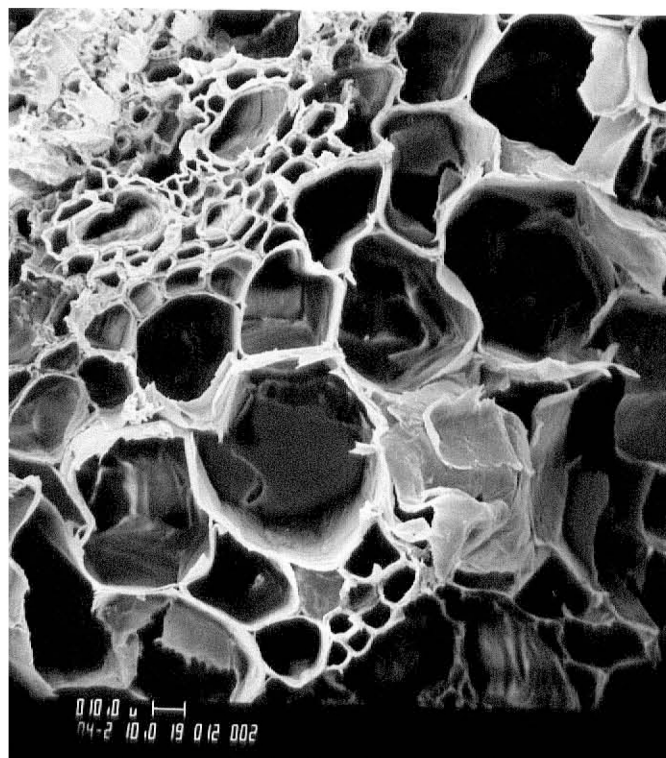


Fig. 8. SEM of sulfite (autoclave) treated wheat straw stem cross-section (x 400)

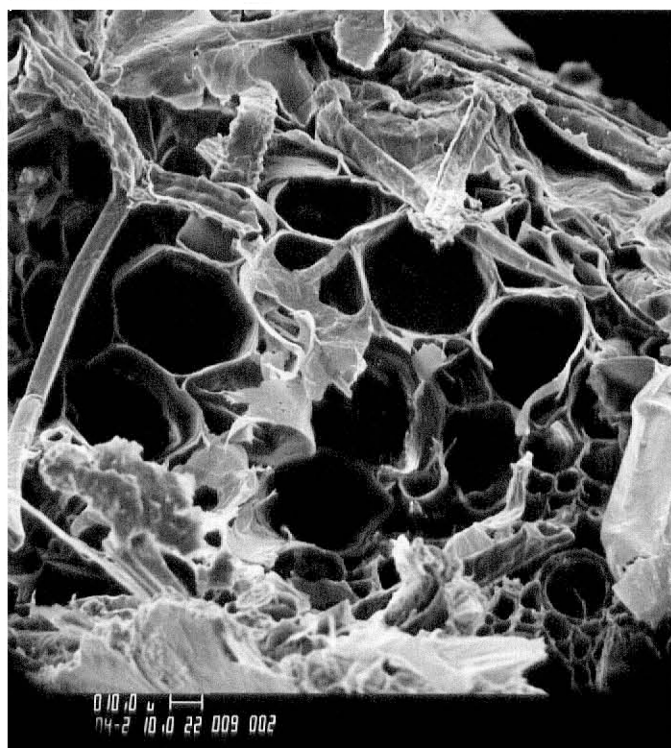


Fig. 9. SEM of peracetic acid treated wheat straw stem cross-section (x 400)



Fig. 10. SEM of ethylene glycol (autoclave) treated wheat straw stem cross-section (x 400)

#### 4.1.7 Economic analysis of pretreatments

Tables 5 and 6 show results of the cost analysis of chemical and physical pretreatments, respectively. The cost of a chemical pretreatment includes only the cost of chemical used (54), and that of a physical pretreatment includes only the cost of energy consumed by laboratory scale apparatus. Therefore, the cost of physical pretreatments would be further reduced for a larger scale of operation.

The costs of chemical pretreatments varied from 4¢/kg of wheat straw for caustic pretreatment to \$11.25/kg of wheat straw for ethylene glycol treatment. The costs of physical pretreatments varied from 1¢/kg of wheat straw for Fitz-milling to \$2.24/kg of wheat straw for roller-milling.

Among the physical pretreatments, ball-milling gives the most promising results in terms of the hydrolysis rate and sugar yield. This pretreatment is clean and easy to operate, but the pretreatment time of 8 hours makes it impractical on a large scale operation. Among the chemical pretreatments, caustic and sulfite pretreatments appear most promising. Caustic pretreatment is a potential candidate for large scale process development based on pretreatment cost, hydrolysis rate, and sugar yield it renders. In addition, ethylene glycol pretreatment is notable because of its effectiveness and possibility of recovery through an appropriate scheme, which will significantly reduce its cost. The chemical pretreatments, however, have disadvantages that should not be ignored. These include use of specialized corrosion resistant equipment, need of extensive washing, and disposal of chemical wastes.

### 4.2 Influence of Structural Features of Agricultural Residues on Enzymatic Hydrolysis

#### 4.2.1 Effect of single pretreatment on structural features and composition of substrate

Table 7 lists the specific surface area, crystallinity index, lignin

Table 5. Cost analysis of chemical pretreatment methods

Type of Pretreatment	Yield of sugar per kg of wheat straw, g/kg	Extent of hydrolysis after 8 hrs, g/l	Cost of chemicals per kg of wheat straw, \$/kg	Pretreatment cost based on sugar, \$/kg
Caustic-AC	341.0	20.5	0.04	0.12
Sulfite-AC	252.9	16.0	0.32	1.26
Hypochlorite-AC	239.9	13.3	0.94	3.92
Peracetic Acid	279.9	20.9	7.51	26.84
Butanol	72.4	4.7	2.86	39.49
Ethylene glycol-AC	241.2	18.2	11.25	46.53
Sulfuric acid	140.5	9.4	0.11	0.78
Standard	70.0	2.1	-	-



Table 6. Cost analysis of physical pretreatment methods

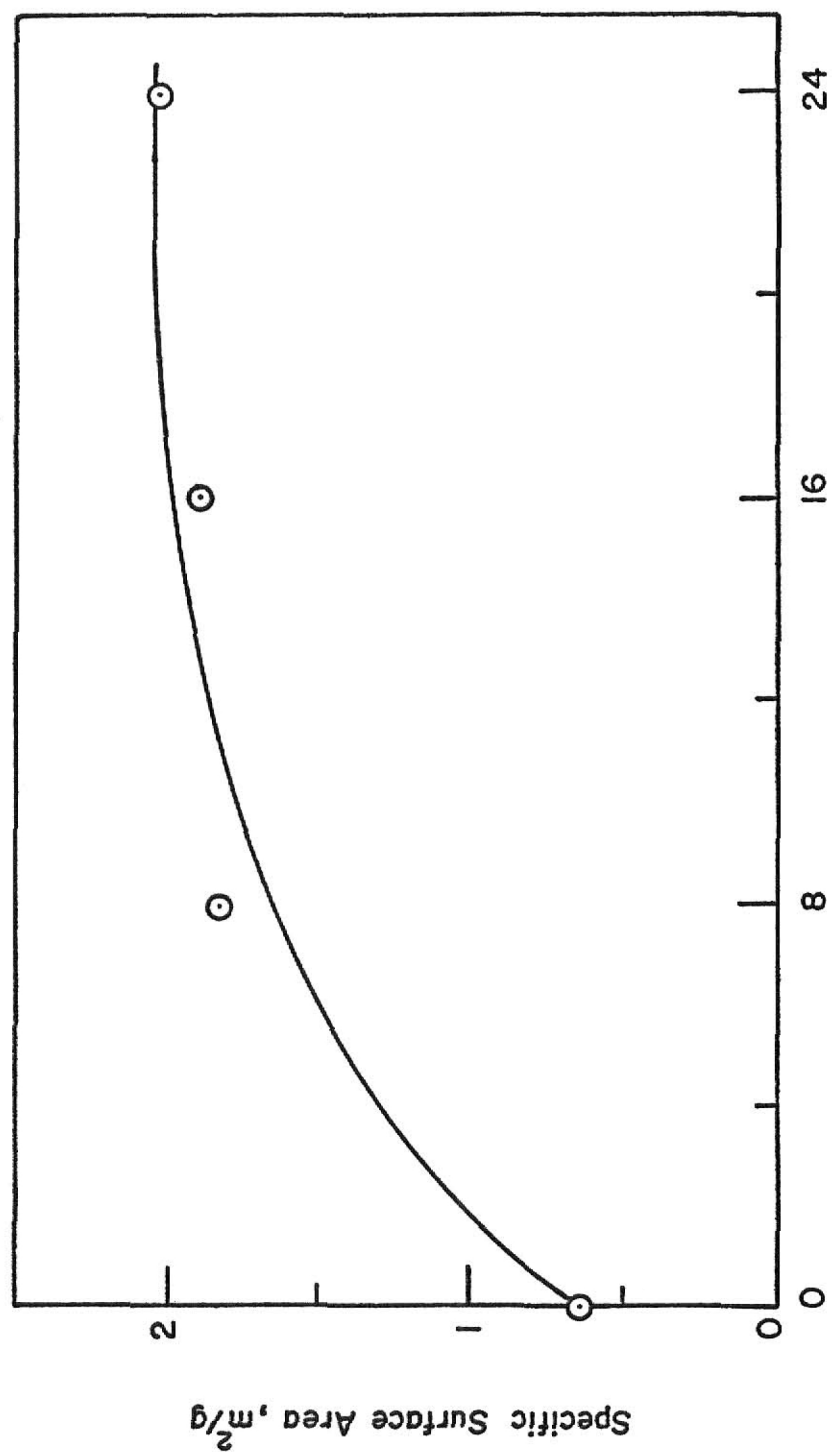
Type of pretreatment	Yield of sugar per kg of wheat straw, g/kg	Extent of hydrolysis after 8 hrs, g/l	Cost of energy consumed per kg of wheat straw, \$/kg	Pretreatment cost based on sugar, \$/kg
Ball-milling, 8 hrs	255.98	9.1	1.48	5.82
Roller-milling, $\frac{1}{4}$ hr	160.0	6.48	2.24	14.0
Fitz-milling, fine	100.0	3.0	0.01	0.1
Extrusion, with pressure	64.29	2.5	0.01	0.16
$\gamma$ -Irradiation 50 Mrads	207.02	5.6	0.1	0.48

Table 7. Effect of single pretreatments on structural features and hydrolysis rate

Type of Pretreatment	Specific surface area, m <sup>2</sup> /g	Crystallinity index	Lignin content, %	Relative extent of hydrolysis after 8 hours
Ball milling				
4 hours	2.3	23.7	11.53	4.0
8 hours	1.8	54.5	11.53	4.8
16 hours	1.9	17.5	11.53	3.6
24 hours	2.0	19.4	11.53	4.4
Fitz milling	0.99	65.6	11.53	1.6
Roller milling	1.2	57.6	11.53	3.3
Caustic soda (fines)	1.7	53.3	5.29	2.4
Peracetic acid (fines)	1.7	28.4	2.60	3.94
Ethylene glycol (fines)	2.9	63.6	3.27	0.68
Standard (fines)	0.64	69.6	11.53	1.0

content, and relative extent of hydrolysis for wheat straw subjected to a variety of pretreatments. The extent of hydrolysis after 8 hours is the reducing sugar produced during the first 8 hours of hydrolysis. The substrate subjected to chemical pretreatments undergoes composition change and hence has a different cellulose content compared to the standard substrate. Therefore, the extent of hydrolysis for the chemically pretreated substrate has been modified by dividing by relative cellulose content of the pretreated substrate. The relative cellulose content of the pretreated substrate is defined as the ratio of cellulose content of pretreated substrate to that of the standard substrate. The relative extent of hydrolysis is the ratio of the extent of hydrolysis of pretreated substrate to that of the untreated standard substrate. The magnitude of the relative extent of hydrolysis is a convenient indicator of the increase in the hydrolysis rate. The various pretreatments employed in this work include ball-milling, roller-milling, Fitz-milling, and chemical treatments with caustic soda, ethylene glycol, and peracetic acid. Their effectiveness in enhancing the enzymatic hydrolysis rate has already been identified in the previous section. The effect of pretreatments on structural features is delineated below.

Specific surface area. The standard substrate wheat straw had a specific surface area of  $0.64 \text{ m}^2/\text{g}$  (see Table 7). This low value may be due to the blocking of capillaries which occurred during substrate preparation using hammer-milling. This blocking of capillaries was evident under the microscope. Figure 11 shows the effect of ball-milling time on the specific surface area. The surface area tended to increase up to about 8 hours of ball-milling time; after which it appeared to level off. The surface area increased from  $0.64 \text{ m}^2/\text{g}$  for the standard substrate to  $2 \text{ m}^2/\text{g}$  for 24 hour ball-milled substrate. The increase in the surface area might be due to drastic fragmentation which resulted from the high shearing and crushing action of ball-



**Ball Milling Time, hrs.**

Fig. 11. Effect of ball milling on the specific surface area of wheat straw

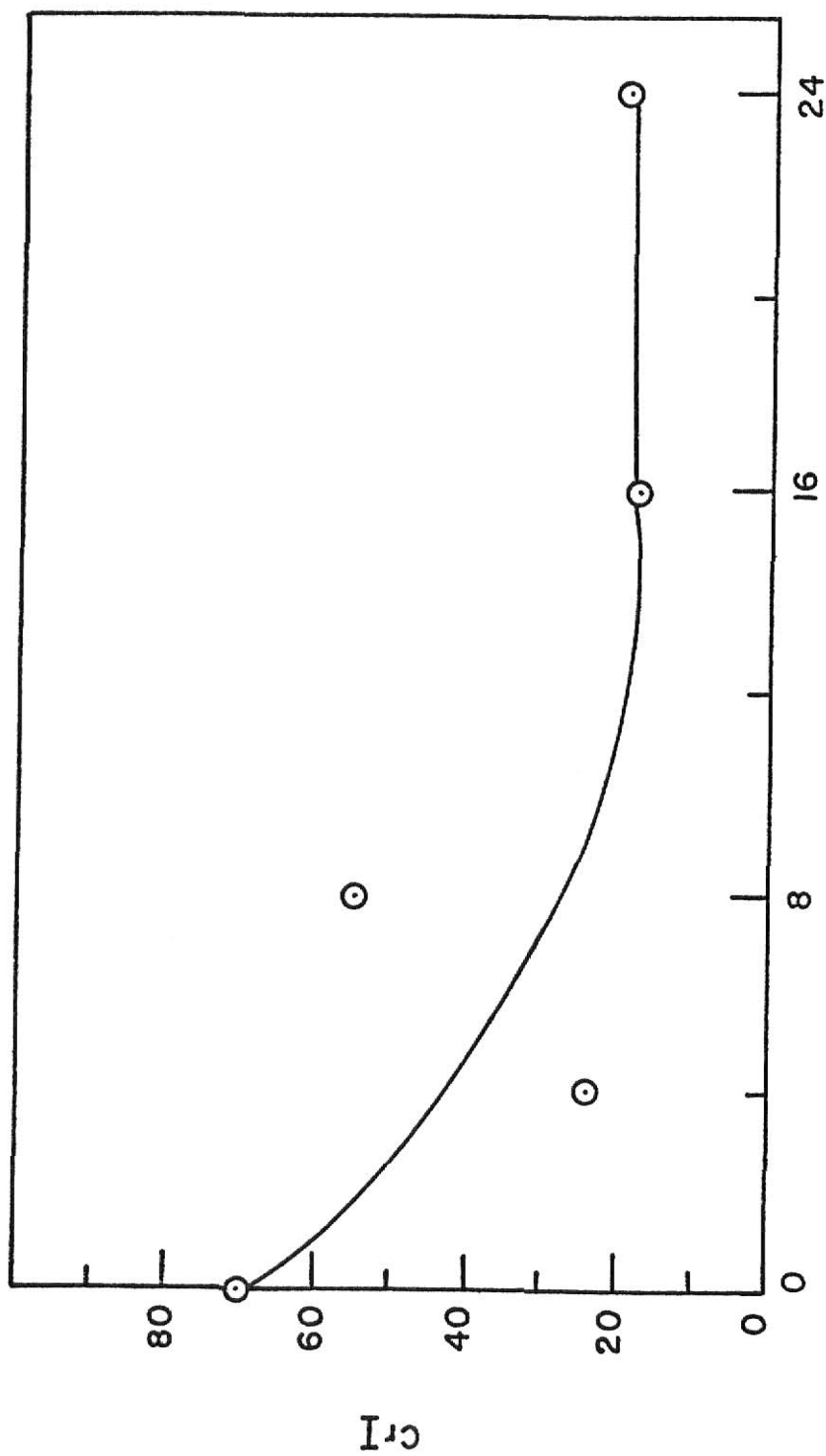
milling. The leveling off of the specific surface area might be due to agglomeration of particles, which was enhanced by decreasing particle size and increasing milling time. This segregation might have been further enhanced by the presence of a small amount of atmospheric moisture in the ball-mill during treatment. Also, extended ball-milling might have caused the collapse of the capillary structure.

Fitz-milling resulted in an insignificant increase in the specific surface area inspite of considerable size reduction; the value observed was  $0.99 \text{ m}^2/\text{g}$  (see Table 7). There are two possible explanations for this. First, the cutting action of the mill might have caused a further blockage of the capillaries and second, Fitz-milling might have caused a collapse of the capillary structure.

The specific surface area of  $1.2 \text{ m}^2/\text{g}$  was obtained upon roller-milling of wheat straw. This low value was possibly due to fusion resulting from heat generated by the shearing action of the rollers. The fusion led to the cake formation that has been observed.

All of the chemical pretreatments led to an increased surface area (see Table 7). The specific surface areas were  $1.7 \text{ m}^2/\text{g}$ ,  $1.7 \text{ m}^2/\text{g}$ , and  $2.0 \text{ m}^2/\text{g}$  for caustic, peracetic acid, and ethylene glycol treated wheat straw, respectively. These increases might be due to an inflation of the lignocellulosic structure upon removal of the lignin glue by the chemical action.

Crystallinity index. The crystallinity index for the standard substrate wheat straw was 69.6. Figure 12 represents the effect of ball-milling time on crystallinity. The crystallinity index declined sharply with increasing ball-milling time, but remained unchanged after about 12 hours. This leveling off might be a result of particle agglomeration, especially in the presence of moisture, which compensated for any decrease in the crystallinity index by the milling action. The crystallinity index after 24 hours of ball-milling



Ball Milling Time, hrs.

Fig. 12. Effect of ball milling on the crystallinity index of wheat straw

was 19.4. Fitz-milling and roller-milling caused relatively insignificant changes in the crystallinity index, the crystallinity indices were 65.6 and 57.6, respectively, for pretreated substrate.

The crystallinity index measured in this work is mainly contributed by cellulose; however, other components such as hemicellulose, lignin may contribute slightly. Chemical pretreatments have a dual effect on crystallinity; first, they cause removal of the amorphous lignin component and second, they loosen the highly packed crystalline through swelling. Caustic soda and ethylene glycol caused a minor reduction in the crystallinity index (see Table 7). This decrease might be mainly due to swelling action. Peracetic acid led to a drastic reduction in the crystallinity index; this might be due to structural swelling and dissolution of the crystalline cellulose component.

Composition of wheat straw. Cellulose, hemicellulose, and lignin contents of wheat straw subjected to a variety of chemical pretreatments are tabulated in Table 8. Notice that caustic soda treatment preferentially attacked the hemicellulose and lignin components; consequently, the treated substrate was richer in cellulose content. The solvent action of ethylene glycol extracted most of the hemicellulose and caused substantial delignification. The substrate was thus enriched in cellulose content. Peracetic acid treatment appears peculiar in that, inspite of its effectiveness in enhancing the hydrolysis rate, it leads to a low cellulose content. The oxidizing action of peracetic acid caused drastic delignification, but a relatively low decrease in hemicellulose content.

#### 4.2.2 Effect of multiple pretreatment on structural features of substrate

The data obtained with multiple pretreatments are tabulated in Table 9. It contains the specific surface area, crystallinity index, lignin content, and relative extent of hydrolysis. A multiple pretreatment consists of a combination of a physical and a chemical pretreatment. The total number of pretreatments in a combination was limited to two for economic reasons.

Table 8. Effect of chemical pretreatments on composition of wheat straw

Type of pretreatment	Cellulose, %	Hemicellulose, %	Lignin, %
Caustic soda	49.33	19.32	4.05
Ethylene glycol	51.79	1.39	4.68
Peracetic acid	30.74	14.56	2.05
Standard	41.15	26.10	13.05



Table 9. Effect of multiple pretreatments on structural features and hydrolysis rate

Type of pretreatment	Specific surface area, $\text{m}^2/\text{g}$	Crystallinity index	Lignin content, %	Relative extent of hydrolysis after 8 hours
Fitz milling-Peracetic acid	2.5	21.1	1.53	8.6
Fitz milling-Ethylene glycol	5.9	29.7	4.46	2.8
Fitz milling-Caustic soda	2.1	40.0	5.73	6.7
Roller milling-Peracetic acid	1.8	26.9	1.33	10.7
Roller milling-Caustic soda	1.6	60.0	8.18	3.6
Ball milling-Peracetic acid	1.5	24.3	2.05	17.4
Ball milling-Ethylene glycol	1.4	55.0	4.68	3.7
Ball milling-Caustic soda	0.9	13.9	4.05	2.8
Standard (fines)	0.64	69.6	11.53	1.0

Also, the order of pretreatment was physical pretreatment followed by chemical pretreatment. This order is more practical, since the reversed order will necessitate an energy intensive substrate drying prior to physical pretreatment. Physical pretreatments substantially manipulate surface area, crystallinity index, and particle size, whereas chemical pretreatments are effective in disrupting the lignin seal. Hence, it is considered that multiple pretreatments will facilitate obtaining an easily digestible substrate through the modification of all the important structural features. Table 9 indicates that multiple pretreatments are remarkable in enhancing the hydrolysis rate; however they don't appear promising, since hydrolysis rates rarely exceed those achieved by single pretreatments. Even when a multiple pretreatment yields a substrate that is highly susceptible to enzyme attack and allows a high slurry concentration in the reactor, the additive nature of the pretreatment costs may abrogate its economic feasibility. The effect of the multiple pretreatments on the structural features is delineated below.

Specific surface area. The standard substrate wheat straw with a specific surface area of  $0.64 \text{ m}^2/\text{g}$  was subjected to various multiple pretreatments. In the first series of experiments, wheat straw was Fitz-milled, after which it was subjected to a variety of chemical pretreatments. In general, these multiple pretreatments led to a substantial increase in the surface area. Fitz-milled wheat straw underwent a drastic increase in the surface area upon ethylene glycol pretreatment; the specific surface area was  $5.9 \text{ m}^2/\text{g}$  (see Table 9). Notice that the Fitz-milled wheat straw had a specific surface area of  $0.99 \text{ m}^2/\text{g}$ . This drastic increase might have stemmed mainly from structural swelling resulting from ethylene glycol

treatment. Caustic soda and peracetic acid pretreatment of Fitz-milled wheat straw also resulted in increased surface area, but the increases were less notable; the resultant specific surface areas were 2.1 and  $2.5 \text{ m}^2/\text{g}$  respectively (see Table 9).

The second series of experiments consisted of roller-milling wheat straw prior to every one of the chemical pretreatments. Only a slight increase in the surface area was observed; the surface areas were 1.6 and  $1.8 \text{ m}^2/\text{g}$  after caustic soda and peracetic acid pretreatments, respectively (see Table 9). The increase might be attributable to structural swelling. A possible reason for this structural swelling is the removal of lignin.

Wheat straw was subjected to a variety of chemical pretreatments, following 8 hrs of ball-milling in the next series of experiments. Ball-milled wheat straw underwent a decrease in the surface area upon chemical treatments. The surface areas observed upon caustic soda, peracetic acid, and ethylene glycol treatments were 0.9, 1.5, and  $1.4 \text{ m}^2/\text{g}$ , respectively. Notice that ball-milled (8 hours) wheat straw possessed a specific surface area of  $1.8 \text{ m}^2/\text{g}$  prior to chemical pretreatments. This decrease in the surface area might be a direct consequence of particle agglomeration leading to cake formation.

Crystallinity index. The various multiple pretreatments carried out have already been described in the previous section. The Fitz-milled wheat straw, with a crystallinity index of 65.6, underwent a decrease in crystallinity, in general. This Fitz-milled wheat straw had a crystallinity index of 21.1 upon peracetic acid pretreatment (see Table 9). There are two plausible reasons for this decrease. First, it is the removal of cellulose, which has a crystalline nature and second, it may be due to the structural swelling of the substrate. The removal of amorphous components, like

hemicellulose and lignin, has a tendency to increase the crystallinity index, but it seems that the factors stated earlier predominantly manipulate the crystalline structure of the substrate. Ethylene glycol treatment of Fitz-milled wheat straw led to a significant decrease in the crystallinity index; CrI observed was 29.7. Here, the decrease might be primarily due to structural swelling which was manifested through the drastic increase in the surface area (see Table 9). Fitz-milling of wheat straw followed by caustic pretreatment provided a substrate with a crystallinity index of 40.0. Here also, structural swelling might be responsible for the decrease.

Roller-milled wheat straw provided a substrate with crystallinity indices of 26.9 and 60.0 upon peracetic acid and caustic pretreatments, respectively. Notice that, the roller-milled wheat straw had a CrI of 57.6 prior to chemical pretreatments. The decrease in crystallinity index upon peracetic acid pretreatment might be due to the removal of crystalline cellulose component.

A similar trend was observed for ball-milled wheat straw subjected to a variety of chemical pretreatments, except for caustic pretreatment for which unexpectedly a low crystallinity index of 13.9 was observed. The pretreatment of ball-milled wheat straw with caustic soda resulted in a hard cake formation. Crushing of this cake was necessary to prepare sample suitable for crystallinity index determination. The destruction of crystallinity by crushing might be plausible reason for this low value of crystallinity index.

Lignin content. In a multiple pretreatment, wheat straw was subjected to a physical pretreatment prior to a various chemical pretreatments. A physical pretreatment inflicted a considerable size reduction, and hence, an

increased degree of delignification was expected possibly due to an increase in the accessibility of lignin to the chemicals. However, lignin data for multiple pretreatments represented in Table 9 shows no major change in lignin content when compared to that for single pretreatments (see Table 7). This may mean that size reduction of wheat straw by physical pretreatments does not result in significantly increased accessibility of lignin to chemicals.

#### 4.2.3 Relationship between structural features of the substrate and its hydrolysis rate

Lignocellulosic biomass is structured substrate, and hence, its heterogeneous enzymatic digestion is influenced by its structural features, such as surface area, crystallinity, and lignin content (13, 15, 48). To understand the mechanism of the hydrolytic reaction, therefore, it is important to investigate the influence of an individual structural feature on the hydrolysis rate. To accomplish it, it would be desirable to vary one particular structural feature at a time, maintaining other structural features unchanged. Unfortunately, for native lignocellulosics, altering one structural feature results in substantial changes in others (10). Physical pretreatments, while manipulating the crystallinity of lignocellulosics, alter the surface area. Decreasing the crystallinity possibly exposes the surface hidden inside the highly ordered crystalline structure. Similarly, chemical pretreatments substantially alter all the important structural features simultaneously. Here, removal of natural glue in the form of lignin possibly results in structural swelling leading to an increased accessible surface area and a decreased crystallinity.

These facts indicate that the structural features are interrelated. Thus, it would be necessary to understand the nature of these interrelationships among the structural features, prior to studying the influence of an individual structural feature on the relative extent of hydrolysis.

Figure 13 plots the crystallinity index of wheat straw, both treated and untreated, against the specific surface area using data presented in Tables 7 and 9; reduction in the crystallinity index tends to increase the specific surface area. This tendency would be expected since reduction in the crystallinity would expose the surface hidden inside the highly packed crystalline structure. The lignin content of wheat straw is plotted against the specific surface area and the crystallinity index in Fig. 14 and Fig. 15, respectively. Unlike Fig. 13, these figures appear to indicate that interaction between the lignin content and surface area or that between the lignin content and crystallinity are extremely complex, if they exist.

The relationship between the relative extent of hydrolysis and the selected set of structural features, namely, specific surface area, crystallinity index, and lignin content, has been examined by means of linear regression analysis using standard SAS package. Due to possible interaction among the structural features, as discussed earlier, following two models have been considered;

$$REH = a_1 (SSA) + a_2 (100-CrI) + a_3 (Lignin) + a_4 \quad (1)$$

and

$$\log REH = b_1 \log (SSA) + b_2 \log (100-CrI) + b_3 \log(Lignin) + \log b_4 \quad (2)$$

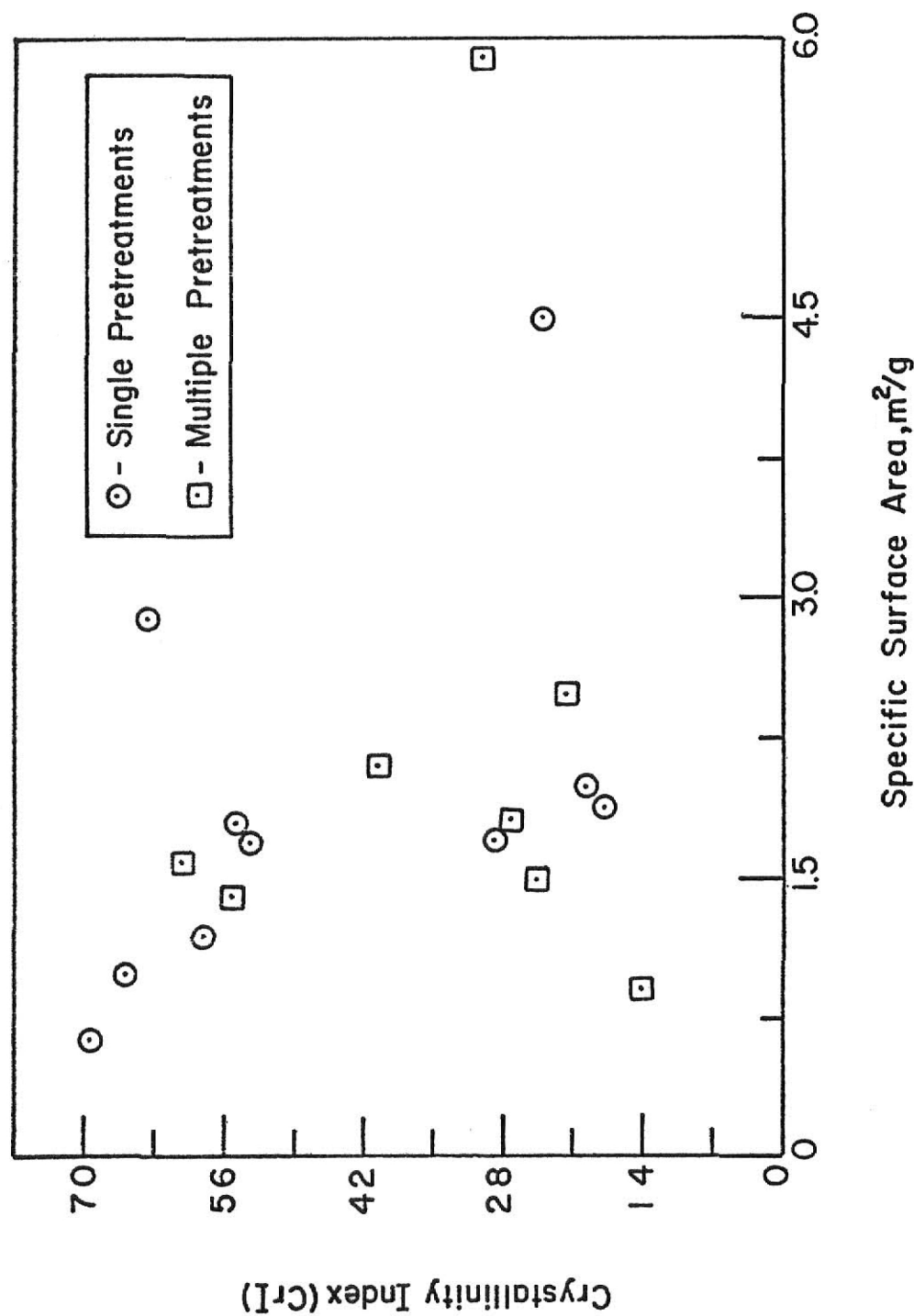


Fig. 13. Interaction between the crystallinity index and specific surface area of wheat straw

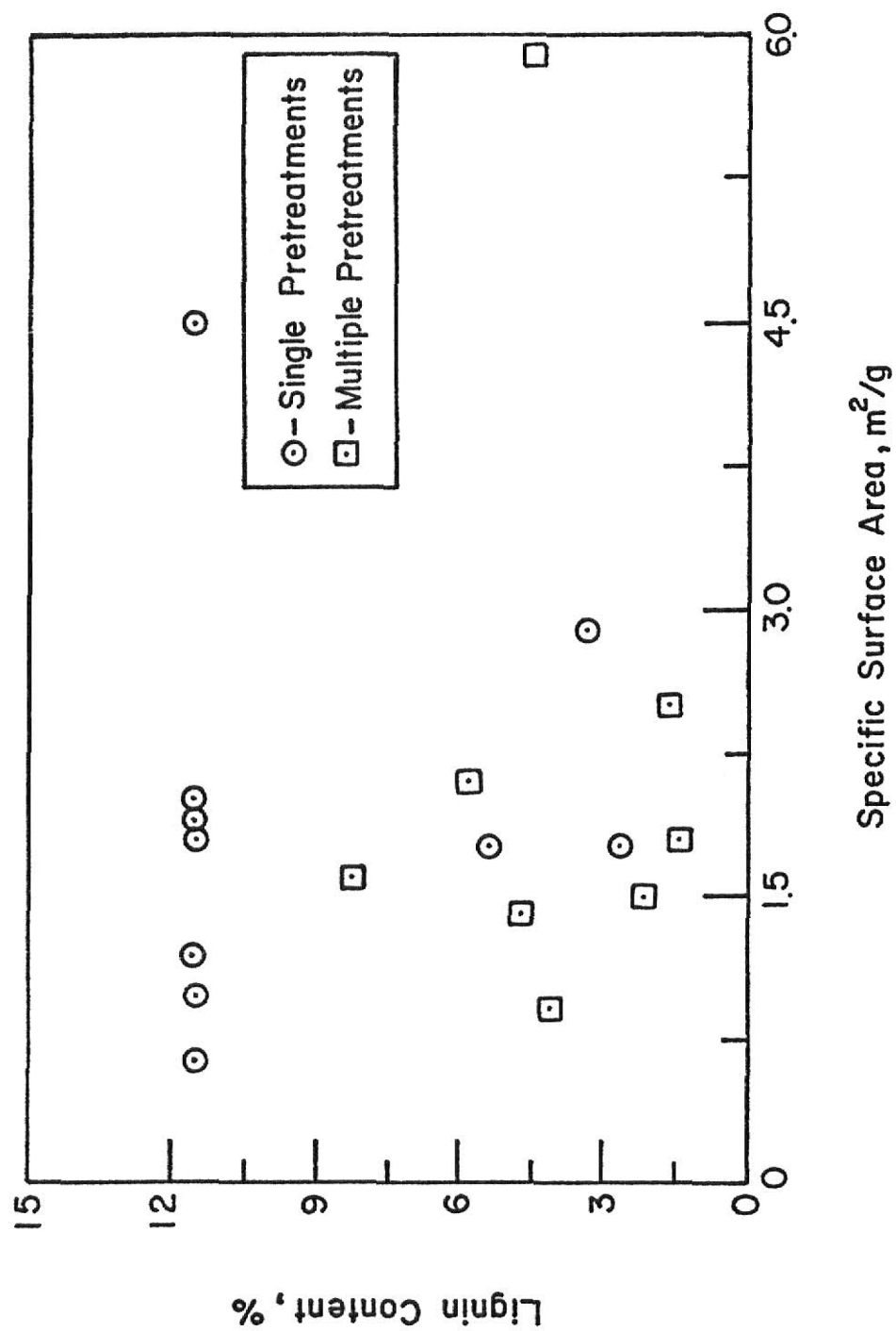


Fig. 14. Interaction between the lignin content and specific surface area of wheat straw



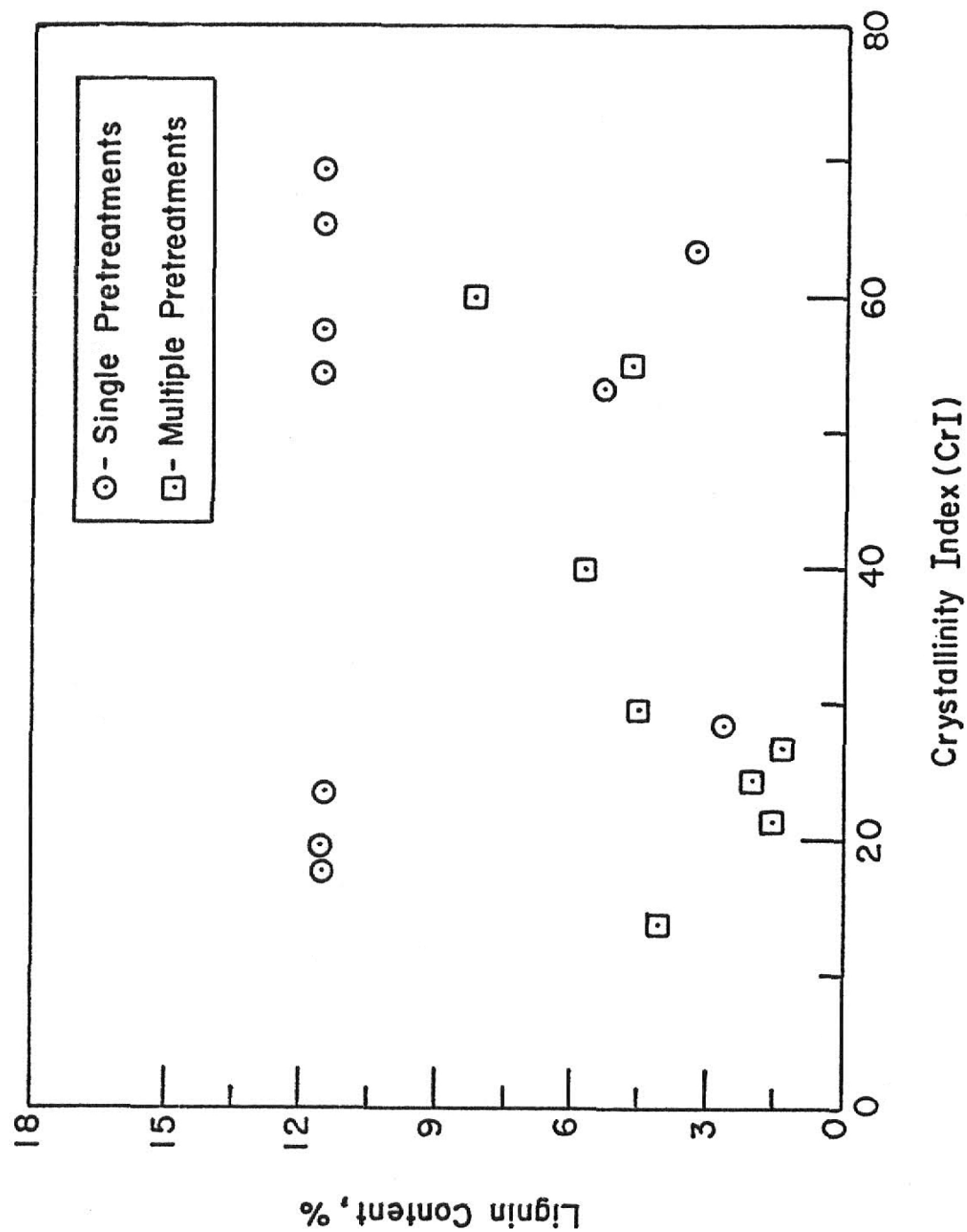


Fig. 15. Interaction between the lignin content and crystallinity index of wheat straw

or

$$REH = b_4 (SSA)^{b_1} (100-CrI)^{b_2} (Lignin)^{b_3} \quad (2a)$$

Here, REH represents the relative extent of hydrolysis, SSA represents the specific surface area of the substrate, (100-CrI) represents the magnitude of amorphous fraction of the substrate, and Lignin represents the lignin content of the substrate. The criteria for goodness of fit is the value of coefficient of regression, R-square, which is the ratio of sum of squares due to regression to the total sum of squares (56).

First, two of the three  $a_i$ 's or  $b_i$ 's,  $i=1, 2, 3$  are considered to be zero in each of the three analyses. Both the models indicate that the specific surface area is more closely related to the relative extent of hydrolysis than the other structural features, as indicated by the significantly higher R-square values for the correlations between the relative extent of hydrolysis and the specific surface area than those for the correlations between the relative extent of hydrolysis and any of the remaining structural features. Increasing surface area appears to increase the hydrolysis rate. This would be expected since the heterogeneous hydrolysis reaction proceeds only when the enzyme - substrate complex is formed. Therefore, direct physical contact between the cellulase molecules and lignocellulosic substrate is a prerequisite to the hydrolysis reaction. Hence, it would be reasonable that the reaction rate be a function of the surface area of the substrate that is accessible to the enzyme.

Increasing crystallinity index appears to diminish the hydrolysis rate. This seems logical, since the highly packed crystalline component of cellulose in lignocellulosics is structurally resistant to enzymatic degradation. Besides, the orderly crystalline structure of cellulose prohibits the entry of large enzyme molecules, thus limiting the accessibility of the substrate.

Increasing lignin content appears to decrease the relative extent of hydrolysis. This is because the enzymatic degradation of cellulose is hindered by the presence of physical barrier in the form of lignin.

In the next step, one of the three  $a_i$ 's or  $b_i$ 's,  $i=1, 2, 3$ , is considered to be zero in each of the three analyses. In general, the second model gives greater R-square values than the first model. The relationship between the relative extent of hydrolysis and the pair of the structural features, namely, the specific surface area and crystallinity index, yields a R-square value of 72.95%. Similarly, the relationship between the relative extent of hydrolysis and the pair of structural features, namely, the specific surface area and lignin content, gives a R-square value of 80.42%. The R-square value for the relationship between the relative extent of hydrolysis and the pair of structural features, namely, the crystallinity index and lignin content is 53.09%. In other words, the exclusion of the specific surface area reduces the R-square value considerably. Thus, it appears that the specific surface area influences the relative extent of hydrolysis more profoundly than the other structural features.

Finally none of the  $a_i$ 's or  $b_i$ 's,  $i=1, 2, 3$ , is considered to be zero to examine the relationship between the relative extent of hydrolysis and the complete set of structural features. The first model yields a R-square

value of 63.91%, whereas that for the second model is 81.1%. Hence, the relationship represented by the second model has been selected; the resultant expression is

$$REH = 1.807 (SSA)^{1.062} (100-CrI)^{0.189} (Lignin)^{-0.268} \quad (3)$$

This relationship is represented in Fig. 16. It indicates that an increase in the surface area and reduction in the crystallinity and lignin content enhance the relative extent of hydrolysis; the specific surface area is the most influential structural feature, which is followed by the lignin content, which is, in turn, followed by the crystallinity. The effects of the crystallinity index and lignin content might be imbedded in the specific surface area to some extent due to interaction among the structural features as discussed before.

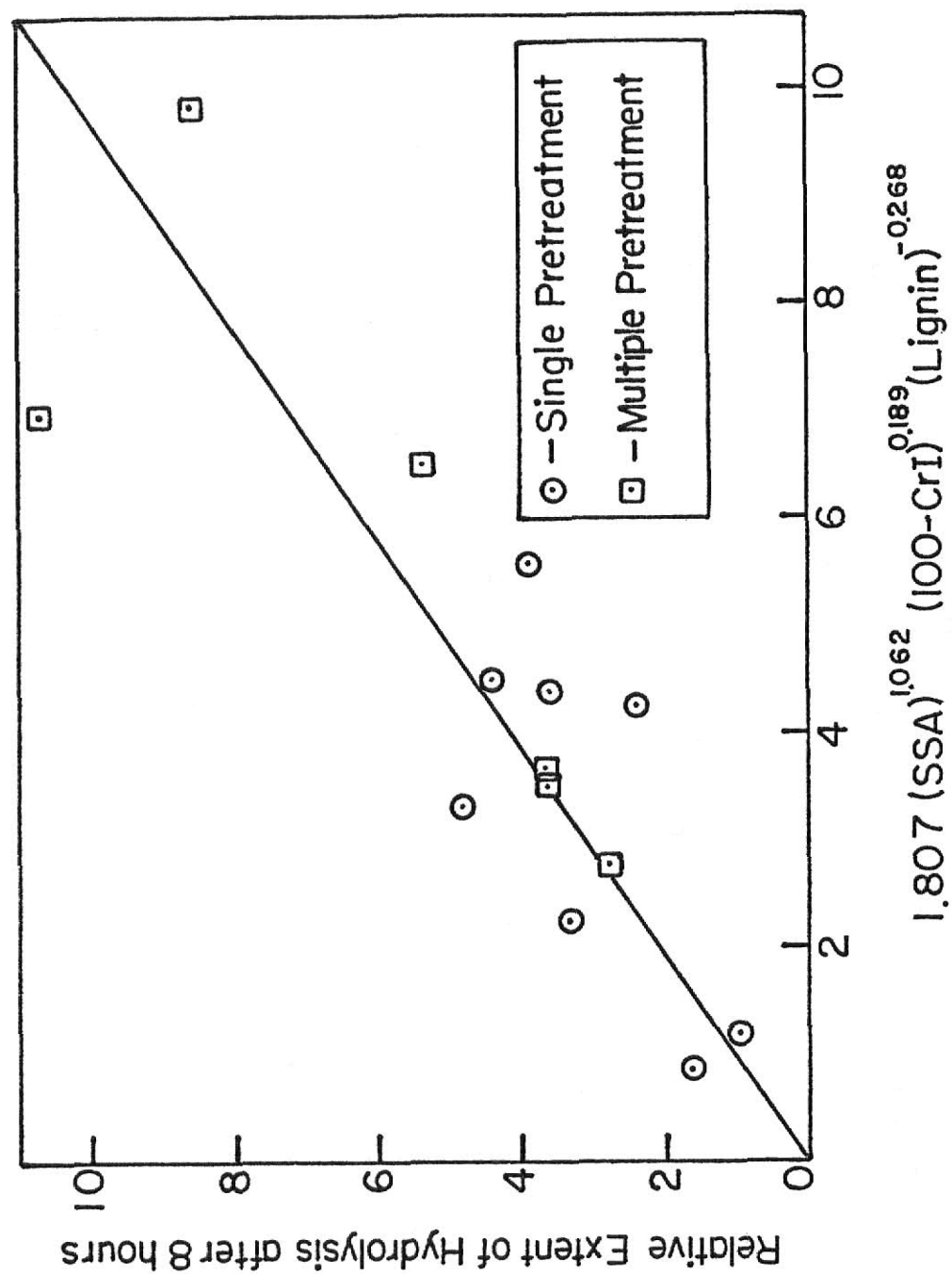


Fig. 16. The relative extent of hydrolysis after 8 hours as a function of the specific surface area, crystallinity index, and lignin content of wheat straw

## 5. CONCLUSIONS AND RECOMMENDATIONS

The relative effectiveness of several promising lignocellulosic pretreatment methods was evaluated. Among physical pretreatments, ball-milling and roller-milling and among chemical pretreatments, treatment with caustic soda and ethylene glycol are highly effective in enhancing the hydrolysis rate. The preliminary structural feature study indicates that although the lignin content is a relatively more important structural feature, yet only 50% delignification results in a maximum hydrolysis rate. Also reduction in size and crystallinity play only a relatively limited role in enhancing the hydrolysis rate of wheat straw, unlike Solka Floc (pure cellulose). Physical pretreatments are relatively less effective in enhancing the hydrolysis rate; hence, chemical pretreatments deserve more emphasis in the future. Caustic soda appears to be a potential candidate for large scale application because of its excellent capability to increase the hydrolysis rate and its low cost. The effectiveness of various multiple pretreatments has been examined. The multiple pretreatments, although effective in enhancing the hydrolysis rate, seem to lack commercial appeal since the hydrolysis rates obtained in most cases were below those achieved by the single pretreatments. An overall structural feature study, including specific surface area, crystallinity index, and lignin content, has been conducted for the substrate subjected to both single and multiple pretreatments.

The results thus obtained indicate that ball milling pretreatment is effective in increasing the specific surface area and decreasing the crystallinity index. Treatment with ethylene glycol is highly effective in increasing the specific surface area, in addition to a high degree of delignification. Among multiple pretreatments those involving peracetic acid treatment generally have lower crystallinity index and lignin content

values. The relationship between the hydrolysis rate and the set of structural features indicates that an increase in the surface area and a decrease in the crystallinity and lignin content enhance the hydrolysis rate; the specific surface area is the most influential structural feature, followed by the lignin content, which is in turn followed by the crystallinity.

The presence of hemicellulose in the substrate may hinder the hydrolysis along with lignin, so measurement of hemicellulose content may prove beneficial. A more intensive economic analysis, including capital cost and operating cost, may prove useful to evaluate the economic viability of a pretreatment scheme on a large scale.

The future research should include:

- (i) similar pretreatment study on other lignocellulosics such as corn stover and poplar wood to generalize the conclusions reached.
- (ii) an optimization of caustic soda pretreatment, due to its promising results in this study, in terms of operating conditions and amount of caustic soda to be used. This may provide an ideal pretreatment scheme.
- (iii) an integrated processing scheme utilizing all the major components of lignocellulosics, including cellulose, hemicellulose, and lignin needs to be synthesized. This will ensure an economic success of biomass utilization process, thus opening a new path for alternate energy development.

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THE EFFECT OF PRETREATMENTS ON THE RATE OF ENZYMATIC  
HYDROLYSIS OF WHEAT STRAW AND ITS STRUCTURAL FEATURES

by

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## ABSTRACT

In this work an evaluation was made of a wide variety of physical and chemical pretreatment methods for enhancing the rate of enzymatic hydrolysis of wheat straw. The structural features of wheat straw, including specific surface area, crystallinity index, and lignin content, were measured to understand the mechanism of the enhancement in the hydrolysis rate upon pretreatment. It has been found that, in general, chemical pretreatments were more effective than physical pretreatments. Although the hydrolysis rate was dependent strongly on the crystallinity index, the lignin content of the substrate appeared predominant in controlling the rate of enzymatic hydrolysis. The hydrolysis rate increased with an increase in the degree of delignification only up to 50%; it remained unchanged thereafter. Scanning electron microscopic observations were made to gain further insight into structural modification of the substrate. The effectiveness of several multiple pretreatments, each involving a physical pretreatment followed by a chemical pretreatment, was evaluated by combining the effective pretreatments already identified. It has been found that, in general, multiple pretreatments were not promising, since the hydrolysis rates rarely exceeded those achieved by single pretreatments. Ball milling pretreatment was found to be effective in increasing the specific surface area and decreasing the crystallinity index. Treatment with ethylene glycol was highly effective in increasing the specific surface area, in addition to a high degree of delignification. Peracetic acid pretreatment was highly effective in delignifying substrate. Among multiple pretreatments, those involving peracetic acid treatment generally had lower crystallinity index and lignin content values. The relationship between the hydrolysis

rate and the set of structural features indicated that an increase in surface area and a decrease in the crystallinity and lignin content enhance the hydrolysis; the specific surface area is the most influential structural feature, followed by lignin content.