THE UNIVERSITY OF MANITOBA

CHEMICAL PRETREATMENTS AND ENZYMIC HYDROLYSIS OF CORN HUSK LIGNOCELLULOSICS

by

BRANKA BARL

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ABSTRACT

Corn husk residues from the hybrid Pickseed 2020 were used as a study system to investigate the feasibility of producing soluble sugars from lignocellulosic agricultural residues by enzymic hydrolysis. This material was selected among other corn plant residues because of its high total carbohydrate content (82.7%) and moderate degree of lignification (6.6%). Morphological characterization (SEM, light microscopy) of husk revealed that sclerenchymatous cells of evenly thickened cell walls in addition to vascular bundles of phloem and xylem conductive cells constitute the largest source of cellulose fibers. The compositional heterogeneity and structural complexity of husk necessitated the application of chemical pretreatments using alkaline and acidic solvents under various regimes prior to enzymic hydrolysis. Optimization studies with respect to solvent (NaOH, H_2SO_4, H_3PO_4) concentration (0.2-5.0%) and reaction temperature (25-85^{0}C) were carried out. The effects of pretreatments on husk solubilization, composition, morphological characteristics, physico-chemical properties (crystallinity and thermal behavior) and enzymic susceptibility were monitored.

Chemical analyses of the liquid extracts showed that pentose-containing carbohydrate material, derived from the hemicellulose component, comprised 86 to 93% of the solubilized fraction. Pretreatment with NaOH (5% w/v NaOH, 85^{0}C-2 h) resulted in preferential extraction of hemicellulose and substantial delignification (2.6 vs. 6.6%) along with

an increase in crystallinity (67 vs. 51%) and pronounced swelling of the remaining residues. There was no change, however, in the resistance of residues to pyrolytic degradation. In contrast, acid pretreatment (5% w/w H_2SO_4, 85°C-2 h) brought about extensive depolymerization of the hemicellulose component as revealed by gel chromatography [Fractogel TSK HW-40(s)]; soluble components were mainly xylo-oligosaccharides of $\bar{DP}$<12. In addition, it caused minor delignification (6.1 vs. 6.6%), moderate changes in crystallinity (61 vs. 51%) and a significant enhancement in the thermal resistance of residual cellulosics.

The feasibility of using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) as probes of structural order of lignocellulosics was established in this study. Thermograms of native husk reflected the pyrolysis of its hemicellulose and cellulose components as single exothermic events peaking at 238 and 317°C, respectively. Experimental evidence is provided that chemical pretreatments caused pronounced alterations in both DSC/TGA thermal profiles and kinetics of husk pyrolysis. Despite the chemical heterogeneity of the materials and complexity of the reactions involved, pyrolysis was found to obey first-order kinetics, as assessed from nonisothermal TGA data and using dynamic equations, while the corresponding apparent activation energies ranged between 95-166 kJ/mol. The X-ray crystallinity values of the lignocellulosics were found to exhibit positive relationships with several TGA thermal parameters (maximum rate of weight loss, temperature at 10% weight loss, activation energy).

The effects of various acidic and alkaline solvents on altering husk reactivity were ultimately assessed by enzymic hydrolysis using two

commercial cellulolytic preparations. While the maximum degree of conversion of native husk into sugars was 28%, the corresponding values for its chemically pretreated counterparts were within 42-59% for the acid-treated residues and 96% for the residual material of the 5% w/v NaOH (25^{0}C-2 h) pretreatment. Major monosaccharides, identified by HPLC, were glucose, xylose, arabinose and mannose. It was also demonstrated that 80-90% of the hemicellulose-derived solubilized fractions yielded fermentable sugars upon further hydrolysis by enzymes. The major hydrolysis products identified were xylose and arabinose. Changes in the resistance to pyrolysis of the residues, following enzymic hydrolysis, were observed by DSC. The shifts of cellulose exothermic transition toward higher temperatures in the initial stage of the reaction suggested a rapid hydrolysis of the amorphous cellulose and it is consistent with the patterns of husk hydrolysis kinetics. An overall process scheme of husk lignocellulose saccharification based on the findings of chemical pretreatments and enzymic hydrolysis studies was proposed.

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The financial assistance and leave provided from the Maize Research Institute, Belgrade, Yugoslavia is gratefully acknowledged.

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GENERAL INTRODUCTION

Biomass, accumulated by photosynthetic storage of solar energy in green plants, is an abundant and inexpensive source of renewable energy. Almost half of this material consists of cellulose (28-50%), the other major components being hemicelluloses (20-30%) and lignin (18-30%) (Thompson, 1983). Owing to the current concern over the depletion of fossil fuels and food shortages, a worldwide search for alternative energy and food resources has been undertaken in the last decade. The challenge facing bioconversion research has been to develop low cost technology to render wood and agricultural lignocellulosic residues into a form amenable to degradation of its carbohydrate and polyphenolic (lignin) components. In this respect, the most widely used approach is the enzyme-catalyzed hydrolysis of carbohydrate constituents into fermentable low-molecular weight sugars that could be subsequently converted to chemicals, fuels or assimilated into microbial proteins.

Hydrolysis[1] of native lignocellulose is, however, prohibitively slow due to the low substrate reactivity and low activity of cellulase enzymes (Mandels, 1982). It is well established that heterogeneous chemical reactions of cellulose are controlled largely by the high order of molecular packing of its crystallites; this in turn drastically reduces cellulose accessibility toward enzymes. In addition to the

[1] "Hydrolysis" refers to "enzymic hydrolysis" throughout the thesis unless otherwise noted.

rate-limiting influence of cellulose "fine-structure", a further deterrent to substrate penetrability resides in its interassociation with hemicellulose and lignin. As a result of the compositional heterogeneity and structural complexity of native lignocellulosics it has been recognized that some kind of pretreatment is essential to increase cellulose reactivity toward enzymic degradation. Enhancement in the hydrolysis rates with such pretreatments is generally attributed to structural modification and/or selective removal of cell wall constitutents. Extensive research efforts have been devoted to the role of structural properties, such as crystallinity, surface area, and extent of delignification (Fan et al., 1980; Gharpuray et al., 1983; Puri, 1984; Grethlein, 1985; Lin et al., 1985), on the substrate susceptibility toward cellulases and the overall hydrolysis yield. It should be pointed out, however, that each agricultural residue has its own composition and particular morphology. Consequently, each type of agricultural biomass residue requires an independent investigation of the effects of structural and compositional characteristics on its degradation efficiency. The activity of cellulolytic enzymes is the second parameter to be considered in cellulose hydrolysis. As a result of intensive research in all aspects of cellulase production, characterization and applications, it has been suggested that balanced activity of individual enzyme components within the "cellulase" enzyme complex is a prerequisite for effective hydrolysis. In this respect, complete cellulose solubilization requires the synergistic action of endo- and exoglucanases. Glucose is formed via several consecutive reactions, each of which may be rate limiting. This is mainly due to end-product inhibition (cellobiose and glucose) recognized to control the action of all enzymic components and, therefore, the extent of enzymic hydrolysis.

Overall, in order to develop large scale production of fermentable sugars from native lignocellulosics, the most significant contribution would be the discovery of a more potent cellulolytic enzyme system. Intensive research on fungi from Trichoderma sp. along with other microorganisms is underway to increase the level of cellulase specific activity through mutations (Montenecourt and Eveleigh, 1977; Nevalainen et al., 1980; Ghosh et al., 1982).

In view of the above considerations, the objective of this study was to investigate the effects of chemical pretreatments on the structure, physico-chemical properties and enzymic susceptibility of corn husk residues. Investigations were undertaken to reveal the relative significance of husk composition and its structural attributes in enhancing its susceptibility to hydrolytic enzymes. Changes in crystallinity, thermal properties and morphological characteristics were some of the features monitored in order to assess the extent of the modifications brought about by the solvent during pretreatment. It was further attempted to relate these changes to the ultimate yield of sugars following cellulose hydrolysis. In addition, chemically solubilized extracts, enriched in hemicellulose-derived products, were characterized and subjected to enzymic depolymerization. In order to provide information relevant to the development of a practical saccharification process, commercially available crude cellulolytic and hemicellulolytic preparations without further purification were employed.

Chapter I

POTENTIAL OF AGRICULTURAL WASTE LIGNOCELLULOSICS AS ENERGY SOURCES WITH REFERENCE TO CORN RESIDUES: STRUCTURAL AND COMPOSITIONAL CONSIDERATIONS

1.1 INTRODUCTION

Abundant, annually renewable and low cost agricultural residues, such as corn stover, wheat, barley, oat and rye straw, rice hulls, sugar cane bagasse etc., represent a potential source of waste lignocellulosic material that is underutilized at present. There are no reliable figures available regarding the yield of crop residues since they fluctuate widely. In contrast to grain yield, crop residue yield is seldom measured due to its high bulk and volume. However, researchers have sought to obtain approximate estimates of crop residue yields by establishing a fixed grain-to-straw ratio. Based on these ratios (in the range of one to two depending on the particular crop) and the values for world annual grain production, it can be estimated that more than 420 million tons of corn residues alone are produced in the world every year; most of it is left in the field, burnt or plowed back into soil (Stoskopf, 1985). In addition to corn, wheat and rice also contribute significantly to the total amount of agricultural biomass available.

Although agricultural residues were recognized several decades ago as an inexpensive source of carbohydrates, potentially convertible to liquid fuels and chemicals, little attention has been given to the use of these materials until recently. Continuous depletion of fossil fuels in combination with fluctuating prices, however, stimulated a worldwide search for alternative energy resources over the last decade. In this respect, many processing routes based on the conversion of cellulose, hemicellulose and lignin to a variety of potential end products have been investigated. The most widely used approach toward biomass utilization is the enzyme-catalyzed hydrolysis of cellulose and hemicellulose

to low molecular-weight components that can serve as substrates for fermentation to fuels and chemicals (Vallander and Eriksson, 1985; Ladisch et al., 1983; Clausen and Gaddy, 1983). The cellulose fraction is commonly fermented to ethanol, while hemicellulose is usually converted to butanol, butanediol, carboxylic acids and acetone on fermentation using different microorganisms (Yu et al., 1984a and 1984b; Mes-Hartree and Saddler, 1982; Saddler et al., 1982a and 1982b). The pentose-rich hemicellulose fraction can be also converted into furfural, xylitol or assimilated into single cell protein (Chahal, 1984; Moo-Young et al., 1978; Ek and Eriksson, 1975). Much research in the area of cellulose biodegradation has focused on the use of enzymes produced from the fungus Trichoderma reesei, known to be the most potent producer of cellulolytic enzymes. However, due to the structural constraints of native cellulosic materials, some physical and/or chemical pretreatments were found to be essential prerequisites in order to obtain extensive saccharification. Pretreatment may also result in selective fractionation of lignin, which can be further converted to a variety of useful chemicals as reported by Coughlin et al. (1984). An alternative and conceptually interesting process is the simultaneous saccharification and fermentation of cellulose to ethanol using the cellulolytic fungus Trichoderma reesei and the yeast Candida brassicae (Blotkamp et al., 1978) or mixed cultures of Clostridium thermocellum and Clostridium thermosaccharolyticum (Cooney et al., 1978). The problem of end product inhibition (i.e., glucose) commonly encountered in conventional sequential hydrolysis and fermentation processes is alleviated in this system, since glucose does not accumulate, but is rather fermented to ethanol immediately following saccharification. However, further research is

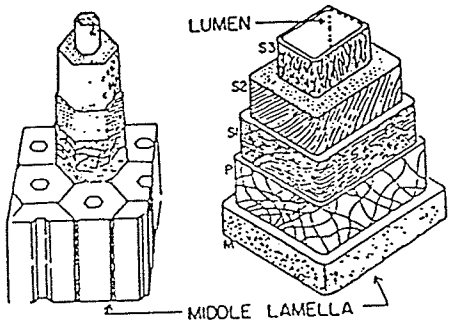
needed in this area as conversion rates to ethanol are still low and economically unattractive.

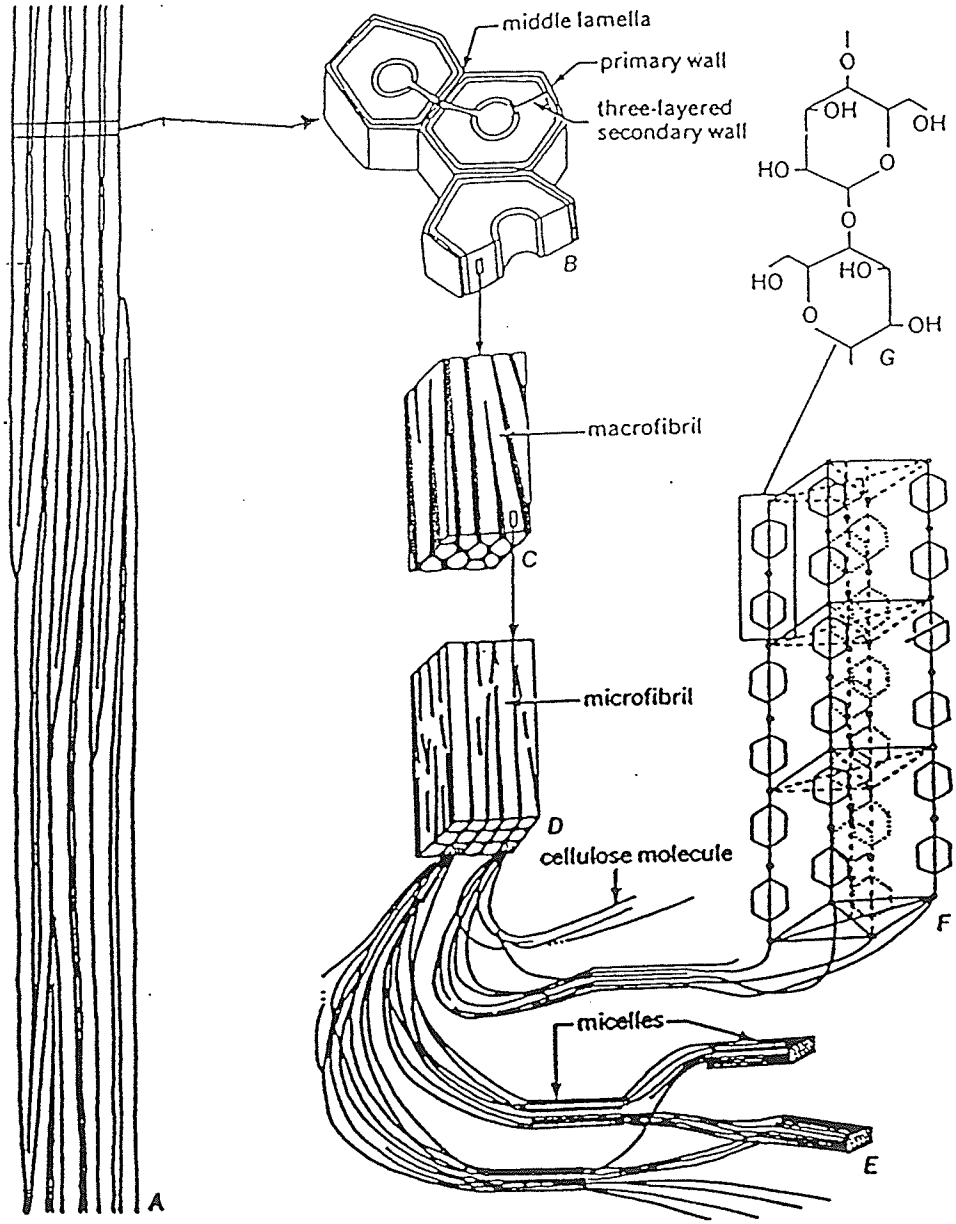
The longest established approach for the conversion of lignocellulose to fermentable sugars has been by mineral acids, usually sulfuric acid; typical glucose yields of approximately 55% can be achieved by this process (Dale and Moreira, 1982; Klyosov, 1986). Several alternatives such as hydrofluoric acid in the vapour phase (Smith et al., 1983; Defaye et al., 1983), and concentrated hydrochloric acid (Goldstein et al., 1983) have been reported to be superior to sulfuric acid. Another processing scheme uses anaerobic digestion to produce either organic acids or methane from biomass by using methanogenic bacteria (Foutch and Gaddy, 1981). Datta (1981) reported that acidogenic fermentation of corn stover after mild alkaline pretreatment can produce volatile organic acids with a respectable yield of 0.5 g acetic acid equivalent/g pretreated straw. Other possible routes for biomass processing are direct combustion, pyrolysis, hydrogenation etc., mostly leading to the production of gaseous and liquid fuels (Klass, 1981).

Research activities in the conversion of biomass, as discussed above, although very intensive in the last decade, have not yet resulted in an economically feasible processing scheme. The reasons are numerous, but the major one is associated with the compositional heterogeneity and structural complexity of the initial lignocellulose material. The major components of agricultural residues are the structural cell wall polysaccharides, primarily cellulose and hemicellulose, which constitute 45-70% of the weight of the dried plant residue (Sloneker, 1976). Cellulose and hemicellulose are deposited in the cell wall of plants in an intimate physical admixture with lignin, a complex aromatic polymer.

As lignification occurs after the deposition of the polysaccharides the lignin precursors can only fill voids between the structural polysaccharides, and by condensation reactions the polysaccharide elements become embedded in lignin (Fengel, 1971). Thus, an enzymatically inaccessible three-dimensional cell wall matrix containing insoluble lignocellulose fibers is formed. The typical structure of plant cell walls is schematically depicted in Figure 1.1 (Esau, 1977; Gilbert and Tsao, 1983); it contains a thin primary wall (P) that surrounds the relatively thick secondary wall. The latter usually consists of three layers designated as S_1, S_2, and S_3; the relatively thin S_1 layer is the first layer deposited, followed by S_2 and S_3 which are deposited in the inner region of the secondary cell wall. Since each of these layers is formed during a particular growth stage of the cell, it contains structural units with a different orientation. The most important of these layers is S_2, since it has the highest concentration of cellulose and makes up much of the secondary wall (Cowling and Kirk, 1976). Within each layer of the secondary cell wall, the cellulose and other constituents are aggregated into long bundles called macrofibrils. The macrofibrils are further composed of smaller cellulose microfibrilar strands. Within the microfibrils there are intermittent highly ordered crystalline areas which are separated by less ordered (amorphous) regions. The ultimate unit-components of microfibrils are elementary fibrils of several dozen linear chains of glucose residues linked by ß-(1,4)-glycosidic linkages. However, the precise manner in which native cellulose molecules are aggregated to form the elementary fibril structure is far from fully understood.

Figure 1.1: Structure of plant cell wall.
A, strand of fiber cells; B, cross section of fiber cells showing the various layers: P, primary wall; S1, S2, and S3, three layers of secondary wall; M, middle lamella and L, lumen; C, fragment from middle layer of secondary wall; D, fragment of a microfibril; E, structure of microfibrils; F, fragment micelle (Esau, 1977; Gilbert and Tsao, 1983).


LUMEN
S3
S2
S1
P
M
MIDDLE LAMELLA

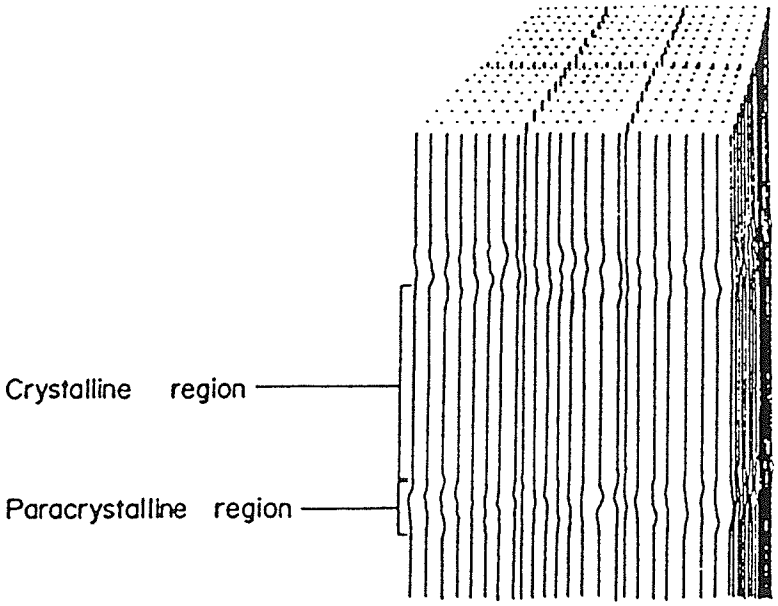

middle lamella
primary wall
three-layered secondary wall
B
macrofibril
C
microfibril
D
cellulose molecule
micelles
E
A
F
G
O
OH
HO
OH
HO
HO
OH
O

Three structural models have been proposed to date for the supramolecular structure of cellulose (Figure 1.2): fringe-micellar (Hess et al., 1957), folded chain (Chang, 1971), and modified fringe-micellar (Rowland and Roberts, 1972) model. In spite of the uncertainty regarding the structural organization of the elementary fibrils, it is generally accepted that ß-(1-4)-glucans are inherently stiff and extended, thus having a strong tendency to associate laterally. This results in formation of a ribbon-like structure via numerous intra- and interchain hydrogen bonds (Ryser, 1985). Apparently, such molecular organization of cellulose renders it highly crystalline and resistant to degradation. In addition to crystallinity, strong association of cellulose with hemicellulose and lignin makes hydrolysis of native agricultural residues very slow and inefficient. Hence, whether hydrolysis is performed by acid, enzyme, or microbial means, some sort of pretreatment of the cellulose-containing material is needed to expose its structure and make it more reactive. Various aspects of hydrolysis of corn husk lignocellulose fibers, including the chemical manipulations designed to overcome the structural constraints posed by the lignin-carbohydrate complex, are elaborated in chapters two and three of this thesis.

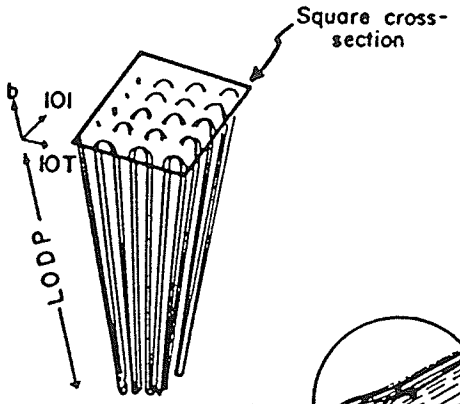
The selection of corn residues as the experimental material for enzymic saccharification into five- and six-carbon fermentable sugars necessitated the compositional and morphological characterization of the raw material. Therefore, the objective of the study presented herein was to evaluate individual corn residues from a compositional viewpoint and to further examine the anatomical features of corn husk that was selected as the ultimate study system. The general morphological characteristics of husk were elucidated by scanning electron microscopy and

Figure 1.2: Three postulated representations of elementary fibrils in native cellulose.
i. fringe-micellar model (Hess et al., 1957) of extended cellulose chains segmented into crystalline and amorphous regions.
ii. folded chain model (Chang, 1971): each cellulose chain is folded within a single cellulose crystallite; regions of folding present the amorphous areas
iii. modification of fringe-micellar model by Rowland and Roberts (1972): (A) coalesced surfaces of high order, (B) readily accessible disordered surfaces, and (C) readily accessible surfaces on strain-distorted, tilt-twist regions.

i


Crystalline region
Paracrystalline region

ii


Square cross-section
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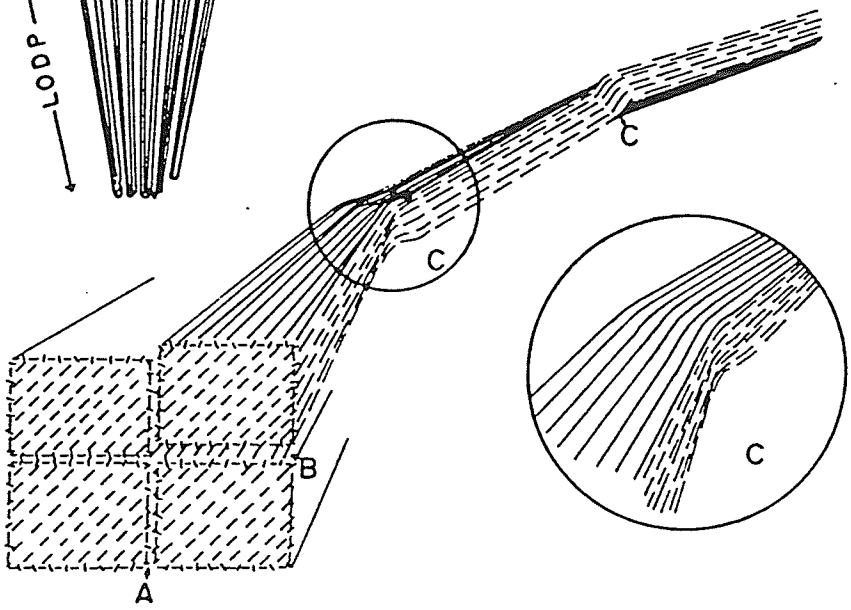
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histochemical tests which are commonly applied in studies of leaf (Esau, 1943) and tree (Biggs, 1985) tissues. In this respect, variously stained specimens were monitored by bright field and fluorescence microscopy.

1.2 MATERIALS AND METHODS

The lignocellulosic material used in this study was corn residues from two commercial corn hybrids: Pickseed 2020 and Pioneer 3995 harvested at maturity in two successive seasons, 1983 and 1984. Following harvesting the corn plant was separated by hand into four types of residue: stalk, cob, leaves and husks which were chopped or cut into smaller pieces prior to drying at 65°C for 48 h. Air dried material was ground in a Udy cyclone mill to approximately 0.3 mm (< 50 mesh) particle size and then subjected to chemical analyses. Chemical composition was expressed on a dry weight basis for each corn residue.

1.2.1 Chemical analyses

Cellulose in native corn husk was determined according to the colorimetric method of Updegraff (1969) and the gravimetric detergent fiber procedure of Goering and Van Soest (1970) using a semiautomatic fiber analyzer Fibertec I (Tecator AB, Hoganas, Sweden). According to the latter method, cellulose is determined from the weight loss upon ashing of lignin free acid-detergent fiber. The hemicellulose fraction, defined as the difference between the neutral-detergent and acid-detergent fiber, was also determined by the method of Goering and Van Soest (1970). Total carbohydrates were calculated by summation of cellulose and hemicellulose contents. Lignin was determined as Klason lignin by the method outlined in TAPPI Standard T22 05-74 method (TAPPI, 1974). All assays were performed in duplicate. The ash and protein (micro-Kjeldahl; Nx6.25) contents of native husk were determined by AOAC (1975) standard methods. All assays were performed in triplicate.

1.2.2 Preparation of husk specimens for microscopy

In order to elucidate the anatomical structure of corn husk and localize the presence of constituents that were chemically identified, morphological analysis of corn husk was undertaken using bright-field, fluorescence and scanning electron microscopy (SEM). Both outer (abaxial) and inner (adaxial) surfaces of air dried mature husk blade were examined initially under a Wild Leitz Stereo Microscope equipped with a 35 mm camera. Specimens were photographed using Kodacolor VR 400 print film and Kodak Ektachrome 800 slide film.

For high-resolution examination with the bright-field and fluorescence microscopes husk blade was cut into approximately 1x1 cm pieces with a razor blade, embedded in methacrylate plastic, sectioned and stained. Sample preparation involved fixation at 25^{0}C using a solution of 5% glutaraldehyde in 0.025 M sodium-phosphate buffer (pH 6.8) for 24 h. Fixation was followed by dehydration using a solvent exchange technique (2-methoxyethanol, ethanol, n-propanol and n-butanol solvent series), infiltration with glycol methacrylate (GMA) mixture and embedding in GMA according to Feder and O'Brien (1968). Transverse and longitudinal sections, 2 μm in thickness, were cut with glass knives on a LKB Bromma 2218 Historange Microtome and stained as described in Table 1.1. All sections were then coverslipped, mounted in immersion oil and observed under a Leitz-Wetzlar Orthoplan Light Microscope equipped with illuminator and filter systems for fluorescence microscopy; the specimens were photographed using films as indicated above.

TABLE 1.1

Staining protocols of corn husk sections.

Stain (concentration; staining time)	Type of microscopy	Component to identify	Reference
Calcofluor white M2R new[1] (0.01% in d.water, 1-2 min)	fluorescence	cell wall polysaccharides	Fulcher (1982)
Toluidine blue O (0.05% in benzoate buffer, pH 4.4, 1-5 min)	bright-field	lignin and polyphenols	Morrison and Dushnicky (1982)
Acid fuchsin (1% in d.water, 1-5 min)	bright-field	proteins and cellular living components by staining/ cell wall polysaccharides and lignin by non-staining	Feder and O'Brien (1968)
Periodic acid-Schiff (PAS) (1% p.a., 10 min;conc. Schiff reagent, 30 min)	bright-field	starch and some complex polysaccharides besides cellulose	Feder and O'Brien (1968)
Sudan black B (saturated in 70% ethanol, 30 min)	bright-field	cuticle and suberized walls	Feder and O'Brien (1968)

[1] A fluorescence filter combination I (exciter filter with transmission max. 365 nm and barrier filter with transmission max.>418 nm) was applied.

For SEM examination, corn husk blade was cut transversely across the major veins with a razor blade and pieces were mounted on metal specimen stubs with Dotite silver paint (Fujikura Kasei Co. Ltd., Tokyo). Another husk sample was ground in a Udy cyclone mill (particle size <0.3 mm) and mounted on metal stub with a double-side adhesive tape. The mounted samples were then coated with approximately 100 Å gold layer and viewed with a JSM-35C (JEOL-Japan) scanning electron microscope operated at an accelerating voltage of 10 kV. Photomicrographs were taken on Plus-X Kodak film.

1.3 RESULTS

1.3.1 Chemical analysis

The chemical composition of two corn hybrids based on the average values obtained in two successive years (1983 and 1984) is presented in Table 1.2. The data indicate that cellulose and hemicellulose comprise the major components of all four types of corn residues. The amount of hemicellulose present in cob and husk is slightly higher than that of cellulose, for both hybrids examined, while the opposite trend is seen in the case of stalks and leaves. Good agreement in the cellulose content between the data obtained by the methods of Updegraff (1969) and of Goering and Van Soest (1970) was observed; the difference was less than 1.1% for all samples tested. All types of residues appeared to have relatively low protein content (<3.6%) with the exception of leaves where it reached 10.2% in the hybrid Pioneer 3995. The distribution pattern of lignin and ash among individual residues was generally the same as that of protein. The highest lignin (13.4%) and ash (14.3%) content were found in leaves of Pickseed 2020 and leaves of Pioneer 3995, respectively. Acid insoluble ash (silica) accounts for approximately 50% of the rather high ash content in leaves, as determined by the method of Goering and Van Soest (1970). Generally, no pronounced variations in the chemical composition of residues between the two hybrids were found. Nevertheless, residues from Pickseed 2020 were found to be of higher cellulose and lower lignin contents, as indicated by the higher cellulose to lignin ratios (Table 1.2). Since husk from Pickseed 2020 exhibited the highest cellulose to lignin ratio among all other residues, it was selected as the experimental material for further investigation.

TABLE 1.2

Chemical composition of corn plant residues expressed in percent dry weight and averaged over two years.

Hybrid	Constituent	Corn residue			
		Husk	Cob	Stalk	Leaves
Pickseed 2020					
	cellulose[1]	38.2	39.1	35.4	30.2
	hemicellulose	44.5	42.1	25.2	29.2
	total carbohydrates	82.7	81.2	60.6	59.4
	lignin	6.6	9.1	11.2	13.4
	protein	1.9	1.7	3.6	8.2
	ash	2.8	1.2	4.6	10.9
	total[2]	94.0	93.2	80.0	91.9
	cellulose/lignin ratio	5.8	4.3	3.2	2.3
Pioneer 3995					
	cellulose[1]	35.2	37.3	35.3	25.9
	hemicellulose	43.0	41.7	23.8	16.9
	total carbohydrates	78.2	79.0	59.1	42.8
	lignin	6.4	9.8	11.6	13.0
	protein	2.0	2.0	2.8	10.2
	ash	2.9	1.0	4.0	14.3
	total[2]	89.5	91.8	77.5	80.3
	cellulose/lignin ratio	5.5	3.8	3.0	2.0

[1] Determined according to Goering and Van Soest (1970).

[2] The remaining percentage is attributed to extractives, waxes, sugars, uronic acids and salts as well as to recovery errors in analytical techniques.

In addition to the Canadian grown corn hybrids, stalk and cob of four Yugoslavian hybrids (SK-42, SK-670_2, SK-704 and Woxsi-616, all from Zemun Polje) were also analyzed for cellulose and lignin content in the initial phase of this study. These hybrids had generally lower cellulose content and approximately the same or higher lignin content. Accordingly, they were considered inferior to the two Canadian hybrids for producing fermentable sugars and, therefore, they were excluded from further investigation.

Although differences for all constituents analyzed between the two harvesting years (1983, 1984) were no higher than 2.6%, there are no data available on compositional variations of these cultivars that are due to seasonal and/or environmental conditions. In this regard, it is of interest to note that variations in cell wall composition within a plant species could be as high as among different species (Theander, 1985). From a chemical composition standpoint, it appears that cob and husk, because of their high total carbohydrate content, are better substrates for hydrolysis to fermentable sugars. In contrast, stalk and leaves with higher contents of noncellulosic components would be more suited for animal feed or the production of lignin-based chemicals.

1.3.2 Morphological analysis

To confirm the results of chemical analyses and qualitatively examine the distribution of the major constituents within the husk tissue, histological studies were undertaken. They were restricted to localization of cell wall carbohydrates, lignin, proteins and protective phytopolymers such as cutin and suberin. Initial examination of husk

surfaces by stereo microscope revealed that the outer surface is highly pubescent in contrast to the inner one, as shown in Figure 1.3b,a. Existence of numerous hairs is probably related to the function of husk as a protective layer in preventing or deterring parasites from attacking developing corn kernels. Parallel venation in husk, typically presented by the principal veins that are interconnected with the smaller lateral veins, is also evident in Figure 1.3a,b. This type of arrangement of conductive elements is commonly found in monocotyledon plants.

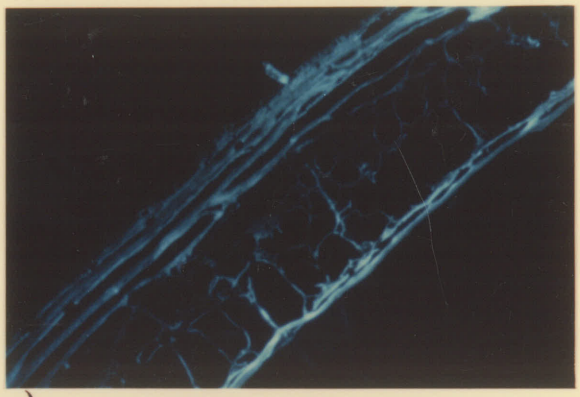
Based on the examination of all stained specimens (Figure 1.3c-f) the general anatomical characteristics of husk structure are as follows. Husk is covered by a layer of epidermis that consists of relatively large cells compactly arranged on both husk surfaces. It is one cell layer thick and includes stomata, large basal cells of hairs plus cork and silica cells on its outer surface as evidenced on all transverse sections of the stained husk specimens. Cuticle is harder to recognize, although it is certainly present on the surface of epidermal cells on both the inner and outer sides of husk. Underneath the epidermis there is a multilayer of sclerenchymatous cells of irregular shape and of evenly thickened cell walls, often lignified. These serve as a supporting tissue and represent a major source of cellulose fibers. Beneath these cells there is one layer of relatively large bundle sheath cells which, in turn, surrounds the vascular bundles, containing phloem and xylem conductive cells. These vascular bundles are the second largest source of cellulose fibers in the husk (Esau, 1977). Phloem and xylem tissue are embedded in mesophyll, which fills the interconnecting

Figure 1.3: Morphological characterization of corn husk; bar=100 μm (c,d,e,f,g) except for a. and b. where bar=1 mm.

a. stereoscope photograph of inner (adaxial) husk surface
b. stereoscope photograph of outer (abaxial) husk surface
c. fluorescence photomicrograph of longitudinal section of husk stained with calcofluor
d. fluorescence photomicrograph of transverse section of husk stained with calcofluor
e. transverse section of husk stained with toluidine blue O
f. transverse section of husk stained with acid fuchsin
g. transverse section of husk stained with periodic acid/Schiff (PAS) counterstained with sudan black.

c-cork cell, p-phloem, x-xylem, os-outer sclerenchyma, is-inner sclerenchyma, m-mesophyll, s-stomata.

a) b)



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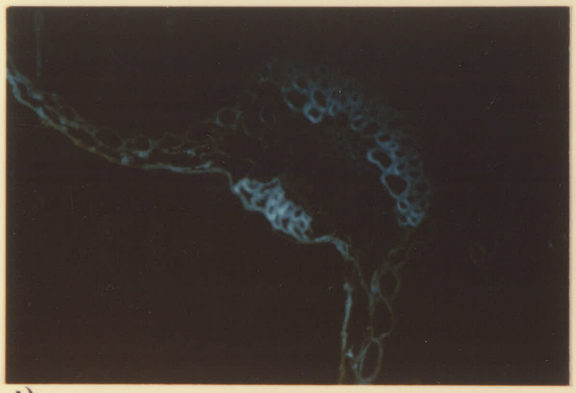

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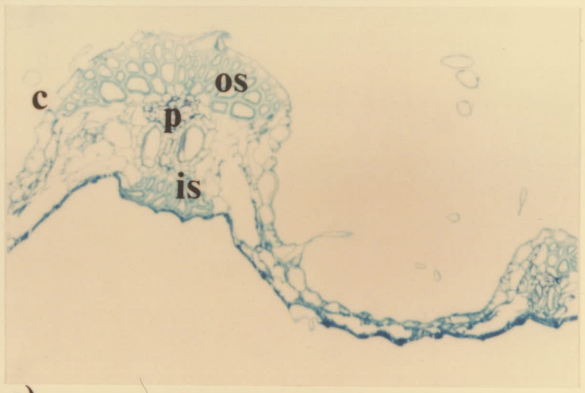

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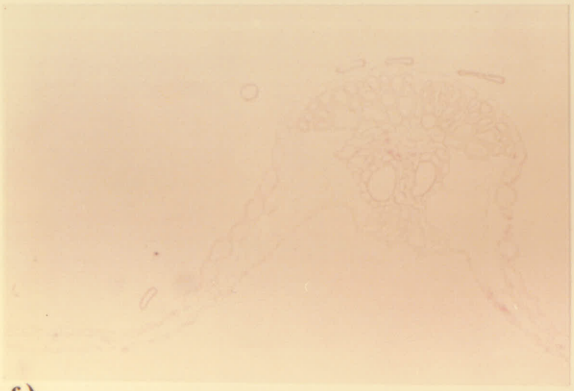

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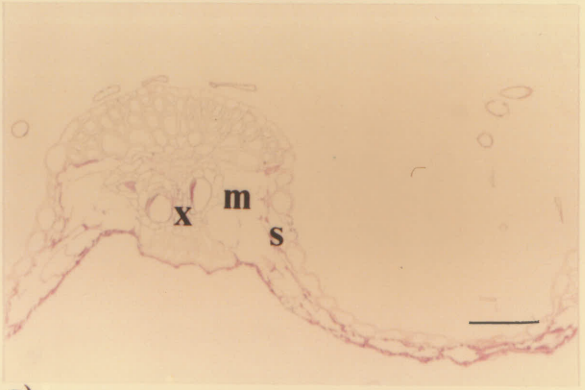


g)

space between the major veins and which is comprised predominantly of photosynthetically active parenchymatous tissue. Mesophyll contains thin-walled living cells and, hence, has a low fiber content. The cellular organization, as described previously, occurs rather regularly and was apparent in all transverse sections of stained specimens.

Histochemical identification of cell wall polysaccharides was conducted by fluorescence microscopy using calcofluor staining. Calcofluor is generally accepted as a specific fluorochrom for ß-glucans. Fluorescent filter combination one (Table 1.1) imparted a pale blue fluorescence of cellulose as expected. Apparently, cellulose was located primarily in the thickened sclerenchyma cell walls, then in xylem and phloem cell walls and occasionally elsewhere (Figure 1.3c,d). Secondary thickening is particularly accentuated in the sclerenchyma cells of the inner husk surface, having little lumen and consisting almost entirely of cellulose fibers.

Detection of lignin, although present in small amounts (Table 1.2), was attempted by staining the specimens with toluidine blue O (Figure 1.3e). However, this dye was not found to be as specific as anticipated. It stained the cell wall material of xylem and sclerenchyma tissue light blue and that of phloem tissue dark blue. In fact lignin was expected to stain blue-greenish according to Feder and O'Brien (1968). Since localization of the stained material using this dye corresponded with the fluorescing regions after staining with calcofluor, these findings imply that the material stained with toluidine blue O has a similar distribution pattern with the cellulosic component or that cellulose itself interacts with the dye. Also, it was interesting to observe that the outer sclerenchyma (corresponding to

outer, hairy husk surface) was stained light blue as compared to the inner sclerenchyma, which stained more intensely. This may reflect differences in the lignification level between these two regions; i.e., the outer zone of the sclerenchyma cell walls is expected to be more lignified. Alternatively, such staining differentiation across the sclerenchyma tissue may be due to a reduced dye penetrability as a result of thickened cell walls or to the thickness of the section per se. Dye impurities could also account for the bluish rather than greenish coloration of the lignified cell walls. Nevertheless, the presence of lignin could not be conclusively confirmed by this staining procedure.

The periodic acid/Schiff (PAS) staining procedure has been suggested as a reliable bright-field indicator of vicinal hydroxyl groups; i.e., it generally stains starch and some complex polysaccharides purple except cellulose (O'Brien and McCully, 1981; Fulcher and Wood, 1983). These staining responses were indeed observed with the husk specimens (Figure 1.3g). When counterstained with sudan black B, the outer sclerenchyma became grayish which may reflect the presence of suberized cell walls. Although the existence of cuticular waxes in corn husk has been confirmed by chemical analysis (Bianchi and Avato, 1984), they were not clearly evident after sudan black B staining. These findings suggest that the cuticular layer is probably very thin and that the waxes associated with it are present in small amounts.

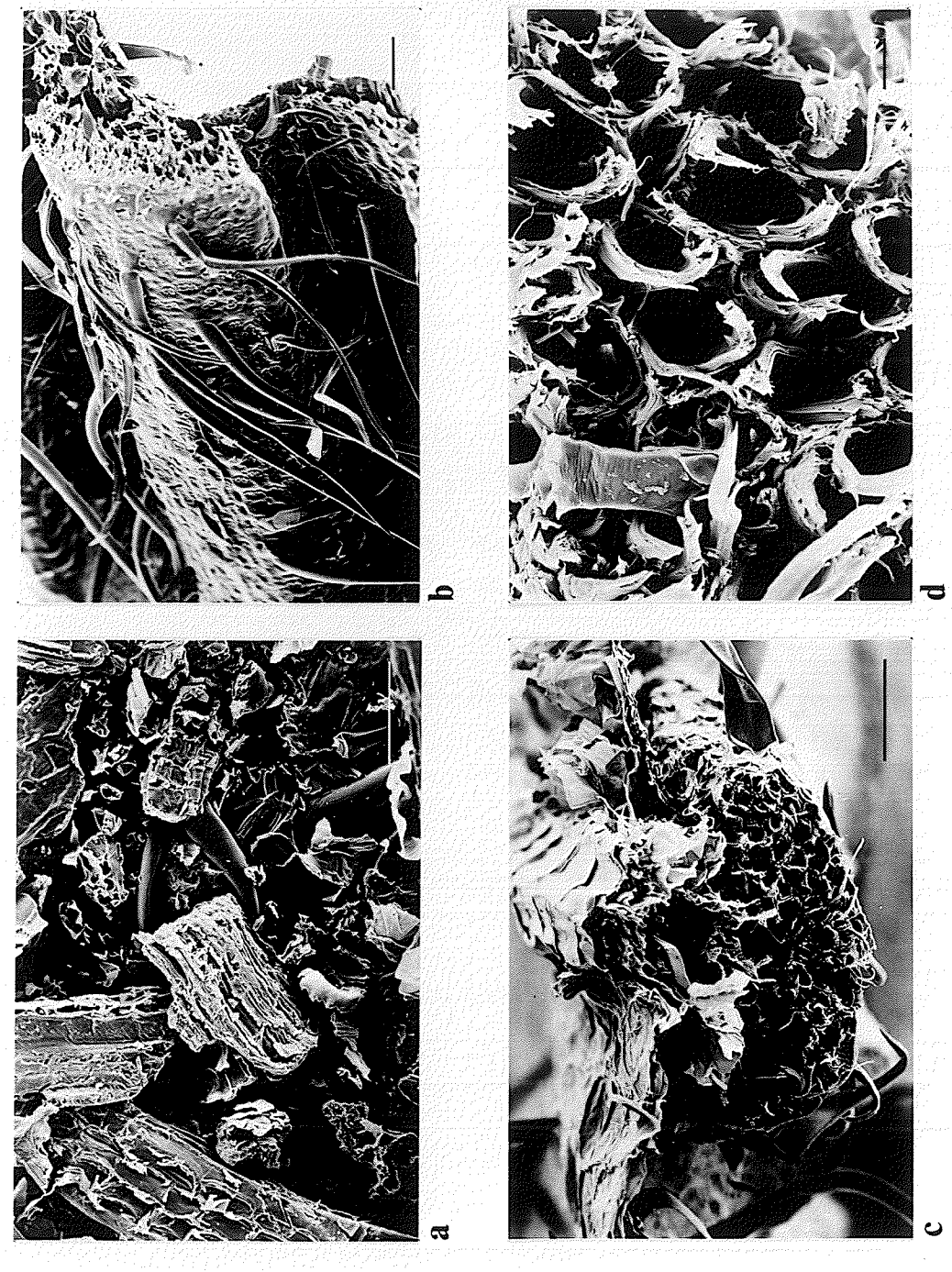
When sections of husk were stained with acid fuchsin, all tissues remained unstained except for small local deposits of material associated predominantly with the phloem tissue, which were stained purple (Figure 1.3f). It is suggested, however, that the light purple colora-

tion of the outer sclerenchyma tissue (Figure 1.3f) results from simple dye adsorption rather than specific dye uptake, since no living organelles are present in this supporting tissue.

In addition to histochemical analysis, corn husk was subjected to SEM examination in order to permit the visualization of its three-dimensional structural organization. In this respect, Figure 1.4a presents a representative photomicrograph of ground husk material showing densely packed vascular bundles which are accompanied by sclerenchyma tissue, smaller pieces of broken mesophyll tissue and single hairs. A longitudinal view of the outer highly pubescent husk surface with numerous stomata cells is shown in Figure 1.4b. When the major husk vein was cut transversely the general anatomical characteristics of its structure, as discussed in the section concerning the histochemical tests, became obvious. The presence of sclerenchyma tissue underneath the epidermal cells, and of the surrounding vascular bundles which are embedded in the mesophyll tissue, has been revealed. The photomicrograph also facilitates differentiation between inner and outer sclerenchyma with regard to appearance and anatomical features, which corresponds to the findings of the histochemical analysis. The higher magnification view of the outer sclerenchyma (Figure 1.4d; transverse section) cells delineates the layered structure of thickened cell walls as well as the interconnecting cellular tissue, i.e., middle lamella.

The data from chemical analyses along with the information from histochemical and SEM examination contributed to an understanding of the overall anatomical features of husk, distribution of the constituents within the husk tissue as well as to the recognition of possible struc-

Figure 1.4: Scanning electron micrographs of native corn husk.
a. typical representation of ground husk; bar=100 μm.
b. outer (abaxial) side of transversely cut husk full of hairs; bar=100 μm.
c. vascular bundle with typical cell arrangement through one of the major veins; bar=100 μm.
d. higher magnification view of vascular tissue cells with thickened cell walls mostly consisting of cellulose fibers; bar=10 μm.


b
d
a
c

tural barriers (lignin, thickened cell wall) that may influence the chemical solubilization and hydrolysis efficiency of husk lignocellulosic fibers.

1.4 DISCUSSION

Compositional and structural considerations regarding the substrate, if substrate availability is assumed, are essential prior to formulation of any processing design in lignocellulosic biomass conversion. In this regard, evaluation of the three basic polymeric constituents, cellulose, hemicellulose and lignin in terms of total content, type of mutual interassociation and distribution within the substrate largely determine the selection and success of an appropriate conversion route. For example, if fermentation potential for production of solvents such as ethanol, butanol, butanediol etc. is to be investigated, total carbohydrate content is a fundamental parameter to consider. The fermentation capacity of a substrate to ethanol is, however, mainly determined by the total amount of cellulose. This is due to the fact that common yeast cannot convert xylose, the major monosaccharide structural element of hemicelluloses, into ethanol. There are a few recent reports, however, on the conversion of pure xylose with Schizosaccharomyces pombe and Saccharomyces cerevisiae, into ethanol (Gong et al., 1981; Wang et al., 1980), but an additional process of converting xylose into xylulose has to precede the main fermentation step. A few organisms, e.g. the yeasts Pachysolen tannophilus (Schneider et al., 1981; Slininger et al., 1982), Candida tropicalis (Jeffries, 1981) and the bacterium Klebsiella pneumoniae (Yu and Saddler, 1982) have been reported to convert xylose directly to ethanol. However, the total conversion rates are very low which make these processes economically unfeasible at present. When the above considerations apply to the concept of exploring the highest possible conversion rate of corn residues into solvents in general, total carbohydrate content obviously becomes very important.

In considering agricultural residues as potential biotransformation substrates, a comparative analysis of different biomass sources appears useful and interesting. In general, forest residues have higher cellulose and lignin, but lower hemicellulose content as compared to agricultural residues. Relative proportions of these three main constituents for some representative cereal crop residues and wood are shown in Table 1.3. The cellulose content of straw originating from four cereal crops varies from 33-44%; hemicellulose varies from 16-36% and silica from 3-13%. Lignin contents are relatively uniform for cereal straws (7-11%), but they are significantly lower from those of hardwood (poplar) and softwood (pine), 22% and 25%, respectively. The data from Table 1.3 suggest that agricultural residues have at least the same, if not better, potential as fermentation substrates for production of ethanol and related solvents (based on total carbohydrate content) than wood residues, although the latter have been more thoroughly investigated to date. Secondly, it appears that corn stalk is of comparable fermentation potential to straw from the slender-stemmed cereals presented in Table 1.3. In addition, corn stover (stalk plus leaves) has been the material of preference in biodegradability studies in relation to other types of corn residues (Gould, 1984; Clausen and Gaddy, 1983; MacDonald et al., 1983). This is presumably due to the fact that stover constitutes the major part of the entire corn plant, approximately 66% (Vetter, 1973). According to Clausen and Gaddy (1983), corn stover is the single most abundant residue available in the world with 150 million tons produced each year. However, it must be emphasized that corn husk and corn cob, owing to their high total carbohydrate content, as shown in this study (Table 1.2), were considered to be more

TABLE 1.3

Comparative chemical composition of cereal crop residues and wood (moisture-free basis).

Source of residue	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Silica (acid-insoluble ash) (%)
barley straw[1]	44	27	7	3
oat straw[1]	41	16	11	3
rice straw[1]	33	26	7	13
wheat straw[1]	39	36	10	6
corn stalk[2]	35	25	11	<4
corn husk[2]	38	44	7	<3
poplar[3]	45	21	22[4]	–[5]
pine[3]	42	23	25[4]	–[5]

[1] Stoskopf, 1985.
[2] Present study (Pickseed 2020, Table 1.2).
[3] Grethlein, 1985.
[4] Refers to lignin with ash.
[5] Value not determined.

suitable as fermentation substrates for ethanol production than corn stover. This also reveals their superiority over other slender-stemmed cereals. Furthermore, husk and cob (comprising 13% and 21% of the corn plant, respectively) should not be neglected as renewable cellulosic resources based on the results of this and other studies (Vetter, 1973). Corn husk is also the most digestable part of the corn plant by ruminants, as reported by Vetter (1973).

In view of the fact that lignin presents the major structural barrier for cellulose hydrolysis by enzymes, as proposed by several researchers (Gould, 1984; Binder et al., 1980; Avgerinos and Wang, 1983), the susceptibility of lignocellulosic material to hydrolysis appears to be related to its cellulose to lignin ratio (C/L). Thus, C/L became a fundamental criterion in predicting the degree of hydrolysis. In this respect, husk was indeed expected to be the most suitable cellulose source having a C/L ratio in the range 5.5-5.8 (Table 1.2). Furthermore, it was considered as an ideal study system for an in-depth investigation of the structural alterations that the material undergoes during chemical and/or enzymic saccharification. Accordingly, husk from corn hybrid Pickseed 2020 having the following composition was selected for further experimentation: cellulose 38.2%, hemicellulose 44.5%, lignin 6.6%, protein 1.9%, ash 2.8%.

The anatomical characteristics of the husk were elucidated using conventional histochemical tests in conjuction with fluorescence microscopy which have been applied in studies of leaf (Esau, 1943), stem (O'Brien and McCully, 1981), and tree (Biggs, 1985), tissues, since no published information on corn husk morphology was available. As corn husk and leaf have common phylogenetic origin, anatomical similarities

were expected and, as such, they provided the base for tissue differentiation in the husk.

An effort to relate husk morphological properties to chemical structure was also attempted. Reasonable agreement between the information obtained by histological analyses and the chemical composition data was found. Calcofluor stain which has been reported to interact with polysaccharides, such as cellulose, substituted celluloses, (1-3)(1-4)-ß-D-glucans, galactoglucomannans (Wood, 1980) has been shown in the present study to bind to cellulose and possibly hemicellulose of the husk cell wall. Cellulose and hemicellulose, found by chemical analysis to be the major components of husk (82.7%), have been confirmed by complementary histochemical tests (i.e., calcofluor and toluidine staining gave positive reactions while acid fuchsin did not stain the cell wall material; Figure 1.3) to comprise the largest part of the husk tissue. Identification of the lignin in the toluidine blue O treated tissue was obscured by the blue coloration of the polysaccharides, which made it difficult to localize lignin in the cell wall. There was, however, an indication that lignin is present in the outer sclerenchyma. The relatively low lignin contents, as found in this study (6.6%), may be one reason for the absence of distinct positive reaction with toluidine blue O. Alternatively, close association of lignin with cellulose, might have prevented stain penetration through the thickened cell wall matrices. Therefore, additional histological evidence, possibly by employing phloroglucinol and hydrochloric acid (2% in 17% HCl) (Jensen, 1962; Biggs, 1985), might be needed to confirm the presence of lignified cell walls in corn husk.

Cutin, suberin and associated waxes were expected to be revealed by staining with sudan black as they have been identified in many plants by microstructural and chemical analyses (Kolattukudy and Espelie, 1985). Bianchi and Avato (1984) have also reported that husks of adult corn plants are covered by waxes which are comprised mainly of esters, sterols, fatty acids, long-chain alkanes and aldehydes. Suberized layers were found in the bundle sheath of corn (Espelie and Kolattukudy, 1979) and are generally deposited around epidermal cells adjacent to silica-containing cells and the base of hairs (Kolattukudy and Espelie, 1985). However, suberized cell walls could not be detected with certainty in this study, as discussed in section 1.3.2. Perhaps they are present in very low levels which requires more specific staining protocols or other techniques for their identification. In contrast to histochemical methods that necessitated tissue fixing and relatively complicated experimental protocols prior to ultimate staining, SEM permitted direct observations of husk tissue structure in its native state.

Chapter II

EFFECT OF CHEMICAL PRETREATMENTS ON PHYSICAL, CHEMICAL AND MORPHOLOGICAL CHARACTERISTICS OF CORN HUSK LIGNOCELLULOSICS

2.1 INTRODUCTION

The application of hydrolytic enzymes offers a very attractive approach for the conversion of complex polysaccharides of plant cell wall to useful products. Despite considerable research effort regarding lignocellulose hydrolysis, however, the actual mechanisms of this biodegradation are poorly understood. This stems largely from the structural complexity of the substrate. Interassociation of cellulose with hemicellulose and lignin represents a major deterrent not only to microbial degradation but also to investigations of the cellulolytic process. Beyond this, also, is the complexity and variability in the organization of the cellulose molecules themselves (Marchessault and Sundararajan, 1983).

Cellulose is a high molecular weight linear polymer of glucose residues connected by ß-1,4-glucosidic linkages. The degree of polymerization ($\overline{DP}$) in some native celluloses has been found to be as high as 15000 (Nevell and Zeronian, 1985). Intra- and intermolecular hydrogen bonds, which are quite weak individually, become a strong associating force within and between cellulose molecules as the $\overline{DP}$ increases (Gilbert and Tsao, 1983). A less obvious force holding the structure together is hydrophobic interaction perpendicular to the sheet of ß-1,4-chains (French, 1985). All these forces can cause neighboring chains to coalesce into crystalline regions which become very tightly packed. As a result, cellulose is not only insoluble in water, but its crystalline regions are virtually inaccessible to most chemical agents; neither water nor even strong acids can easily penetrate the crystallites.

Hemicelluloses are heteropolymers which mainly consist of xylose, galactose, mannose, glucose and arabinose. The hemicellulose component of most agricultural residues and hardwoods is a short chain hetero-1,4-ß,D-xylan ($\overline{DP}$ 50-200) with side groups and branching points. Consequently, it differs from the "smooth" chains of cellulose. This in turn confers much lower tenacity for aggregation (Fengel, 1971). Therefore, hemicellulose is less resistant to hydrolytic degradation as compared to cellulose.

Lignin is a complex three-dimensional phenolic polymer which associates with the cell wall polysaccharides to form a tough rigid structure. The hydrophobic properties of lignin offer resistance to plant cell wall toward hydration and chemical degradation. While the average chemical structure of lignin is known (Adler, 1977; Higuchi et al., 1980), the nature of its association with the cell wall carbohydrates remains uncertain. Three main theories are prevalent: hydrogen bonding between constituents, covalent chemical bonds, and incrustation, where the three-dimensional lignin network encases cellulose, thereby preventing easy access to enzyme molecules. A presently accepted view is that it is largely physical in nature, meaning that lignin and amorphous cellulose form a mutually interpenetrating system of polymers. On the other hand, some covalent links do exist between lignin and hemicellulose (Wardrop, 1971). The nature of chemical bonds anchoring lignin to hemicellulose in the lignocellulosic complex has not been extensively explored. Recent results indicate the presence of ether and 4-O-methyl-glucuronic acid ester bonds to the α-carbons of the lignin monomeric units (Sarkanen, 1980).

In light of the structural complexity of native lignocellulosics some kind of pretreatment is recognized as an essential prerequisite to increase cellulose reactivity toward the cellulolytic enzymes. Enhancement in the rate and extent of hydrolysis by pretreatment is attributed to the modification of the cellulose-hemicellulose-lignin complex by disrupting the interassociation of its components and/or selective removal of some of the cell wall constituents. This results in opening up the structure and thus facilitates the penetration of enzyme molecules into the cellulose matrix (Fan et al., 1980; Gharpuray et al., 1983; Puri, 1984).

A number of different approaches, some of which involve very severe operating conditions, have been applied to improve the accessibility of cellulosics prior to hydrolysis. They include physical treatments such as high pressure steaming (Buchholz et al., 1981; Dekker and Wallis, 1983; Bungay et al., 1983; Brownell and Saddler, 1984), radiation (Han and Ciegler, 1982 a,b; Azuma et al., 1984), mechanical pretreatments (Gracheck et al., 1981; Ryu et al., 1982; Carr and Doane, 1984), biological pretreatments (Hatakka, 1983; Eriksson and Johnsrud, 1985), etc. Among the chemical treatments, dilute acid hydrolysis (Tsao et al., 1982; Knappert et al., 1981; Allen et al., 1983; Grethlein, 1985), organosolv processes (Holtzapple and Humphrey, 1984; Kumakura and Kaetsu, 1983) and alkali digestion (Ohlson et al., 1984; Macdonald et al., 1983; Lai et al., 1983; Farid et al., 1983) have been reported. In addition, combined chemical and physical treatments were also investigated. For example, freeze explosion using liquid ammonia (Dale and Moreira, 1982), radiation in the presence of acids (Kumakura and Kaetsu, 1984), alkali-explosion at elevated temperatures and pressure (Puri and Pearce, 1986),

and explosive pretreatment with high-pressure CO_2 (Puri and Mamers, 1983) have been recently proposed.

Although application of acids is the longest established approach for the conversion of lignocellulose to fermentable sugars, studies on the use of acid pretreatments prior to hydrolysis have been initiated only recently. The following inorganic acids have been examined: sulfuric (Wilke, 1977; Wilke et al., 1981), hydrochloric (Han and Callihan, 1974), and phosphoric (Farid et al, 1983; Fontana et al., 1984). The common characteristic of all acid pretreatments is hydrolysis of hemicelluloses and separation of a pentose-rich fraction. If the conditions are mild enough to avoid further conversion of the released sugars to furfurals, solubilized hemicelluloses may subsequently undergo specific fermentation to ethanol and other chemicals.

Alkali pretreatments, mostly with caustic soda have been initially used to enhance the digestibility of lignocellulosic materials for ruminants (Baker, 1973; Anderson and Ralston, 1973; Han et al., 1975). At present, they are widely used for promoting hydrolysis of agricultural and forest biomass. However, research is still in progress with regard to optimization of alkali pretreatments for various substrates since different materials, having their particular morphologies, respond differently to alkaline solvents.

Autohydrolysis with steam at temperatures about 120°C followed by explosive disintegration of the material, caused by sudden release of the pressure (steam-explosion pretreatment), has been shown to be effective in mechanical disruption of the cellular structure (Puri and Pearce, 1986). It has been, however, suggested that the residence time

at higher temperatures should be kept low to minimize side reactions that can produce byproducts of a noncarbohydrate nature (Mes-Hartree and Saddler, 1983). Autohydrolysis is one of the most promising techniques that has recently emerged.

Many researchers have agreed that certain structural features of lignocellulosics such as cellulose crystallinity, specific surface area, degree of polymerization ($\overline{DP}$), extent of lignification and others affect significantly the hydrolysis of these materials. However, the relative importance of various structural parameters of cellulose to the extent of saccharification has been the subject of extensive debate and is still a matter of controversy (Fan et al., 1980; Dale and Moreira, 1982; Gharpuray et al., 1983; Puri, 1984; Grethlein, 1985; Lin et al., 1985). Since hydrolysis of native lignocellulosics belongs to the category of heterogeneous reactions, it is directly dependent on the available surface area for contact between enzyme and substrate. In this respect, cellulose is a unique substance by having large external surfaces (gross capillaries including the lumen, the pit apertures and pit-membrane of cell wall fibers) and potentially unlimited internal surfaces (spaces between the microfibrils and probably also the ends between the crystallites). The total external surface area for cellulose fiber has been assessed to be approximately 1 m^2g^{-1}, whereas the total internal surface is a function of the pretreatment. Generally, it is several orders of magnitude larger than the former and, at full swelling of the fibers, it may range from 300 to 600 m^2g^{-1} (Cowling and Brown, 1969). Nevertheless, due to steric effects, the accessible surface area to the enzyme is very limited as compared to the total surface. Stone et al. (1969) examined this phenomenon and were first to find a linear rela-

tionship between the initial rate of hydrolysis of cotton pretreated with phosphoric acid and the total surface area that was penetrated by a probe molecule of 30 Å in diameter.

Once contact between enzyme and substrate is established, the relative proportion of the ordered to the disordered regions within the internal structure becomes of primary importance in considering substrate susceptibility to enzymic degradation. Fan et al. (1980) have found that the more accessible amorphous regions of cellulose degrade more easily than the less accessible crystalline parts. Given this relationship, pretreatments leading to decreased crystallinity of lignocellulosics would be expected to increase their digestiblity. It has also been shown that during enzymic hydrolysis, crystallinity of cellulose increases and, consequently, the structure becomes more resistant as hydrolysis proceeds (Baker, 1973).

In addition to the rate-limiting influence of cellulose "fine-structure" on hydrolysis, a further deterrent to substrate penetrability resides in the second major constituent of lignocellulosic materials, lignin. Although the experimental material of the present study, corn husk, is only moderately lignified, as discussed in chapter one, it should be pointed out that retardation of hydrolysis by lignin and other phenolic substances has been widely discussed in the literature. In this respect, it has been suggested that, depending on the source of cellulose, 20 to 65% of lignin needs to be removed in order to achieve considerable increases in susceptibility of the solid substrate to enzymic digestion (Millett et al., 1975; Fan et al., 1981). Most of the delignification pretreatments developed to date are variations of processes originally introduced to the paper industry. For example,

conventional alkaline degradation of native lignin commonly referred to as "kraft pulping", was shown to be applicable in delignifying agricultural residues as well. However, extensive delignification (complete lignin removal has never been accomplished) is accompanied by significant solubilization of pentosans, which is deemed undesirable since the overall conversion efficiency during subsequent hydrolysis and/or fermentation will decrease proportionally. Introduction of anthraquinone additives to the alkaline pulping process promoted lignin fragmentation and retarded lignin condensation (Haggin, 1984), thus resulting in higher extents of delignification. Alkaline solutions of hydrogen peroxide facilitated over 50% solubilization of lignin within 8 h at room temperature and pH 11.5 (Gould, 1984). Such pretreatment also increases the saccharification efficiency of the cellulose-enriched fraction. Unlike other pretreatments, the lignin degradation products released (found to contain a high proportion of low molecular weight carboxylic acids) were not toxic in either enzymic saccharification or subsequent fermentation processes (Gould, 1984).

Lately, delignifications in organic solvents (organosolv pulping) has generated increasing interest. Pannir-Selvam et al. (1983) reported that solvent properties (polarity, solubility and hydrogen bonding capacity) play dominant roles in affecting lignin separation. The maximum delignification of rice straw was achieved at a solvent polarity of approximately 6.5 which is attained by a 50% aqueous butanol solution. Avgerinos and Wang (1983) designed selective solvent pretreatments of cornstover and wheat straw with alkaline ethanol-water mixtures prior to fermentation of carbohydrates by mixed co-culture systems. They observed that, under optimal conditions, up to 67% of the initial

lignin in cornstover could be extracted while 95% of the α-cellulose and pentosans remained insoluble. It has been proposed that release of lignin in all of these organosolv processes is essentially a consequence of the hydrolysis of α-amyl ether and lignin-hemicellulose bonds (Sarkanen, 1980; Holtzapple and Humphrey, 1984).

Microbial delignification (by cooperative action of various fungi and bacteria) is lately gaining attention as an alternative to pulping (Hatakka, 1983; Reid, 1985). However, due to the diversity of inter-monomer linkages (α-aryl ether-, ß-aryl ether-, biphenyl linkage etc.) and monomer units (p-coumaryl, coniferyl, and sinapyl alcohol) within the lignin structure, which in turn require concommitant action of several extracellular enzymes to cause appreciable lignin breakdown (Amer and Drew, 1980), extremely long duration of lignin biodegradative processes is to be expected. Research currently in progress may change the outlook for microbial lignin degradation processes.

Studies attempting to characterize the pyrolytic behavior of cellulosic materials as the function of their compositional and structural properties have not been extensively reported. These studies involve thermoanalytical methods, such as differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA), which are valuable tools to probe the extent and sequence of the physical/chemical transformations encountered under dynamic temperature-time heating protocols (Biliaderis, 1983). In spite of the chemical heterogeneity and structural complexity of agricultural residues, these materials are composed mainly of three polymeric families (cellulose, hemicellulose and lignin) which may undergo thermal decomposition at distinct temperature ranges. For example, it has been reported that hemicelluloses are the least

resistant to thermal degradation, followed by cellulose and lignin (Tang and Eickner, 1968; Bouchard et al., 1985). As such, one would anticipate the compositional and structural attributes of a lignocellulosic material to be reflected in its corresponding thermal degradation profile. However, very few researchers have attempted to relate the thermal behavior to the physicochemical properties of the material under investigation. Furthermore, in most of these studies purified cellulose or small molecular weight compounds (Shafizadeh and McGinnis, 1971; Basch and Lewin, 1973 a,b; Rodrig et al., 1975; Cabradilla and Zeronian, 1976) were employed as study systems. Bouchard et al. (1985) were the first who tried to develop a method for quantitation of the major polymeric constituents in wood residues derived from thermomechanical and chemical treatments by applying thermal analysis techniques. Consequently, it seemed interesting to apply thermoanalytical methods in examining the effect of various chemical pretreatments on corn husk residue and to further explore any possible relationships between thermal properties and structural/compositional features of these materials.

Overall the approach undertaken in the present study was to induce some physico-chemical modifications to the corn husk cell wall material and thus render the cellulose component more reactive toward cellulolytic enzymes. The effectiveness and mechanisms of various pretreatment methods, including both acid and alkaline solvents, were examined. In an effort to elucidate some of the physicochemical and structural alterations of husk lignocellulosics, the thermal properties and morphological characteristics of the modified materials were monitored.

Crystallinity-related parameters, such as crystallinity index and intensity of birefringence, were also assessed before and after the pretreatments. In this respect, an attempt was made to establish relationships between the selected structural parameters as well as to compare the various pretreatment regimes.

2.2 MATERIALS AND METHODS

2.2.1 Solvents and conditions of chemical pretreatments

Native corn husk was dried at 65°C for 48 h and then ground in a Wiley mill (particle size < 1 mm; i.e., <16 mesh) for further treatments designed to increase its susceptibility to enzymic attack. Several chemical pretreatments were chosen which can be classified into three general categories: "one-step" pretreatment using only one solvent, "two-step" pretreatment using two different solvents in consecutive stages and "mechanical/chemical" pretreatment using one solvent in combination with strong mixing action. Table 2.1 summarizes the various types of pretreatments used. Cadoxen (cadmium ethylenediamine hydroxide) consists of ethylenediamine (25% by weight), cadmium hydroxide (5% by weight) and water (70% by weight) and was supplied by Koch-Light Laboratories Ltd., Colnbrook Bucks, England.

All chemical pretreatments were conducted in 100 mL or 250 mL erlenmeyer flasks containing 2-4 g of husk suspended in a solvent at 3.3% w/v concentration on air dry basis. Some of the one-step pretreatments were carried out at selected temperatures in a thermostated water bath under continuous agitation (150 rpm), some in a laboratory autoclave for 15 min or 2 h (time for pressurizing, depressurizing and cooling of the autoclave, approximately 20-30 min, has been excluded) and the rest at room temperature by applying mild stirring with a magnetic bar. Sodium hydroxide, sulfuric acid and phosphoric acid treatments were optimized with regard to solvent concentration and temperature, between 0.1-5.0% concentration and 25-85°C, respectively. On termination of the pretreatment, the sample was filtered through Whatman #4 filter paper and washed with distilled water to remove released solubilized material

TABLE 2.1

Pretreatments used for corn husk lignocellulosics.

Type of pretreatment	Solvent	Conditions (time/temperature)	Equipment
One-Step			
	0.1, 1.0 and 5% w/v NaOH	2 h/25-85°C	water bath shaker
	1% and 10% w/v NaOH	2 h/121°C, 105 kPa	laboratory autoclave
	0.5, 5.0 and 10% w/v NaOH	15 min/121°C, 105 kPa	"
	0.2, 0.5, 1.0 and 5.0% w/w H_2SO_4	2 h/25-85°C	water bath shaker
	0.5, 2.0 and 5.0% w/w H_3PO_4	2 h/25-85°C	"
	14.3% w/w H_3PO_4	2-24 h/25°C	stirring plate
	14.3% w/w H_3PO_4	24-96 h/50°C	water bath shaker
	14.3% w/w H_3PO_4	2 h/85°C	"
	0.5-20.0% w/w CH_3COOH	24 h/25°C	stirring plate
	cadoxen	48 h/25°C	"
	42.5% w/w H_3PO_4	24 h/85°C	water bath shaker
	10% w/v NaOH	2 h/85°C	"
	20% w/v NaOH	24 h/85°C	"
Two-Step			
	0.2% w/w H_2SO_4 / 1% w/v NaOH	15 min/121°C, 105 kPa each step	laboratory autoclave
	0.2% w/w H_2SO_4 / 10% w/v NaOH	"	"
	1.0% w/v NaOH / 0.2% w/w H_2SO_4	"	"
	10.0% w/v NaOH / 0.2% w/w H_2SO_4	"	"
Mechanical/Chemical			
	0.1, 1.0 and 5% w/v NaOH	2 h/25°C	stirring plate
	5% w/v NaOH	5-15 min/25°C	tissue homogenizer
	5% w/v NaOH	5-15 min/25°C	laboratory emulsifier
	5% w/v NaOH	15 min/25°C	ultrasonicator

adsorbed on the husk fibers surface. Washing was continued until the filtrate reached neutral pH. The husk residue was then air dried and stored for further analyses.

Two-step pretreatments were designed as acid/alkali or alkali/acid and conducted in a laboratory autoclave for 15 min time period for each step. After completion of the first step, the slurry was filtered and washed thoroughly with distilled water until neutrality. Then, the husk residues were resuspended in the second solvent and the autoclave treatment was repeated. The final slurry was filtered, washed until alkali- or acid-free (pH 6.0-7.0) and air dried.

In an attempt to examine the feasibility of using shear action, as an alternative to high temperature treatment, combined mechanical and chemical pretreatments were performed. A vigorous magnetic stirrer, a tissue homogenizer-omni mixer (Sorvall, Inc., Norwalk, Conn., USA), a laboratory emulsifier (Silverson Machines Ltd., Waterside, Great Britain) and an ultrasonicator Braun-sonic 1510 (B. Braun Melsungen AG, Great Britain) were used at room temperature for a predetermined time, as specified in Table 2.1. On termination of the treatment, the husk solids were washed and further treated as described above.

2.2.2 Chemical analysis of insoluble husk residue

The insoluble husk residues after chemical pretreatments were characterized in terms of percentage weight residue as well as cellulose, hemicellulose and lignin contents. The weight of residual solids, as an index of the solubilizing capacity of a particular solvent, was esti-

mated based on the weight difference between the native husk sample and the insoluble husk residue remained after chemical pretreatment. Cellulose and hemicellulose were determined according to Goering and Van Soest (1970). Lignin was determined as Klason lignin by the TAPPI Standard T22 05-74 method (TAPPI, 1974).

2.2.3 Crystallinity measurement of insoluble husk residue

The degree of crystallinity of cellulose in untreated and chemically treated corn husk samples was measured by X-ray diffraction using a Philips PW 1051 diffractometer. Air-dried native corn husk, ground in a Wiley mill (particle size < 1.0 mm) was used as control and α-cellulose (Sigma Chem. Co.) as a reference material. Residual husk solids after the pretreatment were washed thoroughly with distilled water, air dried and then subjected to X-ray diffraction analysis. Due to the agglomeration of the particles on drying some of the pretreated residues were further ground (particle size < 1.0 mm) before crystallinity measurements.

The samples were placed on aluminum holders and mounted in the goniometer. An iron-filtered CoKα radiation was used and the diffraction intensity was measured at a voltage of 36kV, current 8 mA and over a range of 2θ 10-36^0. The crystallinity index (CrI) was determined according to Segal et al. (1959). Crystallinity indices were reproducible with a measured standard deviation of 1.5%.

2.2.4 Assessment of structural modifications of husk lignocellulose following chemical pretreatment

The morphological properties of native and chemically altered states of husk lignocellulose material have been examined using both polarizing microscropy (PM) and scanning electron microscopy (SEM). Due to its simplicity, an attempt was made to use PM as a screening technique for evaluation of chemical pretreatments and, as such, it was applied in all stages of this study. Decreases and/or loss of husk cellulose birefringence were expected to occur as a consequence of crystallinity changes brought about by the chemical pretreatments. Alterations of husk fiber surface structure and other possible changes in the internal morphology of husk lignocellulosics were also investigated by high resolution SEM. The native husk was used as a control.

2.2.4.1 Polarizing microscopy

To prepare husk lignocellulosic materials for PM, a small amount of sample was collected in a microbiological innoculating loop and placed on a glass slide. A drop of distilled water was used as the immersion medium. The husk specimens were examined under the fully crossed polarizing filters with a Zeiss Universal Research Microscope equipped with a C35M Zeiss automatic exposure 35 mm camera. Photomicrographs were taken on Kodak Ektachrome 160 ASA film.

With the exception of cadoxen (cadmium ethylenediamine hydroxide; Kochlight Labs, England), extractions of the husk were conducted at 85°C in a water bath shaker for 2-24 h. Pretreatment with cadoxen was carried out at room temperature in a fumehood using magnetic stirring

for controlled mixing. After pretreatment, the slurry was filtered and the residues were washed extensively. Specimens in the wet state were subjected to microscopic examination.

2.2.4.2 Scanning electron microscopy

The extent of structural modifications induced by a selective number of solvents to the corn husk was revealed with a scanning electron microscope (SEM). The native husk samples were prepared for SEM examination as explained in section 1.2.2. Air dried husk residues after chemical pretreatments with either 5% w/v NaOH at 85°C for 2 h in a water bath shaker or 5% w/v NaOH in the laboratory autoclave (121°C, 105 kPa) for 15 min, were ground in a Udy cyclone mill (particle size<0.3 mm; <50 mesh) prior to microscopic examination. Husk samples from the sulfuric (5% w/w, 85°C, 2 h) and the two phosphoric acid treatments (14.3% w/w, 85°C, 2 h and 14.3% w/w, 50°C, 72 h) were not ground prior to SEM since they all appeared powder-like. All samples were mounted onto double-sided tape on metal stubs, coated with approximately 100 Å gold layer and viewed with a JSM-35 C (JEOL-Japan) scanning electron microscope at an accelerating voltage of 10 kV. Photomicrographs were taken at various magnifications (10-2000 times) on Plus-X Kodak film.

2.2.5 Evaluation of the pyrolytic behaviour of husk lignocellulosics by dynamic thermal analysis

Structural alterations of corn husk lignocellulosics caused by chemical pretreatments were followed by monitoring the thermal properties of native and treated husk using differential scanning calorimetry (DSC)

and thermogravimetric analysis (TGA). The residual husk solids after pretreatment were treated identically as described in section 2.2.3. Xylan from oat spelts and α-cellulose, used as reference samples, were products of Sigma Chem. Corp. (St. Louis, MO).

2.2.5.1 DSC analysis

The DSC studies were carried out on a Du Pont 9900 thermal analyzer equipped with a 910 cell base and a pressure DSC cell. The cell base was operated at 10X sensitivity and calibrated with indium. Samples of 3.2-3.8 mg were sealed into aluminum pans by a Du Pont pan crimper; the lids were reversed to minimize thermal lags and thus improve the reproducibility of the thermal profiles. Approximately the same weight of Ottawa sand was used in the reference pan to balance the heat capacity of the sample pan. Samples were heated from 25°C to 500°C in a nitrogen atmosphere (700 kPa) at a heating rate of 10°C min^{-1}. Each sample was run at least three times. Transition peak temperatures generally varied within ±1°C for repeated analyses of the same sample. Data were recorded as heat flow in mW/mg vs. temperature at 1.0 s^{-1} time intervals and stored on floppy disks. Data analyses were performed using the Du Pont software analysis programs.

2.2.5.2 TGA analysis

TGA was performed on a Du Pont 951 TGA unit connected to a Du Pont 1090 thermal analysis system. Samples of 14.5-16.8 mg were run in open aluminum pans and thermograms obtained at a heating rate of 1.5°C min^{-1} over a temperature range of 25°C to 400°C. During each run, the system

was continuously purged with dry nitrogen at a flow rate of 120 cm^3min^{-1}. Each sample was evaluated at least twice and the data were stored on floppy disks with a sampling rate of 1.0 s^{-1}. Data were recorded as weight loss (%) vs. temperature (^0C) (TGA curve), and rate of weight loss (%) vs. temperature (^0C) (dx/dt, derivative curve), respectively. Consequently, the temperature at which sample weight loss of 10% occured (onset of pyrolysis) was directly obtained from the TGA curve while the maximum rate of weight loss (pyrolysis rate) was taken from the derivative curve. Data analyses were performed using the Du Pont software analysis programs.

2.2.6 Kinetic analysis of thermal degradation of husk lignocellulose complex as applied to non-isothermal TGA data

Evaluation of the kinetic parameters from the TGA curves was carried out by employing the dynamic kinetic methods of Broido (1969) and Freeman and Carroll (1958). The former employs the equation:

$$\text{Ln (Ln } 1/y) = -(Ea/R)\ (1/T) + \text{const.}$$

where y is the fraction of the number of initial molecules not yet decomposed, Ea is the activation energy, R is the gas constant and T is the temperature (K). The progress of the reaction(s) can be followed by continuous monitoring of the sample weight. Thus, y is determined as y=(Wt-Winf)/(Wo-Winf) where Wo is the original weight, Wt is the weight at time t, and Winf is the weight at the end of the reaction. A plot of Ln(Ln 1/y) against 1/T provides the Ea from the slope of the curve. The final equation derived by the method of Freeman and Carroll is:

$$\frac{\Delta \log (dx/dT)}{\Delta \log (a-x)} = n - \frac{Ea}{2.303\ R} \frac{\Delta (1/T)}{\Delta \log (a-x)}$$

where 'a' is the initial weight of the material, x is the amount that reacted at temperature T(K), dx/dT is proportional to the rate of the reaction (at a constant heating rate), n is the reaction order and R is the gas constant; Δ refers to the difference between the two subsequent readings of a particular parameter in all cases.

From this equation a plot of $\Delta\log(dx/dT)/\Delta\log(a-x)$ against $\Delta(1/T)/\Delta\log(a-x)$ provides an estimate of Ea and n from the slope and intercept of the curve, respectively. Estimates of Ea generally had a standard deviation of ±7.0 kJ/mol (Broido, 1969) and ±5.0 kJ/mol (Freeman and Carroll, 1958) for repeated analyses of the same sample and data taken over the same temperature range. Actual calculation of the kinetic parameters from the TGA data for a representative sample is shown in Appendix A.

2.2.7 Molecular weight distribution of solubilized or prehydrolyzed husk constituents by gel filtration

The molecular weight (MW) distribution of the components of solubilized and/or prehydrolyzed husk material (chemically solubilized fraction, CSF) from a selected number of chemical pretreatments was investigated by gel filtration. Corn husk (1.5 g) was suspended in 45 mL of one of the following solvents: 5% NaOH (w/v), 5% H_2SO_4 (w/w), 14.3% H_3PO_4 (w/w), 5% H_3PO_4 (w/w). Treatments were carried out in 100 mL erlenmeyer flasks in a water bath at 85°C for 2 h under continuous agitation (150 rpm). The residual biomass solids were then filtered and washed with distilled water. One hundred mL of the CSF were collected and further analysed by gel filtration using Fractogel TSK HW-40(S) (E. Merck, Darmstadt, Federal Republic of Germany). This gel was selected

because of its high chemical stability over the broad pH range encountered in chromatography of the strongly alkaline and strongly acidic samples of this study.

A 50 μL CSF sample containing 100-200 μg of carbohydrates was applied via a sample loop to the top of the column (gel bed 145x0.9 cm). The downward flow rate was maintained at 17 $mL.h^{-1}$ by applying low pressure of approximately 20 Pa. The column was thermostated at 70°C and degassed distilled water was used as eluant. The column was calibrated with standard solutions (0.1-1.5 mg mL^{-1}) of maltoheptaose, maltopentaose, maltotetraose (all supplied by Boehringer Mannheim Biochemicals), cellobiose (Sigma Chem. Co.), glucose (BDH Chemicals Ltd.) and xylose (Sigma Chem. Co.).

Total carbohydrates in the eluate were determined by the orcinol-sulfuric acid method using 70% (v/v) sulfuric acid containing 0.1% (w/v) orcinol as a reagent (Miller et al., 1960). All analytical procedures were carried out by a Technicon AutoAnalyser, as schematically illustrated in Appendix B. One volume of column effluent and 3 volumes of orcinol-sulfuric acid reagent were mixed and air-segmented by a multichannel peristaltic pump. The stream was then passed through a reaction coil (8 min, 93°C) and, after debubbling, the absorbance was measured at 420 nm.

Pentose-containing oligosaccharides in the eluate were determined according to Dische (1962) using the Bial reagent (concentrated hydrochloric acid containing 0.1% (w/v) orcinol and 0.1% (w/v) ferric chloride hexahydrate). The column was calibrated with xylose standard solu-

tions of 0.25-1.5 mg mL^{-1} under the same chromatographic conditions used for total carbohydrates determination. However, the reaction temperature for color development was 85°C and absorbance was measured at 665 nm.

2.2.8 Chemical analysis of solubilized or prehydrolyzed husk material

Total soluble carbohydrate content and pentose-containing oligosaccharide content of husk CSF samples using orcinol-sulfuric acid reagent (Miller et al., 1960) and Bial reagent (Dische, 1962), respectively, were determined on the Technicon Auto-Analyzer system. Instead of column eluate, however, the automatic sampler was loaded with standard solutions or appropriately diluted CSF samples. Total carbohydrates were detected at 420 nm and pentose-containing oligosaccharides at 665 nm, using xylose as standard.

2.3 RESULTS

2.3.1 Optimization of chemical pretreatments

A variety of solvents under various concentration-temperature-time regimes were applied in chemical pretreatments of corn husk, as specified in Table 2.1. The resulting compositional/structural properties of husk lignocellulosics were followed by assessing the degree of solubilized material (D.S.), the degree of structural order, measured by the crystallinity index value (CrI), and the overall morphological characteristics. These parameters were considered suitable for a rapid evaluation of lignocellulose susceptibility to the solvents and, as such, they were monitored throughout this study.

Preliminary investigations indicated that conditions of high temperature and pressure (121°C, 105 kPa, 2h), as suggested in numerous reports (Fan et al., 1981; Dale, 1985; Puri and Pearce, 1986), were not necessary to bring about partial dissolution of husk and alterations of its crystalline properties. Thus, initially applied single-solvent treatments in autoclave for 2 h were replaced by shorter treatments using two different solvents in consecutive stages; each solvent was applied for 15 min. The results of acid/alkali (i and ii) treatments as opposed to alkali/acid (iii and iv) are given in Table 2.2. The order of solvents, when 0.2% w/w H_2SO_4 and 1.0% w/v NaOH were employed (i and iii), did not seem to affect the crystallinity of the residual solids, but resulted in 5.6% difference in the overall D.S. in favour of the acid/alkali treatment (i). When 0.2% w/w H_2SO_4 and 10% w/v NaOH were used in combination, regardless of the solvent order (ii and iv), approximately 69% of the material was solubilized. The alkali/acid treatment (iv) gave,

TABLE 2.2

Effects of two-step[1] pretreatments on corn husk solubilization/crystallinity.

Two-step pretreatment		Solvent	D.S.[2] (%)	Overall D.S.[3] (%)	CrI[4] (%)
None (Control)		-	0	0	52.1
Acid/Alkali					
	i	0.2% w/w H_2SO_4	28.7		64.6
		1.0% w/v NaOH	47.4	62.4±1.2	77.3
	ii	0.2% w/w H_2SO_4	28.7		64.6
		10.0% w/v NaOH	57.6	69.7±2.1	78.9
Alkali/Acid					
	iii	1.0% w/v NaOH	43.9		63.1
		0.2% w/w H_2SO_4	22.9	56.8±1.7	76.0
	iv	10.0% w/v NaOH	66.1		70.7
		0.2% w/w H_2SO_4	8.7	69.0±1.6	82.8

[1] Conditions of each step in a two-step pretreatment: 121°C, 105 kPa, 15 min.

[2] D.S.: Degree of solubilization on the basis of residue weight at each individual step.

[3] Overall D.S.: Degree of solubilization following "two-step" pretreatment expressed as means ± standard deviation (n=3).

[4] CrI: Crystallinity index of the residue as determined by X-ray diffraction (Segal et al., 1959).

however, a residue of higher crystallinity (82.8% vs. 78.9%), which was of a comparable value to the commercial α-cellulose. Chemical pretreatments of cellulose, designed to improve the rate and extent of its hydrolysis, generally yield residues of lower degree of crystallinity (Knappert et al., 1980). In contrast, the chemically pretreated husk residues showed an increase in crystallinity which might simply reflect enrichment of the residues in cellulose. Furthermore, high solubilization levels were achieved in all four pretreatments (Table 2.2). Considering that the D.S. (69.7%) exceeded the amount of non-cellulose components present in corn husk, following pretreatment ii, it was concluded that partial hydrolysis/solubilization of presumably amorphous cellulose also took place. These findings suggested that even milder conditions than those applied in the two-step pretreatments may prove satisfactory in enhancing the cellulose/hemicellulose susceptibility to hydrolysis. Consequently, investigations of 2 h-treatments using NaOH, H_3PO_4 and H_2SO_4 over a wide temperature range (25-85°C) were undertaken. For each solvent, the concentration ranged between 0.1% and 5.0%.

Figures 2.1, 2.2, and 2.3 illustrate the solubilization- and crystallinity index-temperature profiles for all treatments employed. The effects of solvent concentration and temperature were strongly dependent on the nature of the solvent. The D.S. ranged from 13.8 to 62.1% (0.1% w/v NaOH, 35°C and 5% w/v NaOH, 85°C) for the NaOH pretreatments (Figure 2.1). The corresponding D.S. values for H_2SO_4 and H_3PO_4 were in the range of 11-53% and 10-29%, respectively. At low solvent concentration (<1.0%) in all cases, the D.S. remained relatively constant over the entire temperature range examined. This suggests that an increase in

Figure 2.1: Effect of temperature and NaOH concentration on degree of solubilization (D.S.) of corn husk and on crystallinity index (CrI) of residual solids. Pretreatment time: 2 h, bars indicate standard deviations (n=3).

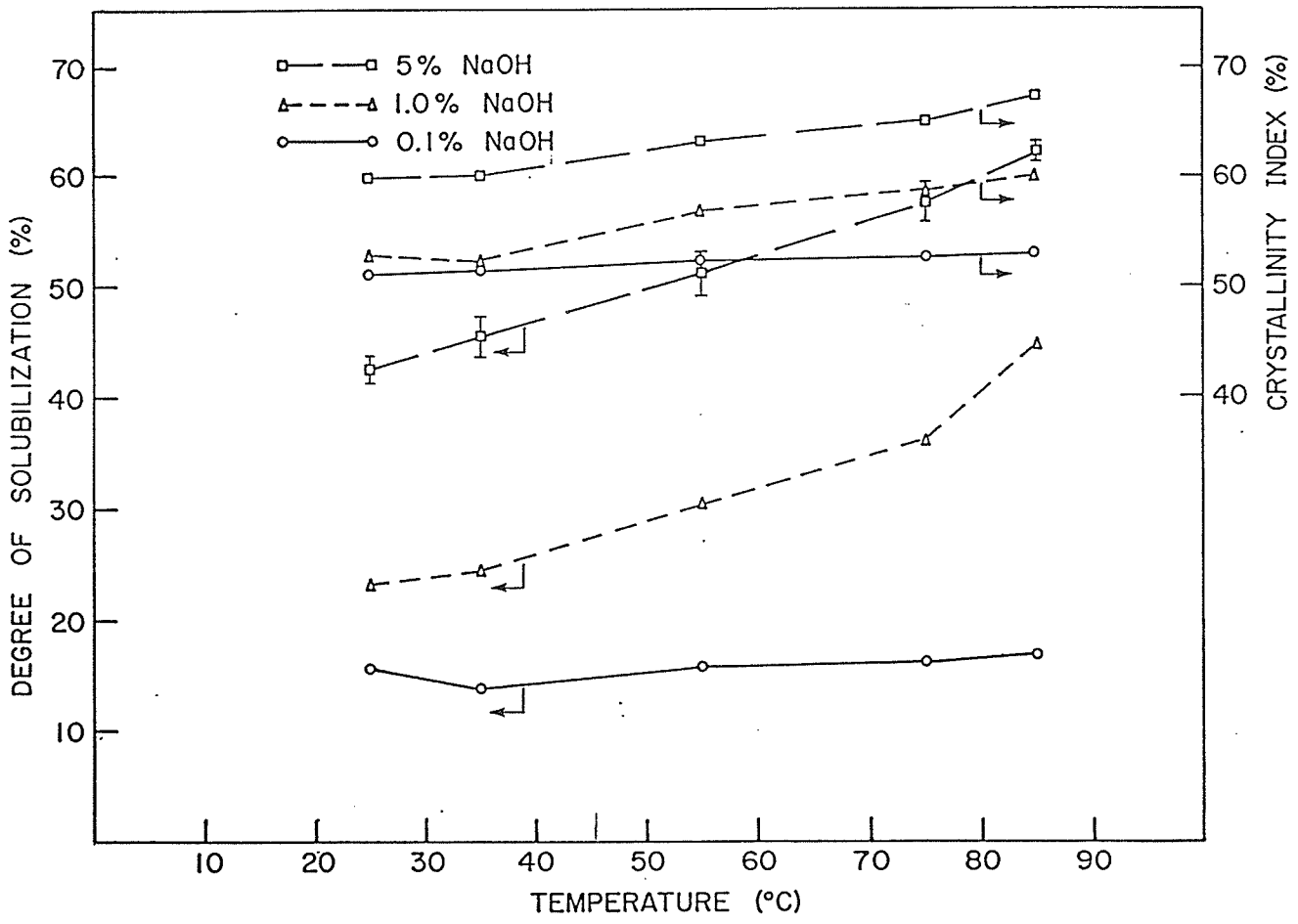

5% NaOH
1.0% NaOH
0.1% NaOH
DEGREE OF SOLUBILIZATION (%)
CRYSTALLINITY INDEX (%)
TEMPERATURE (°C)
10
20
30
40
50
60
70
80
90

Figure 2.2: Effect of temperature and H_2SO_4 concentration on degree of solubilization (D.S.) of corn husk and on crystallinity index (CrI) of residual solids. Pretreatment time: 2 h, bars indicate standard deviations (n=3).

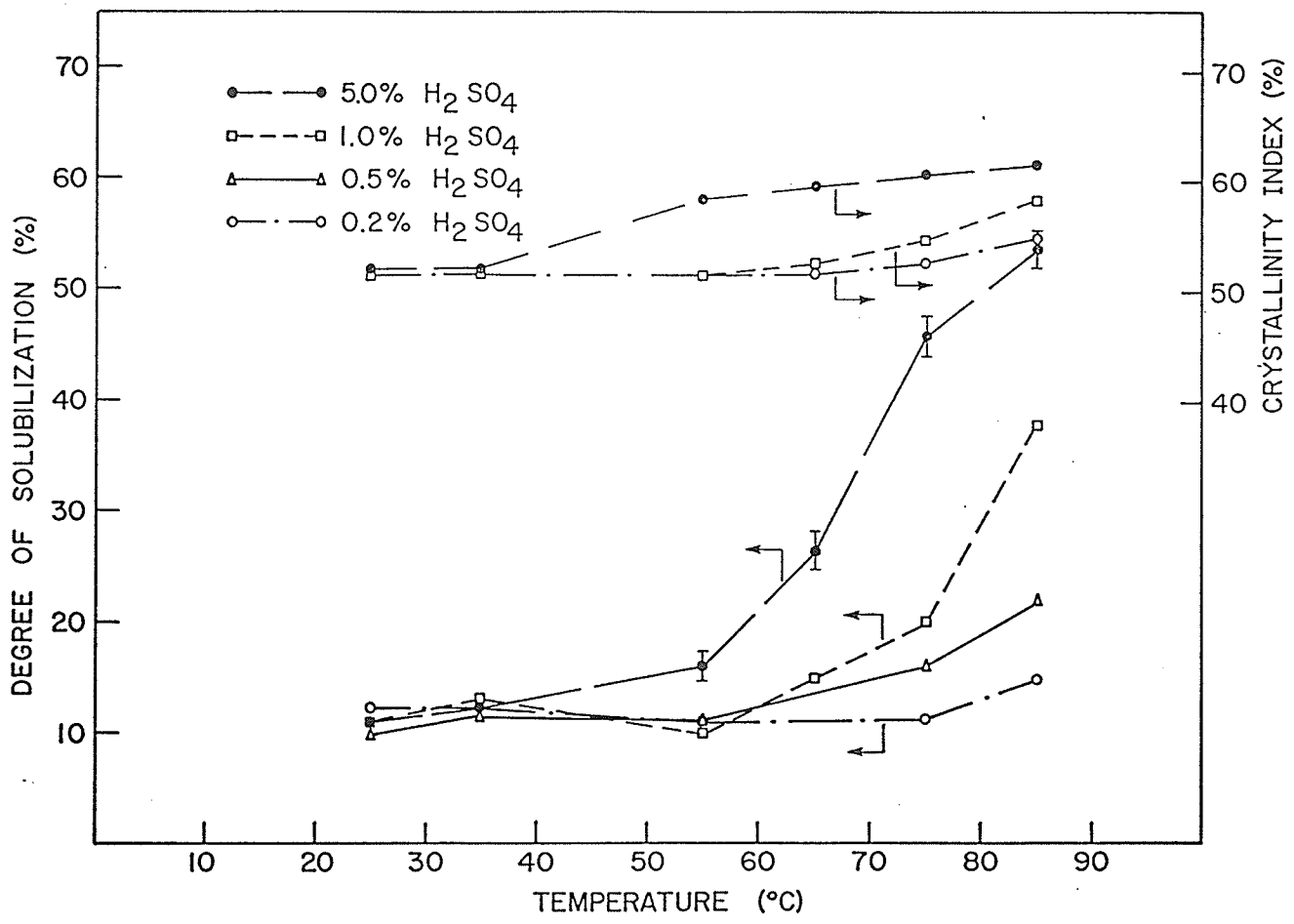
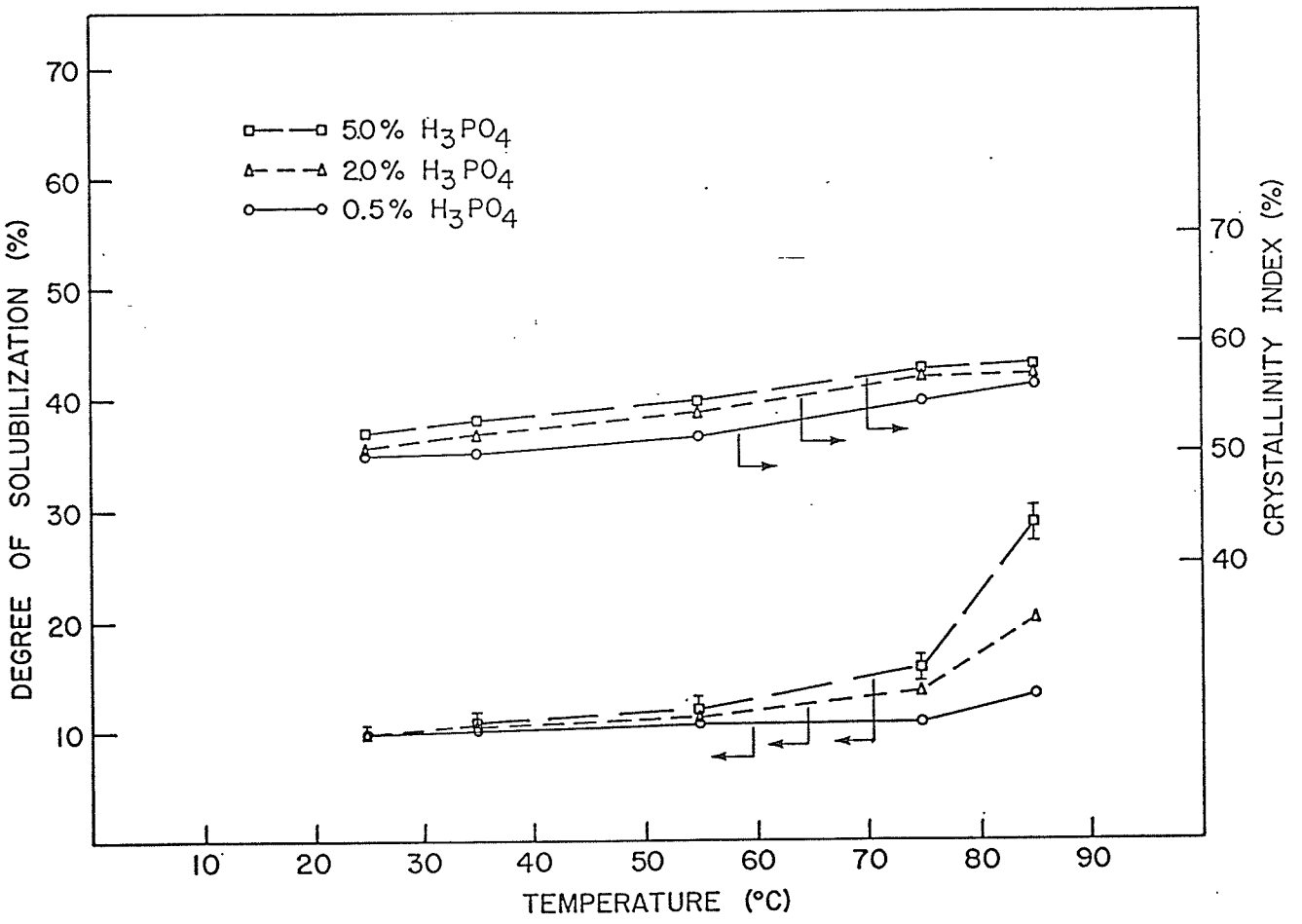

5.0% H_2SO_4
1.0% H_2SO_4
0.5% H_2SO_4
0.2% H_2SO_4
DEGREE OF SOLUBILIZATION (%)
CRYSTALLINITY INDEX (%)
TEMPERATURE (°C)
10
20
30
40
50
60
70
80
90

Figure 2.3: Effect of temperature and H_3PO_4 concentration on degree of solubilization (D.S.) of corn husk and on crystallinity index (CrI) of residual solids. Pretreatment time: 2 h, bars indicate standard deviations (n=3).


5.0% H_3PO_4
2.0% H_3PO_4
0.5% H_3PO_4
DEGREE OF SOLUBILIZATION (%)
CRYSTALLINITY INDEX (%)
TEMPERATURE (°C)
10
20
30
40
50
60
70
80
90

temperature up to 85°C was not effective in intensifying the interactions between solvent and lignocellulose complex. Under these conditions the D.S. reached a constant value of 10-14%. The latter most likely reflects the presence of free sugars and breakdown products of labile hemicellulose components. At solvent concentrations higher than 1.0% the influence of temperature on D.S. was more pronounced and strongly dependent on the nature of the solvent. Thus, at concentrations between 1.0 and 5.0% the limiting temperatures above which the solubilization power became strongly dependent on temperature were 25, 55 and 75°C for NaOH, H_2SO_4 and H_3PO_4, respectively. These observations further indicate the superiority of the alkali as compared to acid pretreatments in dissolving husk lignocellulosics. It should also be noted that 5% w/v NaOH at room temperature appeared to be very effective in selectively extracting the hemicellulose component (D.S.=43%).

The crystallinity changes of the remaining residues after chemical pretreatments were also followed. In all cases, the CrI either remained unchanged or increased (Figures 2.1, 2.2, and 2.3). Alkali pretreatments at elevated temperatures resulted in residues of the highest CrI values (e.g., 67% at 85°C), whereas acid pretreatments did not affect significantly the state of order of residual lignocellulosics.

The simultaneous action of physical and chemical forces on the structural/compositional alterations of corn husk, as an alternative to high temperature treatment, was also investigated. The effects of NaOH, under varying agitation conditions, on D.S. and CrI of husk are presented in Table 2.3. A relatively high degree of solubilization (58.6%) was achieved with 5% w/v NaOH in combination with ultrasonica-

TABLE 2.3

Combined effect of NaOH and shear at room temperature (25°C) on corn husk solubilization and crystallinity.

Solvent	Mixing device	Time (min)	D.S.[1] (%)	CrI (%)
None (Control)	-	-	-	52
0.1% w/v NaOH	Stirring plate	120	15.6	56
1.0% w/v NaOH	"	"	23.2	60
5.0% w/v NaOH	"	"	42.7±1.8	67
5.0% w/v NaOH	Sorvall tissue homogen.	5	36.8	68
5.0% w/v NaOH	"	15	47.1±1.5	64
5.0% w/v NaOH	Silverson lab emulsifier	5	38.1	67
5.0% w/v NaOH	"	15	46.7±2.1	64
5.0% w/v NaOH	Ultrasonicator	15	58.6±1.9	67

[1] D.S.: Degree of solubilization expressed as means of duplicate analyses or means ± standard deviation (n=3).

tion; the corresponding increase in crystallinity of the residue was 15%. As such, ultrasonication was the most effective of all mixing methods tested in bringing about disruption of the lignin-hemicellulose-cellulose complex and therefore its partial solubilization. Overall, all pretreatments were efficient in increasing both D.S. and CrI. The data from Table 2.3 provide the basis for a more extended study on solubilization of lignocellulosics by exposure to various shearing forces.

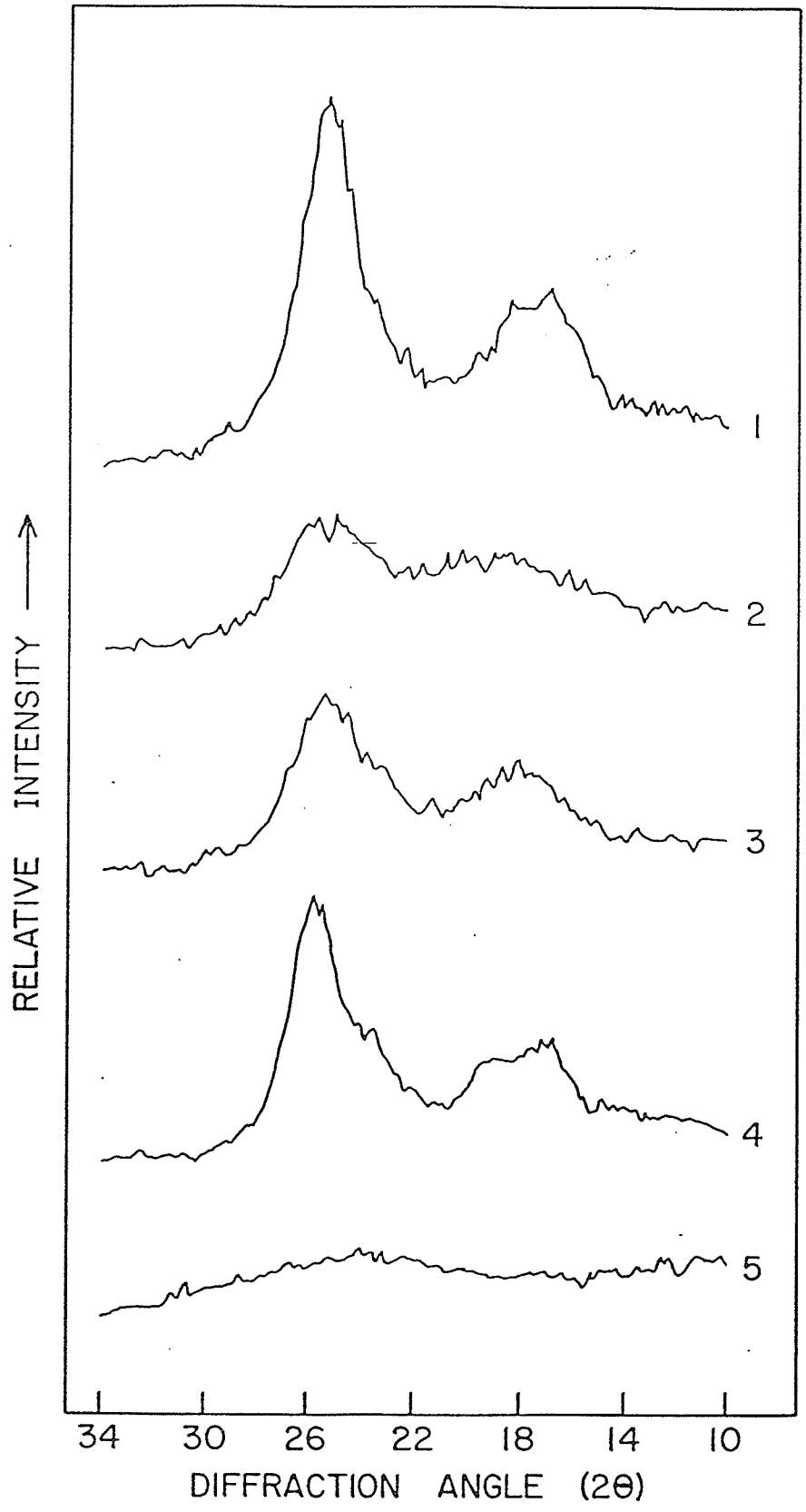
As a result of the above trends in the D.S. and CrI of pretreated husk lignocellulosics as well as the findings regarding the nature of the solubilized material (section 2.6), it was decided that a 2 h treatment at 85°C using NaOH, H_3PO_4 and H_2SO_4 at 5% concentration, would be adequate to maximize the yield of sugars upon subsequent hydrolysis of the residues.

2.3.2 Crystallinity measurements

Typical X-ray diffractograms for cellulose, native husk, selected number of residues after chemical pretreatments and lignin (Klason) originating from husk are presented in Figure 2.4. The X-ray diffraction traces for all samples, besides lignin, revealed the native cellulose type I allomorph whose major and minor peaks occur at diffraction angles (2θ) of 26° and 18°, respectively. The X-ray diffraction of lignin showed no evidence of crystalline order (absence of any diffraction peak). The highest degree of crystallinity (CrI=82.0%), as determined by Segal et al. (1959), was obtained for α-cellulose which was used as standard. Native husk which is a complex of cellulose, hemicellulose and amorphous lignin, was found to be partially crystalline (CrI=51.0%). In general, chemical pretreatments resulted in an increase

Figure 2.4: Typical X-ray diffractograms of husk cellulosics.
1. α-cellulose (Sigma) (CrI=82.0%)
2. native husk (CrI=51.0%)
3. husk pretreated with 5% NaOH/75°C-2 h (CrI=70.0%)
4. husk pretreated with 10% NaOH/Aut-2 h followed by 0.2% H_2SO_4/Aut-2 h (CrI=84.0%)
5. husk lignin isolated as Klason lignin.

Relative intensity expressed in arbitrary units with ordinates shifted to permit superposition of traces.


RELATIVE INTENSITY
1
2
3
4
5
34
30
26
22
18
14
10
DIFFRACTION ANGLE (2θ)

of the degree of crystalline order of the husk as illustrated by the diffractograms of Figure 2.4 (3,4). These results in conjuction with chemical analyses data (Tables 2.2, 2.3, and 2.4) suggest that increases in crystallinity could be attributed to the dissolution of more accessible non-crystalline components of the lignocellulose complex; i.e., hemicellulose and possibly amorphous cellulose, thus resulting in a product with increased concentration of crystallites.

The degree of crystallinity along with the data on solubilization and cellulose content of husk residues after NaOH- and H_2SO_4- pretreatments at various concentrations (e.g., 0.1, 1.0 and 5.0%) and temperatures (25, 55 and 85°C) are presented in Table 2.4. This approach permitted a comparative analysis of the alterations in crystalline structure imposed by acidic and alkaline solvent manipulations under relatively mild conditions and, even more importantly, assessment of the significance of solvent concentration versus temperature on the degree of structural order in the residual husk. Regardless of the solvent applied, CrI and cellulose content of the pretreated residues were increased by increasing the solvent concentration under all temperature/time regimes employed (e.g., 25°C, 2 h; 85°C, 2 h). It was also noted that the increase in crystallinity of the residual husk lignocellulosics paralleled the increase in cellulose content. This observation further suggests that the higher crystallinity might simply be a direct consequence of enriching the residue in cellulose. However, partial conversion of amorphous into crystalline cellulose when exposed to an aqueous environment could also contribute to the observed crystallinity changes (Wadehra and Manley, 1965).

TABLE 2.4

Relationship between the chemical composition and crystallinity of residual solids following various pretreatments.

Pretreatment (solvent/temp-time)	Residue[1] (% wt)	Cellulose[1] (%)	CrI (%)
None (Control)	100	39	51
0.1% w/v NaOH/25°C - 2 h	84	40	51
1.0% w/v NaOH/25°C - 2 h	77	46	55
5.0% w/v NaOH/25°C - 2 h	57±2.0	60±1.3	62
0.1% w/v NaOH/55°C - 2 h	84	41	49
1.0% w/v NaOH/55°C - 2 h	69	51	57
5.0% w/v NaOH/55°C - 2 h	49±1.2	64±1.0	65
0.1% w/v NaOH/85°C - 2 h	83	41	49
1.0% w/v NaOH/85°C - 2 h	55	59	63
5.0% w/v NaOH/85°C - 2 h	38±1.7	72±1.0	70
0.2% w/w H_2SO_4/25°C - 2 h	89	-[2]	49
1.0% w/w H_2SO_4/25°C - 2 h	89	41	50
5.0% w/w H_2SO_4/25°C - 2 h	88±1.3	-	50
0.2% w/w H_2SO_4/55°C - 2 h	89	-	51
1.0% w/w H_2SO_4/55°C - 2 h	87	-	55
5.0% w/w H_2SO_4/55°C - 2 h	84±1.5	-	61
0.2% w/w H_2SO_4/85°C - 2 h	85	-	53
1.0% w/w H_2SO_4/85°C - 2 h	63	-	61
5.0% w/w H_2SO_4/85°C - 2 h	47±1.0	69±0.6	63

[1] Data on percent residue and cellulose content are means of duplicate analyses or means ± standard deviation (n=3).

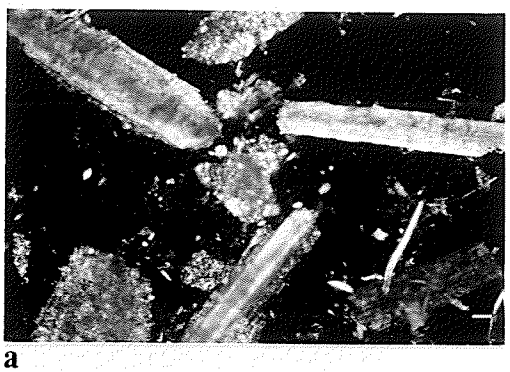
[2] Value not determined.

The data of Table 2.4 also indicate that higher NaOH concentrations caused more pronounced changes in crystallinity of the residual solids as compared to increases in temperature at a particular NaOH concentration. For the latter, the strongest response of 8% increase in CrI was observed at 5% NaOH by raising the temperature from 25°C to 85°C. In contrast, NaOH concentrations between 0.1% and 5% w/v at 85°C resulted in crystallinity changes of 21%.

2.3.3 Polarizing and scanning electron microscopy

Photomicrographs of native and pretreated corn husk residues obtained by polarizing microscopy (PM) are shown in Figures 2.5 and 2.6. The native husk exhibited crystalline, strongly birefringent, parts of densely packed vascular tissue and less birefringent material probably originating from the interconnecting mesophyl tissue (Figure 2.5a). The latter appeared as a transparent-like structure when viewed by PM. When cellulose solvent, like cadoxen (cadmium ethylenediamine hydroxide), was applied to husk at room temperature, loss of physical integrity occurred in the first 3 h of the treatment as shown in Figure 2.5b. Separation of husk fiber bundles into individual fibers in addition to extensive swelling was also apparent at the early stages of the treatment (Figure 2.5c). It is well known that cadoxen has the capacity to penetrate cellulose crystallites. This is evidenced by the numerous sites of attack along the individual cellulose fibers (Figure 2.5d,e) which resulted in loss of birefringence. It is difficult to assess, however, whether loss in birefringence was solely caused by converting crystalline cellulose segments into amorphous, by actual dissolution of cellulose or by a combination of both processes. With respect to decrystal-

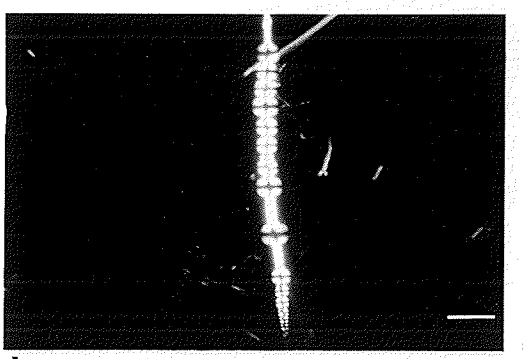
Figure 2.5: Polarizing photomicrographs of native corn husk and husk treated with cadoxen (cadmium ethylenediamine hydroxide) at room temperature.
a. native husk; bar=100 μm.
b. husk treated with cadoxen for 3 h; bar=100 μm.
c. husk treated with cadoxen for 3 h; bar=100 μm.
d. husk treated with cadoxen for 8 h; bar=100 μm.
e. husk treated with cadoxen for 8 h; bar=50 μm.



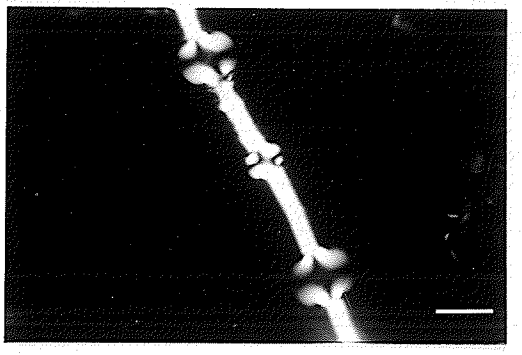
a

b

c

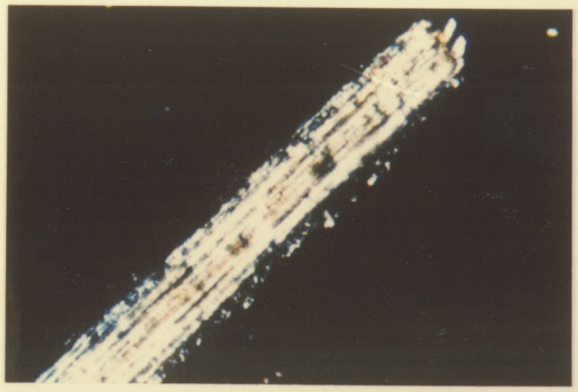


d

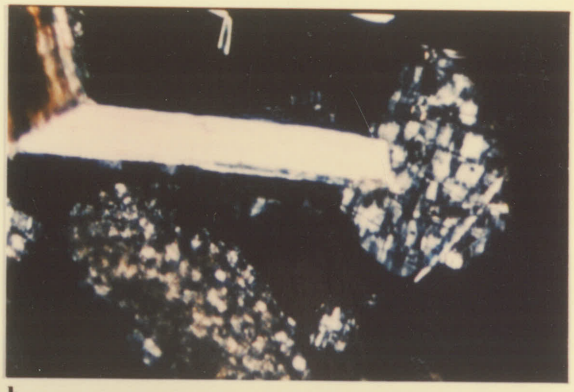


e

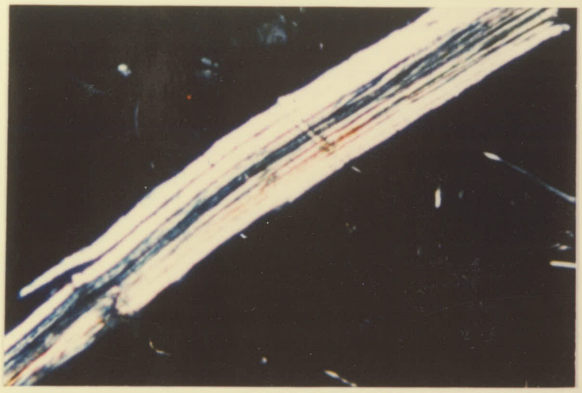
Figure 2.6: Polarizing photomicrographs of corn husk; bar=100 μm.
a. native husk
b. husk treated with water at 85°C for 2 h
c. husk treated with 10% w/v NaOH at 85°C for 2 h
d. husk treated with 10% w/v NaOH at 85°C for 24 h
e. husk treated with 14.3% w/w H_3PO_4 at 85°C for 2 h
f. husk treated with 42.5% w/w H_3PO_4 at 85°C for 24 h.



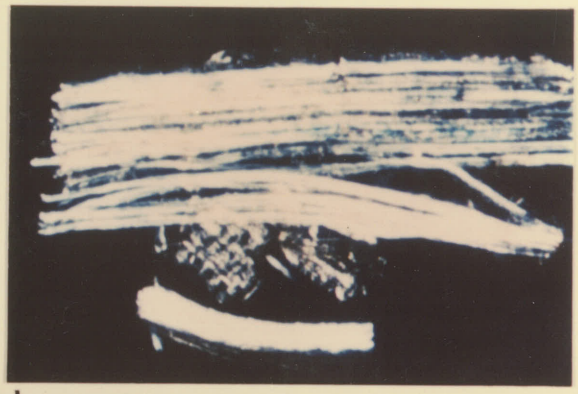
a



b



c



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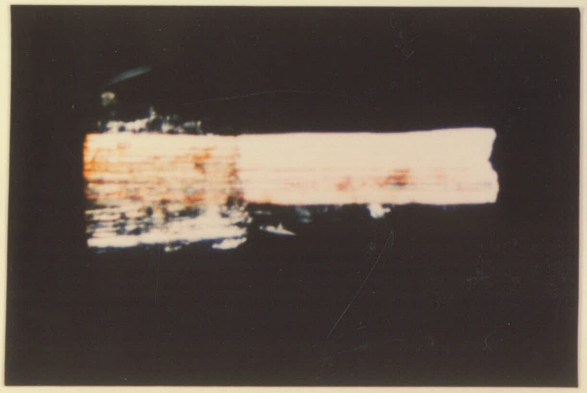
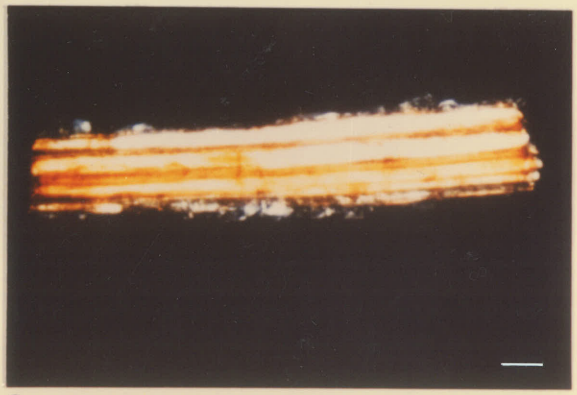


e



f

lization, it is well known that amines can cause intracrystalline swelling of cellulose (Zeronian, 1985). As such, cadoxen would be expected to solubilize cellulose by disrupting its crystal lattice. The micrographs of the cadoxen-treated samples (Figure 2.5d,e) further suggest that this solvent exerts a random mode of attack on the cellulose structure. Although complete dissolution of corn husk by cadoxen was not accomplished, even after 24 h of treatment, presumably due to the presence of non-cellulose constituents, this treatment did bring about extensive solubilization of husk.

In contrast to cadoxen, all other solvents employed were not as effective in decreasing cellulose birefringence. In fact, distilled water, 5% w/v NaOH, 5% w/w H_2SO_4 and 5% w/w H_3PO_4 acids when applied at 85^{0}C for 2 h caused no detectable changes in both structural integrity and birefringence of husk. In view of these findings, the suitability of PM in characterizing the structural and morphological features of lignocellulosic materials after chemical pretreatment was further explored by employing more severe pretreatment conditions (i.e., high solvent concentration and longer reaction time). Under these regimes more pronounced alterations in the structure were anticipated. Representative polarized light photomicrographs of such specimens are shown in Figure 2.6. In comparison with native husk (Figure 2.6a), exposure of husk to water at 85^{0}C for 2 h resulted in no visible change in any of the aspects of the lignocellulosic structure (Figure 2.6b), as discussed above. On the other hand, 10% w/v NaOH caused extensive swelling of the entire fibrillar bundle after 2 h treatment, thus allowing the recognition of individual fibrils (Figure 2.6c). Cellulose birefringence was, however, not affected. When NaOH pretreatment was

carried out for 24 h, defibrillation and extensive cellulose intercrystalline swelling were even more pronounced. Nevertheless, cellulose crystallinity seemed to be completely retained (Figure 2.6d).

The effect of 14.3% w/w H_3PO_4 on the structural features of husk was similar, although less distinctive than that of 10% w/v NaOH (Figure 2.6e). Exposure of husk to 42.5% w/w H_3PO_4 for 24 h at 85°C brought about the most extensive swelling in the husk structure among all H_3PO_4 treatments (Figure 2.6f). The crystalline properties were also slightly changed as evidenced by a decrease in birefringence intensity. Alteration in the colour of reflected light from the husk residue could further imply partial dissolution of the cellulose material. In fact, concentrated H_3PO_4 (85% w/w) is known to solubilize cellulose at room temperature (Jayme and Lang, 1963).

Further characterization of the husk morphology, as affected by the solvent, was carried out by SEM (Figures 2.7 and 2.8). NaOH caused similar morphological alterations when used at two different set of conditions (micrographs are not shown); 5% w/v NaOH/85°C-2 h/ambient pressure and 5% w/v NaOH/121°C-15 min/105 kPa. These findings suggested that high pressure and temperature could be effectively compensated by milder conditions under extended reaction times (120 min vs. 15 min). A low magnification longitudinal view of husk treated with NaOH (Figure 2.7B) illustrates the ability of this solvent to disintegrate densely packed fibrillar bundles into smaller fibrillar assemblies and possibly individual fibrils. The effect is comparable to that shown in Figure 2.5c and it is a consequence of extensive fiber swelling that occurs upon disruption of hydrogen bonding between cellulose molecules. Such

Figure 2.7: Scanning electron micrographs of native corn husk and husk treated with 5% w/v NaOH at 85°C for 2 h.
A. native husk; bar=10 μm.
B. pretreated husk; bar=100 μm.
C. pretreated husk; bar=10 μm.
D. pretreated husk; bar=10 μm.

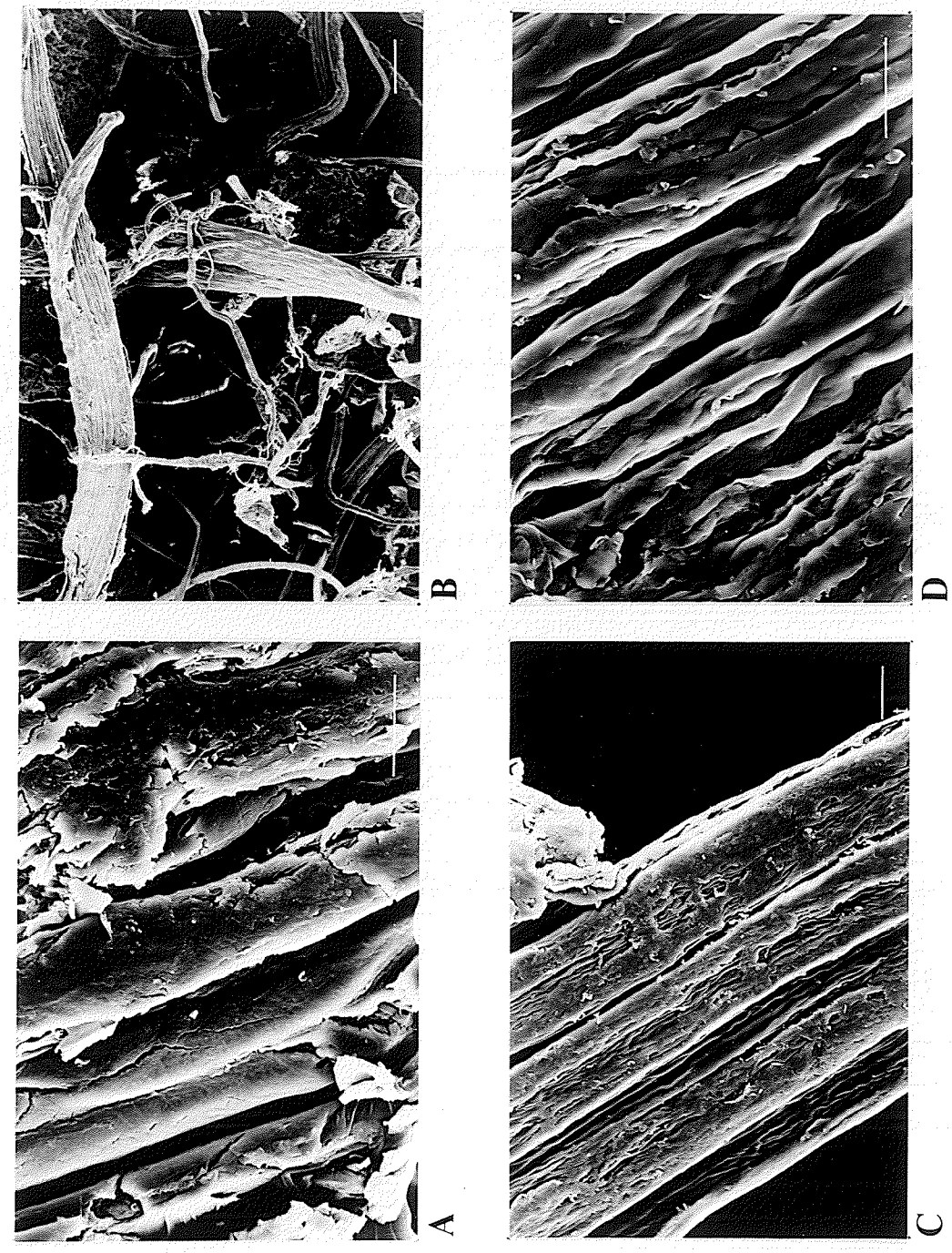
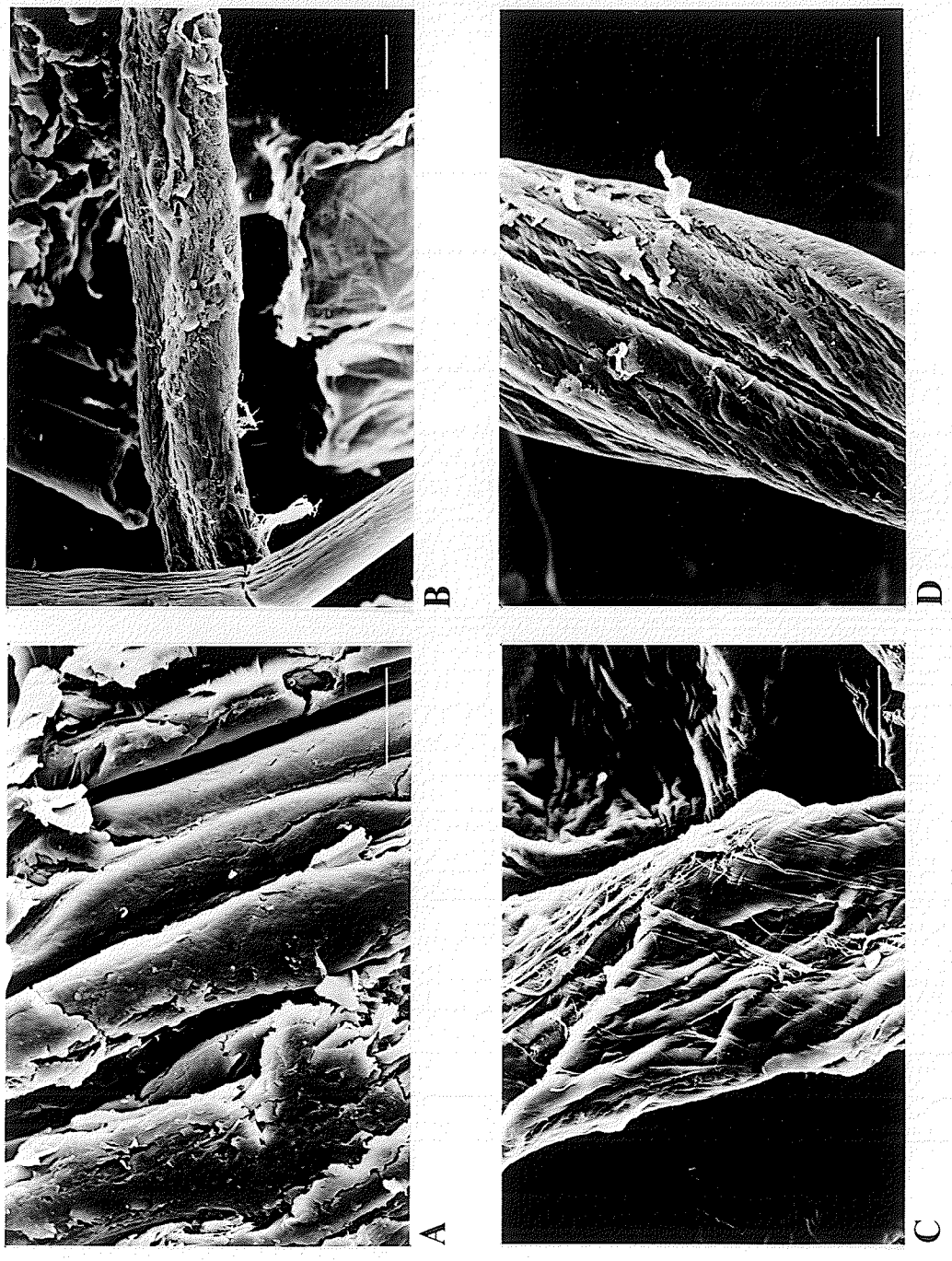

A
B
C
D

Figure 2.8: Scanning electron micrographs of native and chemically pretreated corn husk; bar=10 μm.
A. native husk
B. pretreated husk with 14.3% (w/w) H_3PO_4
C. pretreated husk as in B
D. pretreated husk with 5% (w/w) H_2SO_4, 85^{0}C for 2 h.


B
D
A
C

disorganization would be expected to allow penetration of hydrolytic enzymes and thereby enhance the reactivity and biodegradability of cellulose. The fibrillar surface features as well as the structural regularities within individual fibrils after swelling with NaOH are illustrated in Figure 2.7C. As compared to the untreated sample, which showed relatively smooth surfaces (Figure 2.7A), NaOH caused considerable surface pitting (Figure 2.7C), presumably due to partial removal of matrix material (hemicellulose and lignin). However, the internal fibrillar structure appeared masked by the presence of nonfibrillar material. On the other hand, acid-treated husk fibers exhibited a more pronounced surface erosion as shown by the exposed fiber structural network. This is particularly evident with the H_3PO_4-treated samples (Figure 2.8B,C) where the primary cell wall seemed to be removed. There were no apparent differences in the specimens after H_3PO_4 (14.3% w/w) treatement at 50°C for 72 h and 85°C for 2 h as viewed by SEM. Both H_2SO_4 and H_3PO_4 appeared to affect the fiber surface in a similar way, thus exposing the three-dimensional organization of the fiber network.

In conclusion, the SEM studies suggested that acids are more effective in degrading the matrix material, while NaOH tends to swell the entire structure and thus disintegrate it into smaller fibrillar elements.

2.3.4 Thermal degradation studies of chemically pretreated husk

The pyrolytic decomposition of residual corn husks following some selected chemical pretreatments was investigated using DSC and TGA. The chemical composition and degree of crystallinity of husk residues examined by thermal analysis are presented in Table 2.5. The effect of

TABLE 2.5

Chemical composition and crystallinity index of corn husk residues.

Sample	Pretreatment (solvent/temp.-time)	Residue (% wt)	Hemicellulose (%)	Cellulose (%)	CrI (%)
(i)	Native husk	100	45	39	51
(ii)	1.0% w/w H_2SO_4/55°C - 2 h	90	44	41	57
(iii)	5.0% w/w H_2SO_4/85°C - 2 h	47	8	69	61
(iv)	14.3% w/w H_3PO_4/50°C - 72 h	66	28	49	59
(v)	0.5% w/v NaOH/121°C - 15 min	57	29	58	61
(vi)	5.0% w/v NaOH/121°C - 15 min	36	13	75	67

chemical pretreatments on the thermal behavior of corn husk residues along with the representative DSC decomposition curves of two commercial samples of xylan and α-cellulose (used for comparative purposes) are illustrated in Figure 2.9. The general appearance of all profiles obtained indicated that the recorded thermal events of the pyrolytic processes were exothermic. The peak temperatures [I, II, and III, as specified in curve (i)] for all samples are presented in Table 2.6. The thermal curve of native husk (i) is characterized by three exothermic peaks at 238, 317, and 450°C, respectively. Interestingly, the peak temperatures of the first two transitions corresponded well to those of xylan (240°C) and α-cellulose (317°C). Since single exotherms were exhibited by both xylan and α-cellulose, the peaks I and II of sample (i) most likely reflect the decomposition processes of the hemicellulose and cellulose constituents in native husk. Pretreatment of husk with both alkali or acid resulted in solubilization or hydrolysis of the hemicellulose component as evidenced by the chemical composition of the residual solids (Table 2.5). These results indicated that selective removal of the hemicellulose fraction is dependent on the conditions employed during pretreatment; i.e., it increases with temperature and time of the treatment as well as with the concentration of the solvent used. From the DSC thermal curves (Figure 2.9), it was also evident that there was a decrease in the magnitude of the transition I after removal of the hemicellulose fraction during solvent pretreatment. On the other hand, the size of transition II increased, as expected, because of enrichment of the residue in cellulose. The reaction enthalpies associated with the decomposition of the individual constituents were not measured due to the convolution of peaks I and II. Following

Figure 2.9: Typical DSC thermal curves for the pyrolytic degradation of xylan, α-cellulose, and corn husk residues.

Samples weights from top to bottom (mg): 3.50, 3.58, 3.56, 3.20, 3.43, 3.67. Samples i, iv, iii, and vi as designated in Table 2.5. Heating rate $10^{\circ}C\ min^{-1}$.

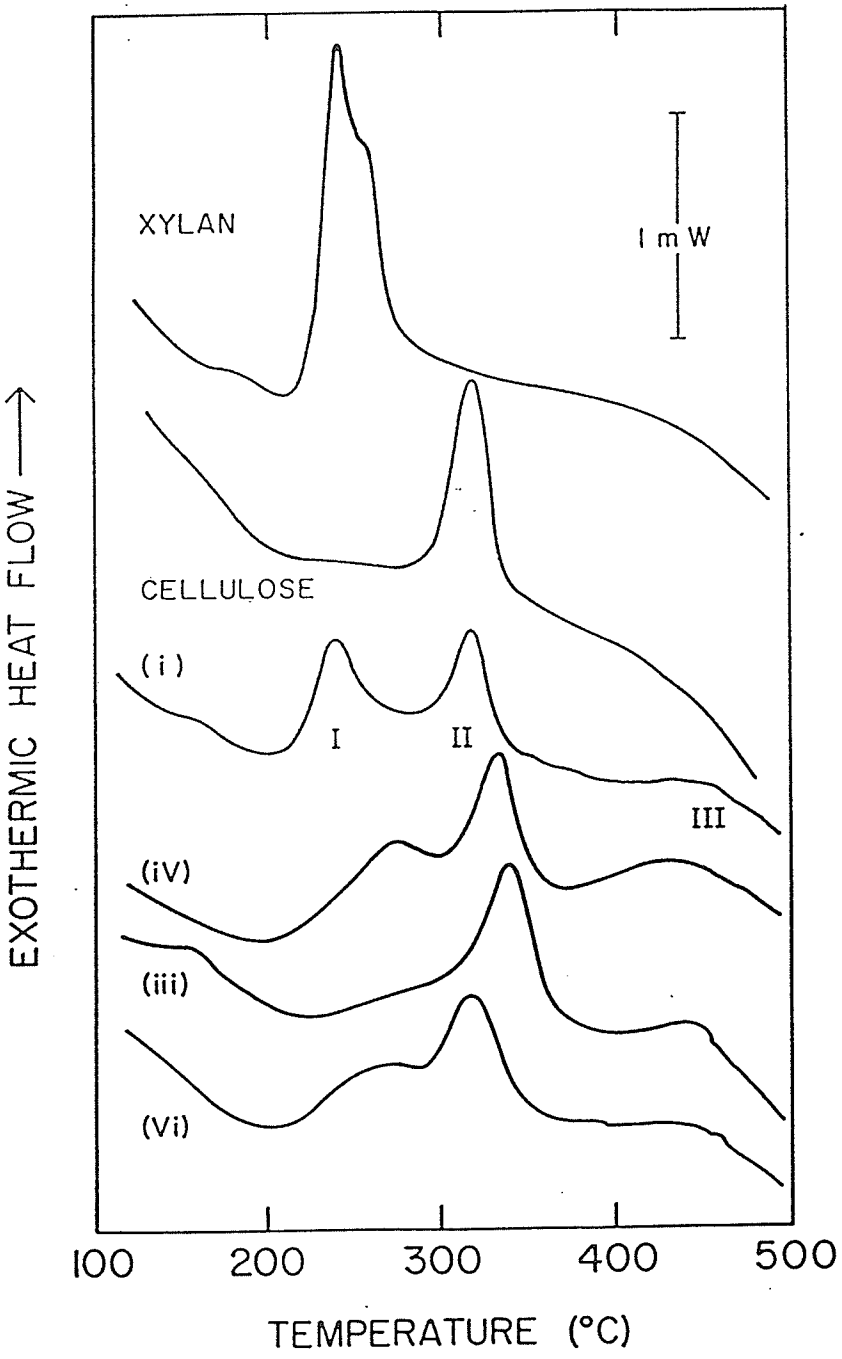

XYLAN
1 m W
CELLULOSE
(i)
I
II
III
(iV)
(iii)
(Vi)
EXOTHERMIC HEAT FLOW ⟶
100
200
300
400
500
TEMPERATURE (°C)

TABLE 2.6

Thermal analysis data (DSC, TGA) of α-cellulose, xylan and corn husk residues.

Sample[1]	DSC exoth. peak temp. (°C)			Kinetic parameters (TGA)				
	I	II	III	Temp. range[2] (°C)	Ea[2] (kJ/mole)	n[2]	Temp. range[3] (°C)	Ea[3] (kJ/mole)
(i)	238	317	450	295-327	59 (-0.96)[4]	0.97	295-327	56 (-0.99)
(ii)	266	334	437	292-325	95 (-0.97)	0.87	301-334	83 (-0.97)
(iii)	-	339	441	299-325	152 (-0.99)	0.99	302-334	135 (-0.99)
(iv)	275	332	434	290-326	86 (-0.98)	0.85	290-326	102 (-0.99)
(v)	232	315	438	303-326	101 (-0.94)	0.87	303-333	117 (-0.98)
(vi)	249	318	436	303-321	166 (-0.99)	0.94	303-331	147 (-0.99)
α-cellulose	-	317	-	326-346	178 (-0.98)	0.98	326-346	195 (-0.99)
xylan	240	-	-	263-296	148 (-0.99)	0.97	263-292	120 (-0.99)

[1] Sample (i) - (vi) as designated in Table 2.5.

[2] Freeman and Carroll equation.

[3] Broido's equation.

[4] Numbers in parentheses are the correlation coefficients for the corresponding kinetic plots.

the second transition, a small exotherm (III) was observed at 430-460°C for most of the corn husk residues. Secondary decomposition/charing reactions of cellulose and/or thermal degradation of lignin could account for this peak.

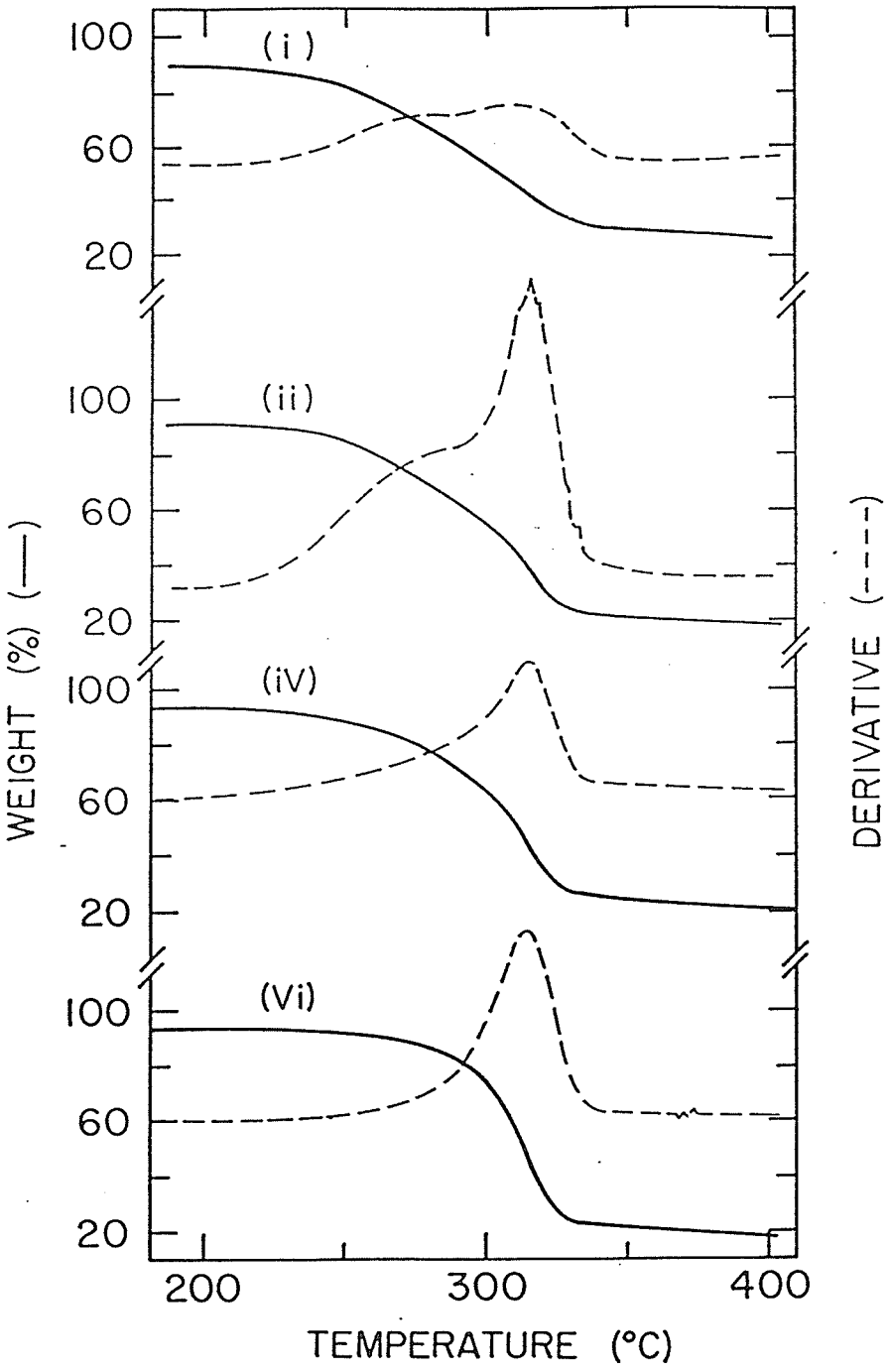
Another interesting aspect of the DSC curves, within the series of samples examined, has been the response of the transition II peak temperature to the various chemical pretreatments. Hydrolysis of corn husk residues with acids imparted a marked increase (15-22°C) in the temperature of this transition as shown in Table 2.6 (samples ii-iv). On the other hand, alkali treatment had no significant effects on the pyrolysis temperature of the cellulose component (samples v and vi). These results could be explained by considering the semicrystalline nature of native cellulose and the action mechanism of the various solvents employed on the lignin-hemicellulose-cellulose complex during pretreatment. In this respect, after heterogeneous acid prehydrolysis, a significant increase in the crystalline material of the solid residues would be expected since the less ordered regions are amenable to acid hydrolysis. This, in turn, could elevate the onset of the thermal decomposition to higher temperatures and decrease the pyrolysis rate, as suggested by Basch and Lewin (1973a,b) and Cabradilla and Zeronian (1976). In contrast to acid treatments, alkali causes substantial delignification and inter- and intra-crystalline swelling of the cellulose fibers, besides hemicellulose extraction. Under alkali conditions, however, cleavage of glycosidic bonds in the intercrystalline region is expected to be minimal. Since the cellulose component of these samples did not show any changes in thermal resistance (transition II tempera-

tures, Table 2.6), it is reasonable to assume that the presence of the amorphous regions, which decompose at lower temperatures (Sefain and El-Kalyoubi, 1984), influences the pyrolytic behavior of the entire cellulosic structure.

The thermal properties of corn husk lignocellulosics were also investigated by TGA. Characteristic TGA thermal curves (both weight loss and first-derivative profiles) are shown in Figure 2.10. All samples exhibited a slight weight loss (7-10%) due to elimination of physically adsorbed water below 100°C, a very slight gradual loss in weight between 100 and 250°C, and major losses due to thermal decomposition between 280 and 340°C. Finally, there was a slight decrease in weight in the region of 340-450°C, indicative of further decomposition reactions involving char formation. For native husk, the major weight loss appeared to take place in two distinct consecutive stages, as shown by the two inflections in the rate of weight loss curves (i.e., derivative TGA). It is suggested that this thermal behavior mainly reflects the decomposition of hemicellulose and cellulose components occurring at two different temperature regions. The effects of chemical pretreatments were also apparent in the TGA decomposition profiles. For example, decreased proportions of the hemicellulose fraction, due to chemical pretreatments, were reflected in the TGA curves by a shift in the onset of the pyrolytic events toward higher temperatures. Furthermore, the major weight losses were confined within a narrower temperature range and thus yielded a sharper decline in the main stage of the thermal decomposition process. These effects were particularly evident with samples iii and vi; their derivative TGA curves also showed a single peak, as one would anticipate with one-component (cellulose) system.

Figure 2.10: Typical TGA thermal curves for the pyrolytic degradation of corn husk residues, weight loss (%) and first-derivative, dx/dt (arbitrary units).

Sample weights from top to bottom (mg): 15.99, 16.62, 15.25, 15.43. Samples i, ii, iv, and vi as designated in Table 2.5. Heating rate $1.5^{0}C\ min^{-1}$, flow rate of dry N_2 120 $cm^3\ min^{-1}$.


100
60
20
(i)
100
60
20
(ii)
100
60
20
(iV)
100
60
20
(Vi)
200
300
400
TEMPERATURE (°C)
WEIGHT (%) (——)
DERIVATIVE (----)

Kinetics of thermal decomposition of husk cellulosics were determined from dynamic thermogravimetric data by employing the equations of Broido (1960) plus Freeman and Carroll (1958). Representative plots of the treated data according to these methods are shown in Figures 2.11 and 2.12. Table 2.6 presents estimates of the kinetic parameters (activation energy Ea and reaction order n) as well as the temperature range of the data used for kinetic treatments. It should be pointed out that kinetic analysis for each particular sample was based on the data collected in the temperature range over which the major weight loss occurred. Consequently, it varied from one to another sample depending on its susceptibility to pyrolysis which in turn was related to the chemical pretreatment applied. As can be seen, plots for both equations gave significant linear relationships: $r=-0.94$ to -0.99, $p<0.005$ (Freeman and Carroll); $r=-0.98$ to -0.99, $p<0.001$ (Broido). The actual data treatment for calculating the kinetic parameters of a representative sample is shown in Appendix A. Despite the complexity of all reactions involved, the pyrolytic decomposition of all samples appeared to obey first-order kinetics as indicated by the values of n, which were within 0.85 and 0.99; note that the extrapolated intercepts of plots (n) in Figure 2.12 are near unity. The values for the apparent activation energy of α-cellulose and xylan were 195 and 120 kJ mol^{-1}, respectively. From Table 2.6, it is also obvious that Ea increased considerably after chemical pretreatment. The higher values derived for the pretreated samples most likely reflect the structural/compositional differences of these materials; i.e., removal of hemicellulose, preferential hydrolysis of amorphous cellulose and structural modifications of cellulose crystallites. The relationships between X-ray crystallinities and TGA thermal parameters were also explored. Plots of crystallinity (%)

Figure 2.11: Plots of Ln[Ln(1/y)] vs. $10^3/T$ (K^{-1}) using Broido's equation for the pyrolytic degradation of corn husk residues.

Samples i, iv, iii, and vi as designated in Table 2.5.

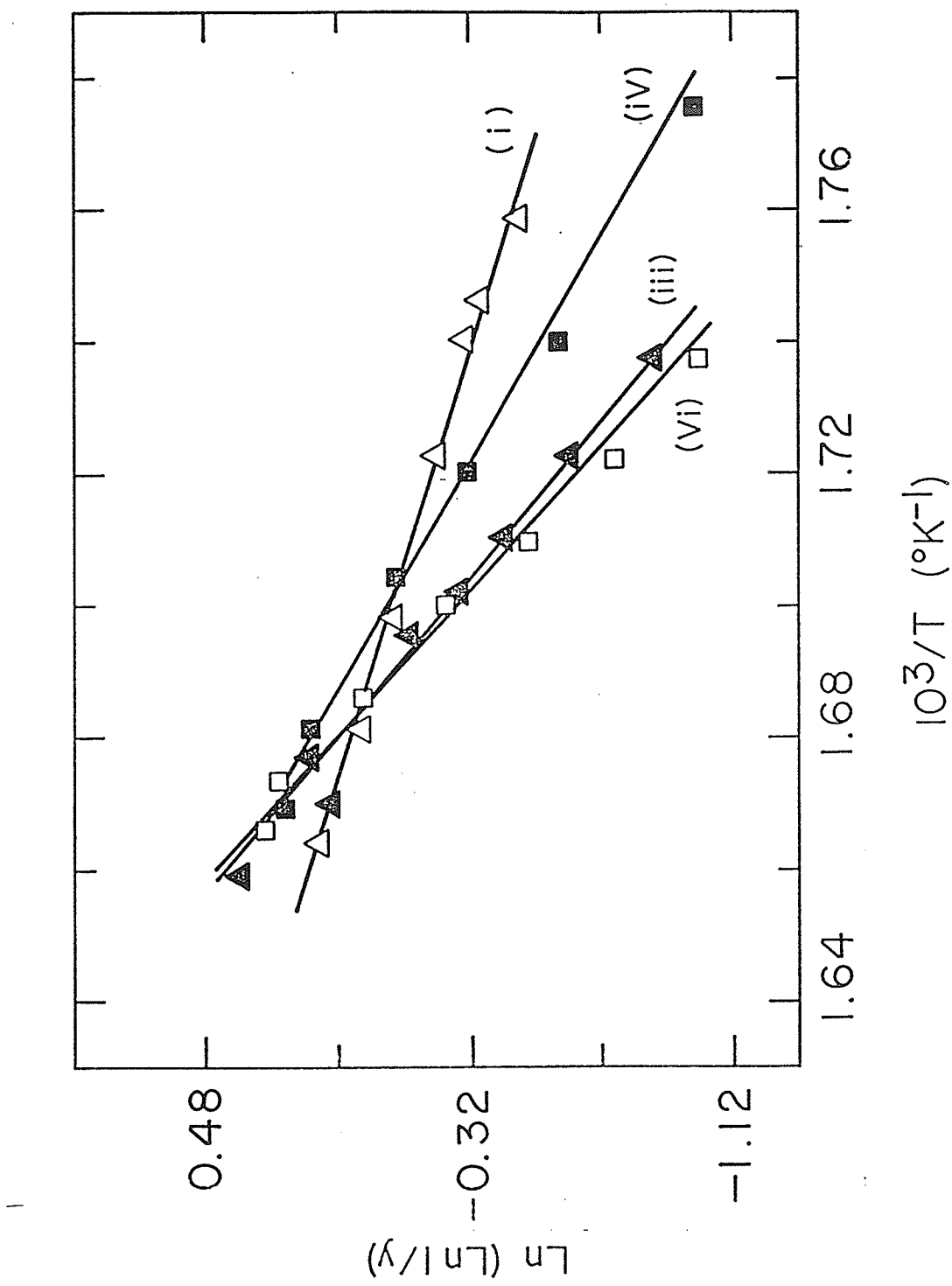
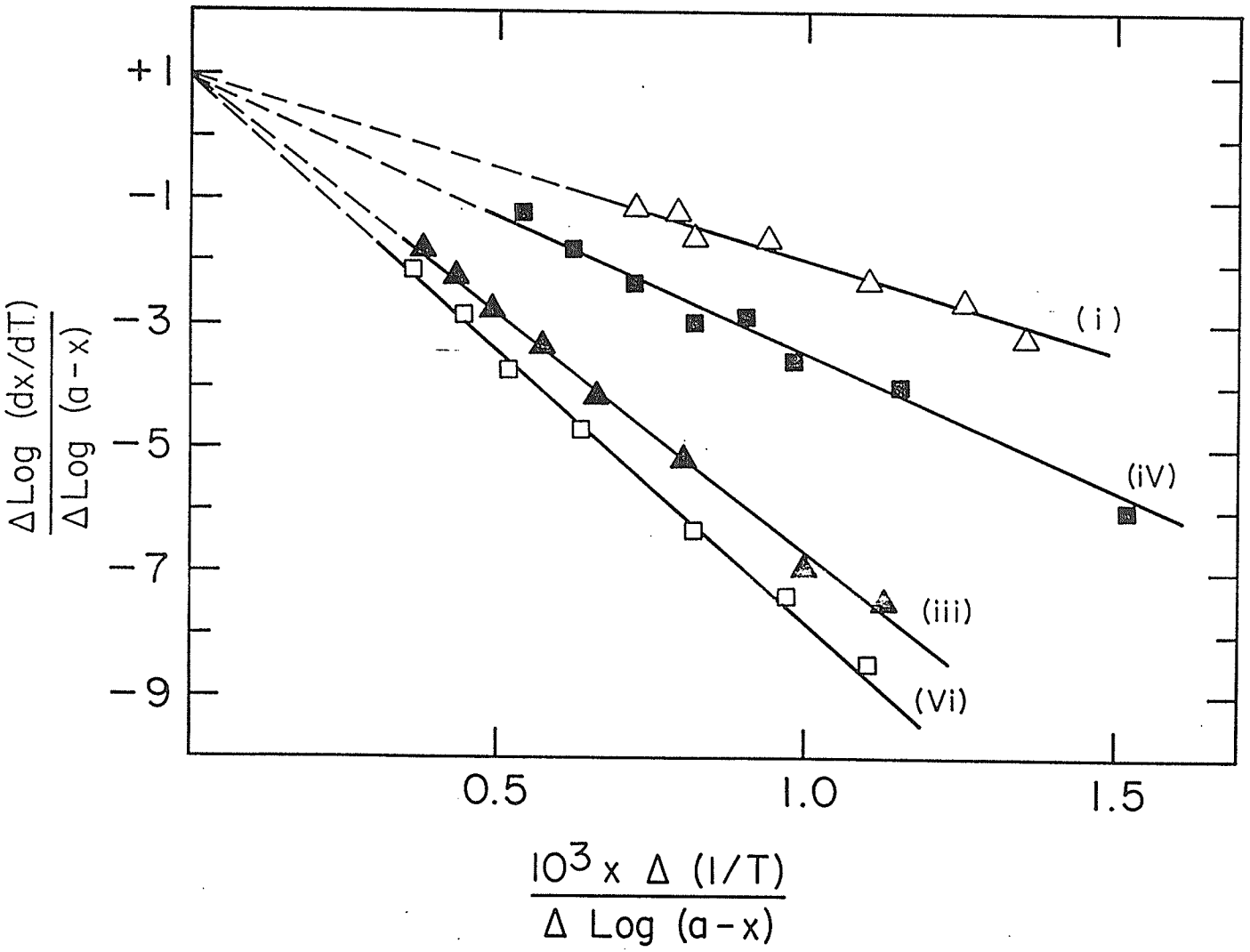

0.48
-0.32
-1.12
Ln (Ln 1/y)
1.64
1.68
1.72
1.76
$10^3/T$ (°K-1)
(i)
(ii)
(iii)
(vi)

Figure 2.12: Plots of $\Delta \log(dx/dT)/\Delta \log(a-x)$ vs. $10^3\Delta(1/T)/\Delta \log(a-x)$ using the Freeman and Carroll equation for the pyrolytic degradation of corn husk residues.

Samples i, iii, iv, vi as designated in Table 2.5.


+1
−1
−3
−5
−7
−9
0.5
1.0
1.5
(i)
(iV)
(iii)
(Vi)
ΔLog (dx/dT) / ΔLog (a−x)
10³ x Δ (1/T) / Δ Log (a−x)

versus maximum rate of weight loss, temperature at which the first 10% loss in weight of the dried cellulosic material occurs, and apparent activation energy (Broido, 1969) are shown in Figure 2.13. With regard to Ea, as the degree of crystallinity increases, there is a concomitant increase in activation energy. Linear regression analysis for these plots yielded correlation coefficients of 0.98 and 0.93 for the maximum rate of weight loss (dx/dt) and the temperature at 10% weight loss, respectively. High values for the latter two parameters are indicative of selective enrichment of the samples in cellulose (i.e., the system becomes more homogeneous) as a result of the chemical pretreatment(s).

2.3.5 Molecular weight distribution of CSF by gel filtration

The objective of this study was to examine the carbohydrate composition (pentose- versus hexose-containing oligosaccharides) as well as the molecular weight distribution of the solubilized carbohydrate material after various chemical pretreatments. Total carbohydrates (TC), pentose-containing carbohydrates (PC) and the PC/TC ratios for four different prehydrolyzates are presented in Table 2.7. All solvents examined solubilized selectively PC, as suggested by the high PC/TC values (85.8-92.8%). This is indicative of the susceptibility of the hemicellulose component to extraction/degradation by either acid or alkali yielding mainly arabinoxylan oligomers. These findings are consistent with the data reported in previous studies on composition of hardwood and wheat straw hydrolyzates (Yu et al., 1984a; Cunningham and Carr, 1984). There were also differences in the capacity and selectivity of the solvents in solubilizing the carbohydrate material of

Figure 2.13: Relationships between crystallinity index (%) and TGA kinetic parameters: maximum rate of weight loss (dx/dt,o), activation energy (Broido's equation, Δ), temperature at 10% weight loss (●).

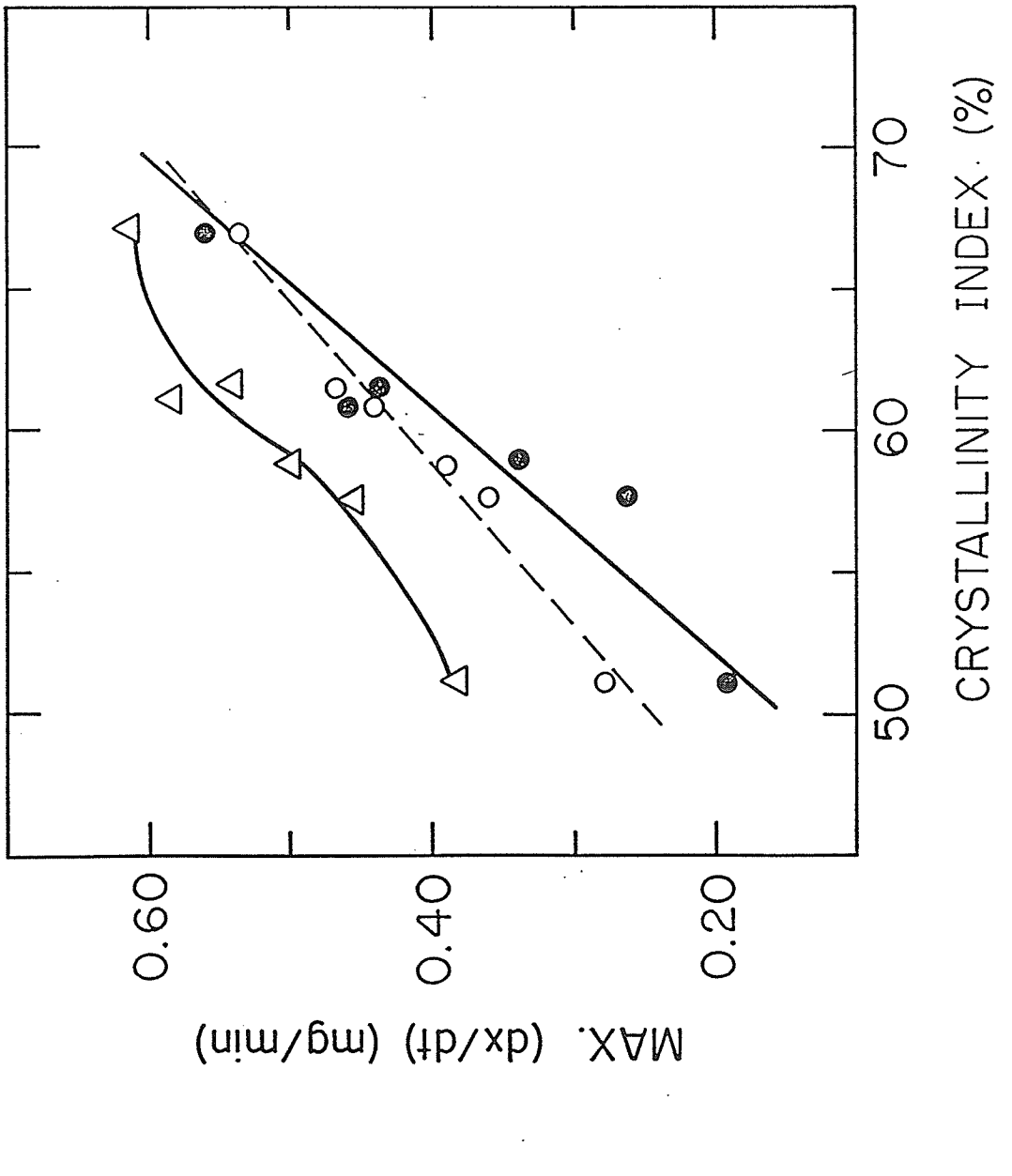

MAX. (dx/dt) (mg/min)
0.60
0.40
0.20
50
60
70
CRYSTALLINITY INDEX. (%)
290
270
250
Temp. at 10% wt. Loss (°C)
150
100
50
Act. Energy , Ea (kJ/mol)

TABLE 2.7

Chemical analysis of prehydrolyzates obtained by treatment of corn husk with various solvents at 85°C for 2 h.

Solvent	TC[1] ($mg.mL^{-1}$)	PC[2] ($mg.mL^{-1}$)	PC/TC ratio (%)
5.0% w/v NaOH	4.07 (32.8)[3]	3.77 (56.5)[4]	92.8
5.0% w/w H_2SO_4	2.82 (22.7)	2.42 (36.3)	85.8
5.0% w/w H_3PO_4	1.09 (9.0)	0.94 (14.1)	86.6
14.3% w/w H_3PO_4	2.97 (23.9)	2.57 (38.5)	86.4

[1] Concentration of total carbohydrates (TC) of prehydrolyzate as determined by the orcinol-sulfuric acid (Miller et al., 1960) and using xylose as standard.

[2] Concentration of pentose-containing oligosaccharides (PC) of prehydrolyzate as determined by the Bial reaction (Dische, 1962) and using xylose as standard.

[3] Numbers in parentheses represent the amount of TC in the prehydrolyzate as percent of the carbohydrate content of husk.

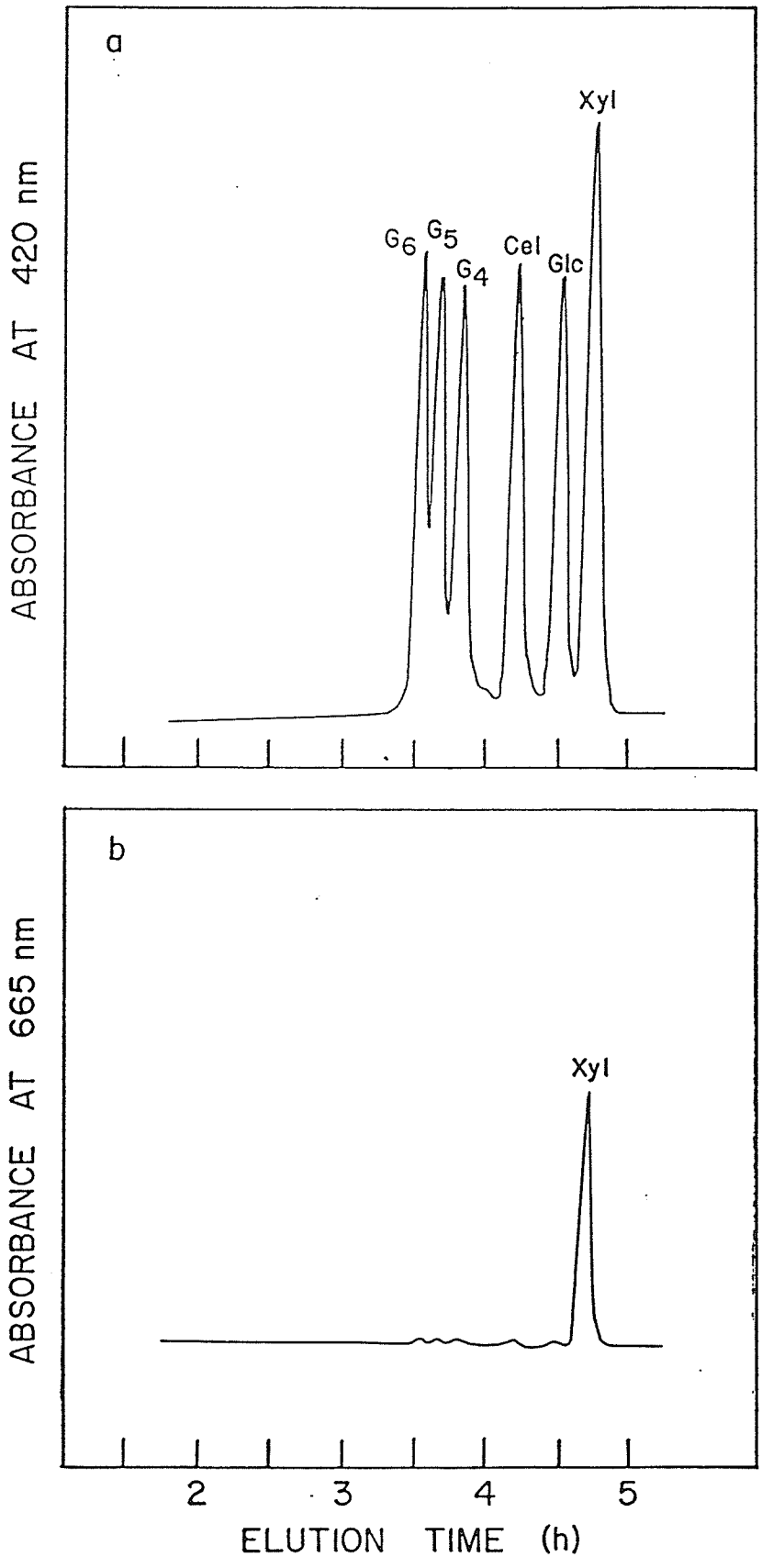
[4] Numbers in parentheses represent the amount of PC in the prehydrolyzate as percent of the hemicellulose content of husk.

husk. In this respect, NaOH was more effective in extracting both TC and PC than H_2SO_4 and H_3PO_4 under the experimental conditions of this study. Furthermore, 14.3% w/w H_3PO_4 exhibited equal hydrolyzing potential to 5% w/w H_2SO_4, as shown by the yield values of the liberated soluble carbohydrates (approx. 2.9 $mg.mL^{-1}$) given in Table 2.7.

The molecular weight distribution of the components of CSF as specified in Table 2.7, was also investigated by gel filtration chromatography. Both orcinol-sulfuric acid, a non-specific carbohydrate reagent, and the Bial reagent, specific for pentose-containing carbohydrates, were used to characterize and quantitate the gel filtration fractions. It must be pointed out here that, since pure standards of the xylo-oligosaccharide series were not available, column calibration was carried out by chromatography of well characterized maltooligosaccharides. Representative elution profiles of the standard carbohydrate mixture containing xylose, glucose, cellobiose, and malto-oligosaccharides are shown in Figure 2.14. Considering that the nature of the monomeric units as well as the type of linkages in the oligosaccharide molecules determine their hydrodynamic volume and thus their retention times, xylo-oligosaccharides would not be expected to have equal elution times with the corresponding malto-oligosaccharides of identical $\overline{DP}$s. Nevertheless, due to the consistency of the linkage (β-1,4), at least in the linear main chain of the oligomers, it seems plausible to suggest that the sequence of the eluted carbohydrate components would correspond to their degree of polymerization. An effective separation of both standards and husk solubilized carbohydrates was achieved using the Fractogel TSK HW-40(S) in relatively short time (5

Figure 2.14: Gel filtration elution profiles of the standard carbohydrate mixture containing xylose, glucose, cellobiose, maltotetraose, maltopentaose and maltohexaose on a Fractogel TSK HW-40(s) column eluted with water. Post-column derivatization and carbohydrate analysis was performed by orcinol-sulfuric acid (a) and Bial (b) reagents.

Xylose (Xyl), glucose (Glc), cellobiose (Cel), maltotetraose (G_4), maltopentaose (G_5) and maltohexaose (G_6). The Fractogel TSK HW-40(s) column (145x0.9 cm) was eluted with water (70°C, flow rate 17 $mL.h^{-1}$).


a
Xyl
G6
G5
G4
Cel
Glc
ABSORBANCE AT 420 nm
b
Xyl
ABSORBANCE AT 665 nm
2
3
4
5
ELUTION TIME (h)

h). This gel exhibited good resolution, as evidenced by the chromatogram of Figure 2.14a; even glucose and xylose were eluted as narrow peaks. The specificity of the Bial reagent for assaying pentose-containing carbohydrates in the eluate is demonstrated in the chromatogram of Figure 2.14b; a single peak, corresponding to xylose, was detected under the standard chromatographic conditions and post-column derivatization conditions employed.

The elution profiles of CSF following NaOH, H_2SO_4 and H_3PO_4 pretreatments are shown in Figures 2.15 and 2.16, while the percent distributions of the eluted carbohydrate components through the Fractogel TSK HW-40(s) column are summarized in Table 2.8. When compared to the acidic materials, alkaline CSF exhibited different distribution. A high MW pentose-based carbohydrate fraction, excluded from the gel, constituted the main component of the NaOH extract. On the other hand, low MW oligosaccharides dominated the elution profiles of the acidic CSFs. These findings are consistent with the view that glycosidic linkages in cellulose and hemicellulose are susceptible to acid-catalyzed hydrolysis but remain relatively intact upon alkali treatment under mild conditions of extraction (Nevell, 1985). In addition to the high MW carbohydrate fraction, small amounts (5.7% of the solubilized material) of a hexose based tetrasaccharide were detectable in the elution profile of the NaOH extract (Figure 2.15a). This constituent might have been present, as such, in the initial tissue and thus extracted by the alkali. It is also apparent from the chromatograms of Figures 2.15(c,d) and 2.16 that considerable amounts of xylose and low MW xylodextrins were released during acid hydrolysis. Between H_2SO_4 and H_3PO_4 hydrolyzates, the

Figure 2.15: Gel filtration elution profiles of husk prehydrolyzates following NaOH (5%w/v, 85°C, 2 h) (a and b) and H_2SO_4 (5%w/w, 85°C, 2 h) (c and d) pretreatments on Fractogel TSK HW-40(s) column.

Chromatograms a and c refer to total carbohydrates, b and d to pentose-containing carbohydrates; $\overline{DP}$ refers to the average degree of polymerization of the components detected. Chromatographic conditions as in Figure 2.14.

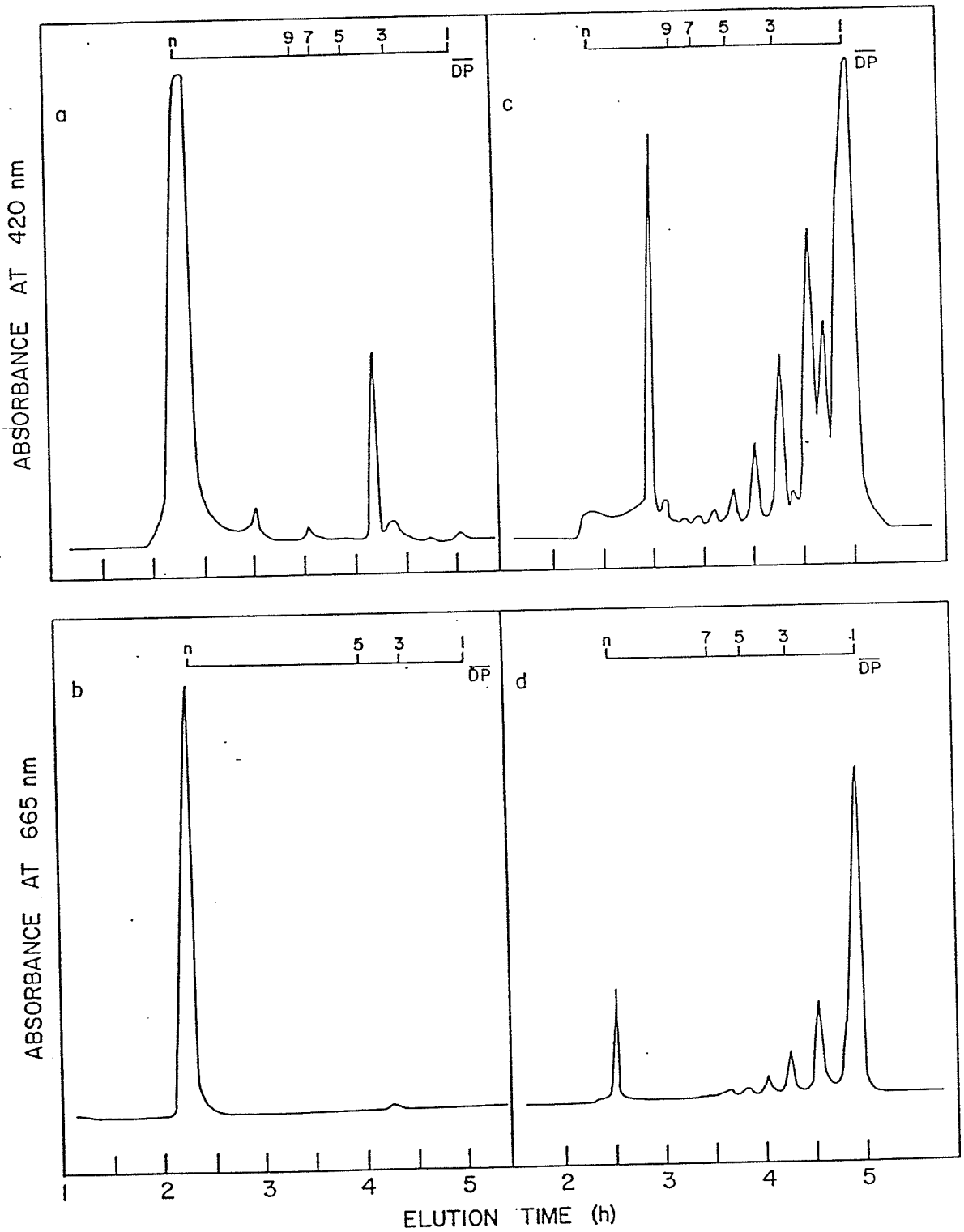

a
c
b
d
n
9 7 5 3 1
5 3 1
7 5 3 1
DP
ABSORBANCE AT 420 nm
ABSORBANCE AT 665 nm
1 2 3 4 5
2 3 4 5
ELUTION TIME (h)

Figure 2.16: Gel filtration elution profiles of husk prehydrolyzates following 14.3% w/w H_3PO_4 (85°C, 2 h) (a and b) and 5.0% w/w H_3PO_4 (85°C, 2 h) (c and d) pretreatments on Fractogel TSK HW-40(s) column.

Chromatograms a and c refer to total carbohydrates, b and d to pentose-containing carbohydrates; $\overline{DP}$ refers to the average degree of polymerization of the components detected. Chromatographic conditions as in Figure 2.14.

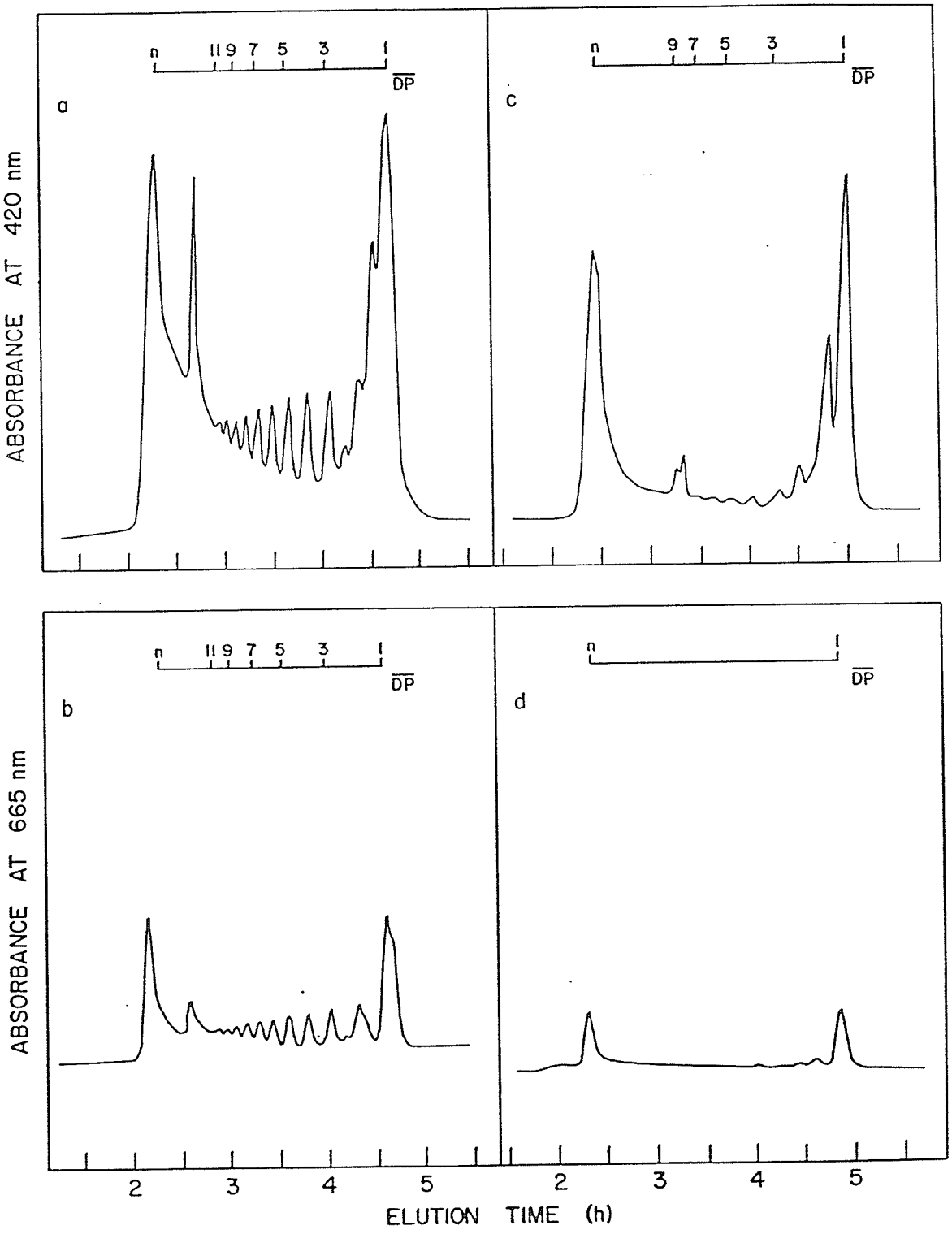

a
n 11 9 7 5 3 1
DP
c
n 9 7 5 3 1
DP
b
n 11 9 7 5 3 1
DP
d
n 1
DP
ABSORBANCE AT 420 nm
ABSORBANCE AT 665 nm
2 3 4 5
2 3 4 5
ELUTION TIME (h)

TABLE 2.8

Percent distribution of total carbohydrates and pentose-containing fractions of chemically solubilized/prehydrolyzed husk material (85°C/2 h).

$\overline{DP}$ of carbohydrate	Relative amount of each product							
	5% w/v NaOH		5% w/w H_2SO_4		5% w/w H_3PO_4		14.3% w/w H_3PO_4	
	I[1]	II[2]	I	II	I	II	I	II
1	0.5	-	50.1	64.3	41.9	41.1	27.5	22.2
2	-	-	15.4	14.6	3.3	8.0	6.1	8.4
3	2.6	-	7.3	6.6	2.4	4.0	4.4	4.8
4	5.7	0.9	3.9	3.4	1.7	2.9	4.4	4.5
5	-	-	2.0	1.9	1.6	trace[3]	3.7	4.1
6	-	-	1.0	1.1	1.4	-	3.4	3.7
7	0.5	-	0.8	-	1.6	-	3.2	3.4
8	-	-	0.6	-	-	-	2.7	3.1
9	-	-	0.5	-	4.2	-	2.1	2.6
10	-	-	0.8	-	-	-	2.1	2.1
11	1.8	-	10.6	-	13.2	-	1.9	2.3
12	-	-	-	-	-	-	trace	trace
>12	88.9	99.1	6.5	8.0	28.6	43.8	38.3	38.6

[1] Total carbohydrates as determined by the orcinol-sulfuric acid reagent (Miller et al., 1960).

[2] Pentose-containing carbohydrates as determined by the Bial reagent (Dische, 1962).

[3] Trace refers to amounts <0.3%.

zates, the former exhibited a narrower range in $\overline{DP}$ for the eluted components which implies a higher degree of depolymerization of husk hemicelluloses. Furthermore, the profile of the H_2SO_4-CSF revealed that the amount of high MW fragments, above the gel exclusion limit, is very low as compared to their H_3PO_4 counterparts (Figure 2.16; Table 2.8).

2.4 DISCUSSION

2.4.1 Relationships between type of chemical pretreatment and some structural and compositional features of residual husk lignocellulosics

Concentration and type of solvent as well as reaction temperature, pressure and time were some of the parameters varied in the optimization studies of chemical pretreatments of husk, as discussed in sections 2.3.1 and 2.3.2. Based on the solubilization data (Tables 2.2, 2.3 and 2.4) and the microscopic observations on treated husk residues (section 2.3.3), it appears that high pressure and temperature could be effectively compensated by milder conditions over extended reaction times; e.g. treatments for 15 min in the autoclave vs. 120 min at room temperature. Moreover, there have been indications from our preliminary studies and those published by Puri and Pearce (1986) that the reaction time could be reduced even further with no apparent impact on the final digestibility of the lignocellulosic substrate. Therefore, such pretreatments seem attractive because of the relatively mild operating conditions, simplicity and low energy intensiveness.

NaOH at 5% w/v was found to be superior, among the solvents examined, in solubilizing the husk material (Figures 2.1, 2.2 and 2.3). This may be attributed to the disruption of the cellulose-lignin-hemicellulose complex and disintegration of fiber bundles by swelling (Figure 2.6) along with alkali-mediated reactions involving individual components of the complex. Depolymerization of cellulose, hemicellulose and lignin are known to take place simultaneously under alkaline conditions (Lachenal and Monzie, 1985). Degradation and dissolution of lignin occur mainly through reactions at its phenolic groups, causing rupture

of phenolic ether bonds, which are alkali sensitive. Hydrolytic and condensation reactions can also take place (Adler, 1977). The extent of lignin solubilization is largely dependent on the concentration of alkali and temperature; more severe conditions promote more extensive delignification (Puri and Pearce, 1986). Depolymerization of hemicellulose and cellulose occur mainly through end-group "peeling" reactions (Johansson and Samuelson, 1977; Kenner and Richards, 1957). It has been also recognized that by proper selection of operating conditions the extent of both lignin and hemicellulose degradation could be minimized in order to avoid accumulation of toxic non-carbohydrate condensation products (Mes-Hartree and Saddler, 1983). As shown in Figure 2.1, changes in NaOH concentration resulted in more pronounced solubilizing action than temperature increases. The observed trends in alkali concentration dependence of the solubilized material might be related to swelling of cellulose, although the latter does not necessarily imply dissolution. Zeronian and Cabradilla (1973) reported similar findings on cotton. A progressive increase in swelling was evident up to 4N NaOH at 21°C and did not further change by additional increase in alkali concentration. However, for a given concentration of NaOH, the degree of swelling decreased with increasing temperature. For example, at 3N NaOH the maximum extent of swelling was observed at 0°C, whereas at concentrations of 4N and 5N, the corresponding maxima were at 25°C and 100°C, respectively. It is expected that swelling of cellulose would make the lignin and hemicellulose components of the husk more accessible as well as expose capillary surfaces of the cellulose structure. This, in turn, should permit accelerated diffusion of solvent or enzyme into the molecule as well as the removal of degradation products from the complex. However, even under the most severe pretreatment conditions of

Kraft cooking (Sundquist, 1985), there is a considerable amount of residual lignin in the form of cross-linked lignin and/or lignin-carbohydrate complexes.

The x-ray diffraction measurements on the solid husk residues revealed changes in crystallinity. In addition to the enrichment of the sample in cellulose, due to selective removal of the hemicellulose component, there also may have been an increase in crystallinity due to cellulose recrystallization. This latter effect has been reported by Watherwax (1977) and Zeronian (1985). It has been hypothesized that this arises from the formation of intermolecular H-bonds between mobile cellulose chain segments in the swollen structure after the removal of lignin and hemicellulose (Fan et al., 1980). In this respect, it has been postulated that crystallization is mediated by water plasticization of the amorphous cellulose (Betrabet and Paralikar, 1978).

Wet milling plus delignification (Dale, 1985), disk refining plus ammonia as well as attritor milling with organosolv (Detroy et al., 1980) have been claimed as effective pretreatments for release of crystalline aggregates of cellulose fibrils and structural alterations of cellulose. The synergistic effect obtained by combining mechanical and chemical pretreatments could be explained by the simultaneous alleviation of more than one of the barriers to heterogeneous hydrolysis of native lignocellulosic residues. The data obtained in this study when 5% w/v NaOH was combined with several mixing treatments at room temperature for a relatively short time (Table 2.3) are in good agreement with such a postulate. For example, husk when treated with 5% w/v NaOH in the ultrasonicator for 15 min was solubilized to an extent of 58.6% and gave a residue with CrI=67%. Comparable results were obtained when the

sample was treated with the same solvent for 15 min in the autoclave (Table 2.2) or 2 h at 85°C (Table 2.4). A more detailed techno-economical analysis would be necessary, however, to show which one of the above processes is the least energy intensive and feasible from a practical viewpoint.

As compared to NaOH, H_2SO_4 exhibited limited solubilization power, particularly at temperatures below 55°C and concentrations lower than 1% (Figure 2.2). At higher solvent concentrations, however, the extent of solubilization was more temperature-dependent. For example, treatment with 5% w/w H_2SO_4 gave a solubilization curve with a steep slope in the range of 55-85°C (Figure 2.2). These effects were even less pronounced with H_3PO_4 of similar concentrations, presumably because of its lower dissociation constant. Nevertheless, the use of H_3PO_4, as compared to much stronger acids, has the advantage of reduced coproduction of undesirable degradation products of lignin and hemicellulose (i.e., furfural and phenolic derivatives) which are inhibitory to the growth and fermentation ability of xylose-utilizing yeasts (Fontana et al., 1984).

The effects of temperature on husk solubilization by acids can be explained considering the mechanism of acid hydrolysis, which is thought to involve the catalytic scission of the glycosidic bonds by H_3O^+ ions and temperature, with higher temperatures promoting rapid reaction rates (Be Miller, 1967). Secondly, disordered regions readily take up H_3O^+, thus being more prone to acid hydrolysis. This explains the preferential hydrolysis of hemicellulose by acids. In contrast, most of the glycosidic linkages in cellulose, due to its supramolecular crystalline structure, are inaccessible to H_3O^+. Therefore, acid hydrolysis is

confined to the crystallite surfaces and the intercrystalline amorphous junction zones. Clausen and Gaddy (1983) have recently reported similar trends regarding temperature and solvent concentration effects on corn stover hydrolysis by strong mineral acids. They have also noted that formation of furfural derivatives from pentoses was more pronounced at high temperatures than at high concentrations of acid. The increase in crystallinity of H_2SO_4-treated husk (Figure 2.2) is consistent with the results of chemical analysis (Table 2.5) which suggested hemicellulose removal. This effect most likely reflects the enrichment of residual solids in cellulose and agrees with the findings reported by Grethlein (1985). Moreover, several authors have found that during acid hydrolysis of cellulose (Hermans and Weidinger, 1949) and granular starch (Wu and Sarko, 1978) partial crystallization of amorphous material might take place.

Acid pretreatment, as envisaged by electron microscopy, caused pronounced surface erosion on the fibers (Figure 2.8), thus exposing the internal fiber structural network. Consequently, the residual husk would be expected to exhibit more reactive sites for adsorption of cellulolytic enzymes and thus be more susceptible to hydrolysis. The effects of NaOH on native husk were also recognized morphologically by the increased swelling and fragmentation of cellulose fibers (Figure 2.6c,d and Figure 2.7). NaOH therefore increases the cellulose surface area and, as a result, its reactivity. Although the x-ray diffraction analysis of the alkali-pretreated samples was by no means conclusive as to the possibility of structure modifications in the crystallites themselves, various conflicting literature reports on the subject point out

the complexity of the processes involved (Cowling and Brown, 1969; Knappert et al, 1980; Fan et al., 1980; Puri, 1984; Grethlein, 1985).

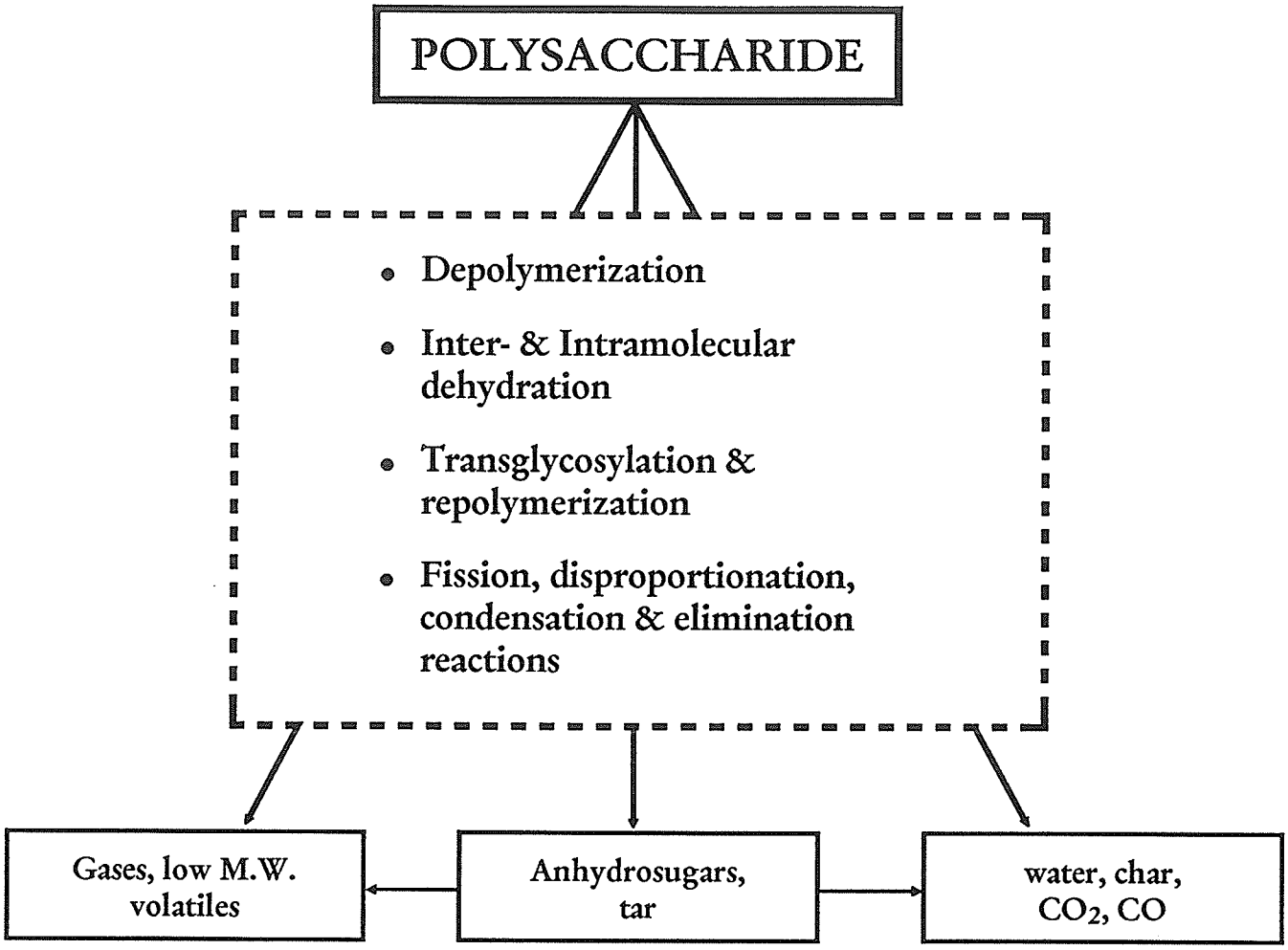
2.4.2 Pyrolytic degradation of residual husk

Because of their sensitivity, speed and simplicity, thermal analysis techniques (DSC and TGA) have been widely employed in studies of the transitional behavior of natural polymers. These methods have provided valuable insight into the order-disorder phenomena of granular starch (Donovan, 1979; Biliaderis et al., 1980), thermostability and denaturation characteristics of proteins (Privalov, 1974; Murray et al., 1981), as well as polymorphic changes of fats (Hagemann et al., 1972; Lovegren and Gray, 1978). However, the thermal behavior of cell wall carbohydrates and lignin has not been extensively explored. Therefore, the feasibility of using DSC and TGA to assess the effects of various solvents on corn husk lignocellulosics was the objective of this study. From the thermal curves of Figure 2.9 it is evident that the transition temperatures of individual husk components are clearly distinguishable; hemicellulose being the least thermostable, followed by cellulose and lignin. The corresponding peak temperatures were 238, 317 and 445°C, respectively. The last, high temperature, peak (445°C), however, cannot be unequivocally ascribed to lignin, since no purified lignin was analyzed as a control. Nevertheless, it has been shown that lignin undergoes major changes at temperatures above 350°C (Shafizadeh and McGinnis, 1971; Bouchard et al., 1985). The fact that lignin decomposes mainly into aromatic compounds, which are generally formed at higher temperatures than the non-aromatic oxygenated degradation products of cellulose (Shafizadeh, 1983), further supports the above argument. The

experimental data obtained in this study are in agreement with previously published decomposition temperatures for different species of wood and wood residues derived from thermomechanical and chemical treatments (Bouchard et al., 1985).

It must be emphasized here that the thermal response of the recorded pyrolytic events of husk lignocellulosics was exothermic. This, however, does not mean that all reactions occuring during thermal degradation have an exothermic character. Instead, it implies that the net thermal response of the various competitive reactions is exothermic. Most studies on purifed cellulose and model small molecular weight compounds indicate that pyrolytic degradation leads to a variety of products that can be derived by more than one pathway, as shown in the over-simplified scheme of Figure 2.17 (Shafizadeh and McGinnis, 1971; Shafizadeh et al., 1971; Shafizadeh and Fu, 1973; Shafizadeh and Lai, 1975; Furneaux and Shafizadeh, 1979; Liskowitz et al., 1980; Shafizadeh, 1983). The suggested mechanisms for molecular rearrangements/ transformations that take place include cleavage of glycosidic linkages (via free-radical formation and/or transglycosylation), formation of anhydro sugars (mainly levoglucosan; i.e., 1,6 anhydro-ß-D-glycopyranose) and tar (via transglycosylation, condensation, and dehydration), plus decomposition into carbonaceous char and volatiles (via dehydration, disproportionation and fission reactions). Some of these reactions are endothermic (transglycosylation and volatilization of the depolymerization products), while others are exothermic in character (dehydration and charring). Furthermore, dehydration and charring are favored at low temperatures and in the disordered regions,

Figure 2.17: Generalized competing pathways for pyrolysis of carbohydrates.


POLYSACCHARIDE
Depolymerization
Inter- & Intramolecular dehydration
Transglycosylation & repolymerization
Fission, disproportionation, condensation & elimination reactions
Gases, low M.W. volatiles
Anhydrosugars, tar
water, char, CO_2, CO

while depolymerization by transglycosylation and levoglucosan production take over at higher temperatures and usually occur in the crystalline parts of the molecule (Basch and Lewin, 1973b; Broido et al., 1973; Cabradilla and Zeronian, 1976; Shafizadeh, 1983). Overall, the extent to which dehydration and char formation competes with that of levoglucosan production will determine the net thermal response (DSC) of the system undergoing pyrolytic degradation. As such, the thermal behavior observed for the samples of the present study suggests that the decomposition pathway of dehydration and charring was predominant. It is of interest here to note that Shafizadeh (1971) reported endothermic responses on pyrolysis of cottonwood and its components. These conflicting findings may reflect differences in various physical and chemical parameters (heating rate, thermal lags of the thermoanalytical device, pressure and atmosphere under which decomposition takes place, sample size, purity and homogeneity of the carbohydrate material) which affect the degradation pathways (Chen, 1974; Jain et al., 1985).

From the thermal curves of Figures 2.9 and 2.10 it is also evident that chemical pretreatments caused pronounced changes in the DSC transition characteristics and the corresponding TGA profiles. Acid treatments resulted in a significant shift of cellulose pyrolysis to a higher temperature, which is likely a consequence of changes in both chemical and structural organization of the residual cellulosic material. A shift from 317°C (native husk) to 339°C (H_2SO_4 -treated sample; Table 2.6) along with corresponding increases in crystallinity from 51% to 61% (Table 2.5) were observed. The major source of stability in cellulose is hydrogen bonding, which is less extensive in the amorphous parts of the structure. Therefore, thermal decomposition commences in these

regions at much lower temperatures and with higher rates (Basch and Lewin, 1973a, 1973b; Cabradilla and Zeronian, 1976). In this respect, on partial removal of amorphous chain segments by hydrolysis, chain mobility on the exposed faces of the crystallites is enhanced and thus perfection (annealing) may occur. The increased resistance to pyrolysis of husk residues following acid pretreatments could thus be attributed to the increased proportion and/or perfection of cellulose crystallites. Unlike the acid-pretreated samples, alkali pretreatments seemed to cause no major changes in the thermal resistance of cellulose, as shown by the transition II temperatures (Table 2.6). Although these residues were found to have increased crystallinities (Table 2.5), the amorphous regions of cellulose appear to remain relatively intact under alkali conditions. It is, therefore, plausible to suggest that the effect from the presence of the amorphous regions of cellulose exerts considerable influence on the pyrolytic behavior of the entire cellulosic structure; i.e., they tend to reduce the thermal stability of the polymer.

The chemical pretreatments also affected the TGA pyrolytic profiles of the residues in terms of slope of the weight loss curves vs. temperature and onset of the pyrolytic events (Figure 2.10). Both parameters proved to be closely related to the chemical composition of the examined materials, i.e., the higher the cellulose to hemicellulose ratio, the steeper the slope and the higher the temperature for the onset of pyrolytic degradation. As with the DSC thermal curves, this behavior reflects the fact that the two polymeric families, cellulose and hemicellulose, have distinct decomposition temperatures (Bouchard et al.,1985).

In view of the differences in pyrolytic behavior, as assessed by TGA, between amorphous and crystalline cellulose (Basch and Lewin, 1973a and 1973b; Cabradilla and Zeronian, 1976) as well as the observation that appreciable changes in crystallinity did occur upon acid or alkali pretreatments (Table 2.5), it seemed of interest to explore any relationships between x-ray crystallinities and TGA thermal parameters. With regard to Ea, calculated from plots obtained by application of Broido's plus Freeman and Carroll's equation (section 2.2.6), it was found that samples of higher degree of crystallinity exhibited greater activation energies. This implies that more energy is required to break down ß-glucosidic linkages in cellulose crystallites than in amorphous parts of the polymeric structure. Commercial xylan was also found to have lower activation energy than cellulose (120 vs. 195 kJ/mol, as determined by the Broido's equation; Table 2.6). Literature data on activation energies for pyrolysis of pure cellulose lie generally in two groups of approximately 80-120 and 200-240 kJ/mol, as reported by Basch and Lewin (1973a) and Sefain and El-Kalyoubi (1984). The former values represent the activation energy of the less ordered regions of cellulose while the latter are characteristic of cellulose crystallites. However, impurities and the presence of other constituents present can catalytically influence the pyrolysis of cellulose and thus overshadow the effects of the structural features on the pyrolytic behavior of this polysaccharide (Basch and Lewin, 1973b).

A significant correlation was also found between crystallinity of the husk sample (%) and maximum rate of weight loss from the TGA thermal curves (Figure 2.13). These data indicate that highly crystalline

and/or more homogeneous materials are expected to lose their weight within a narrower temperature range than amorphous or heterogeneous carbohydrate samples. This is evident from the slope of TGA curves (e.g. sample vi vs. i; Figure 2.10). A similar relationship between the temperature at which the first 10% weight loss of the dried cellulosic materials occurs and crystallinity was also found. These findings clearly demonstrate the role of composition and degree of molecular organization on the thermal stability and pyrolytic pathway of cellulosic samples.

With regard to application of thermal analysis techniques to quantitation of lignocellulosics, Hornof et al. (1979) reported on a relationship between the energy released on pyrolysis and the lignin content of different pulps. Although it has been difficult so far to measure accurately the areas of the DSC peaks I and II in this study, because of the convolution of these transitions, DSC, using well defined conditions, may prove a rapid and simple method to determine the relative proportions of the two main polymeric components present in native and chemically pretreated cellulosics. Existing quantitative methods of analysis for cellulose, hemicellulose, and lignin are tedious and based on wet chemistry. However, additional development of the thermal methodologies is required to permit assessment of the analytical potential of DSC in quantitating these polymeric constituents.

In summary, experimental evidence was provided on the sensitivity of pyrolytic decomposition patterns of cellulose and hemicellulose to compositional and structural alterations of husk lignocellulosics brought about by chemical pretreatments. The alkali and acids applied caused major changes in both pyrolysis kinetics and DSC/TGA profiles.

Despite the chemical heterogeneity of the materials and complexity of the reactions involved, pyrolysis was found to obey first-order kinetics. Several relationships between the structural/compositional features and TGA thermal parameters were found. In light of these findings, it appears that DSC and TGA, in combination with other methods, provide useful information on structural modifications caused in lignocellulosic materials by chemical pretreatments.

2.4.3 Chemical characteristics of husk CSF

When the problem associated with biomass utilization is approached from the standpoint of selective fractionation of the lignocellulosic complex into its major components, pretreatments are usually designed to release hemicellulose. Because of the branched structure of hemicellulose and thus its limited intermolecular association by H-bonding (Timell, 1967), most solvents, under mild conditions, preferentially attack this polymer. Consequently, recovery of hemicellulose could be accomplished in the form of an enriched solvent-solubilized fraction. Although hemicellulose exhibits variability in terms of both structure and monosaccharide composition, certain hemicelluloses are characteristic of the particular source of plant biomass. For instance, the hemicellulose of hardwoods and most cereals is an arabino-1,4-ß-D-xylan, while glucomannans dominate the softwoods (Poutanen et al., 1986). In the case of corn cobs, the main side constituents on the ß-D-xylopyranosidic backbone are L-arabino and D-glucuronic acid (Donnelly et al., 1973). The alkali-soluble hemicelluloses of corn leaf and corn stalk have been shown to be arabinoglucuronoxylans of similar

structure, having $\bar{DP}$ of about 55 and 70, respectively (Dutton and Kabir, 1972). According to Krull and Inglett (1980), neutral hemicelluloses of husk consist of 56% xylose, 13% arabinose and 29% glucose.

Different hemicellulose-derived soluble fractions can be obtained from complex lignocellulosics depending on the pretreatment conditions and hemicellulose composition. Cunningham and Carr (1984) reported, for example, that dilute acid treatments (1-3% w/w H_2SO_4, 130°C-1 h) of wheat straw yielded xylose in the solubilized fraction, whereas alkali extraction (4% and 10% w/v NaOH, steeping overnight at room temperature) gave a xylan-rich fraction. Analysis of water-extracted hemicelluloses of birchwood, after steaming at 170 and 210°C for 10 min, showed that xylose was the predominant sugar, while in the case of straw, oligomeric sugars were of higher concentrations than xylose (Puls et al., 1985). With agricultural residues (corn stover, wheat and barley straw), the solubilization of pentosans under similar conditions to those in the previous study was less complete than in aspenwood and a large portion of it remained in the water-insoluble fraction (Yu et al., 1984a and 1984b). Complete recovery of hemicellulose in the form of either high or low MW constituents could not also be achieved under the conditions employed in the present study. For the alkali-pretreated samples, this may be due to the stability of the hemicellulose-lignin complex (acetal and benzyl ether linkages are not alkali sensitive; Adler, 1977; Higuchi et al., 1980). Acid hydrolysis, on the other hand, is not selective and incomplete depolymerization as well as formation of secondary dehydration products from the released monosaccharides could be responsible for the apparently low yields of PC in the hydrolyzates. According to the data presented in Table 2.7, the maximum amount of PC found in the solu-

bilized fraction (3.77 mg/mL for the 5% NaOH pretreatment) accounted for 56.5% of the PC content of native husk. The yields for the other hydrolyzates were even lower. The low recovery values for hemicellulose and its depolymerization products could also arise from two additional reasons. First, the water extraction of solubilized, but adsorbed on the husk residues, carbohydrate constituents might have been incomplete, thus leading to only partial quantitation. Second, some higher MW xylo-oligosaccharides might have been undetected due to their poor solubility. A progressive decline in solubility by increase in $\overline{DP}$ has been reported for both xylo- and cello-oligosaccharides (Heyraud and Rinaudo, 1978; Gum and Brown, 1977). Furthermore, it appears that the differences in PC/TC ratios among the various CSF, as shown in Table 2.7, reflect the presence of various amounts of liberated hexoses. Similar trends in carbohydrate composition of prehydrolyzates from corn stem were reported by Buchala and Meier (1973).

With regard to MW distribution of the CSF components, differences in the action of acids and alkali were evident from the gel filtration chromatographic profiles. Although the most effective hemicellulose extracting solvent (5% w/v NaOH) caused virtually little, if any, chain depolymerization (at least not below $\overline{DP}$=12), low MW xylo-oligosaccharides dominated the acidic prehydrolyzates (Figures 2.15 and 2.16). The latter could include side chain substituents (L-arabinose) and depolymerization products of the xylan backbone. The magnitude of individual contributions of xylose and arabinose to the pentose monomer containing peak (Figure 2.15d and 2.16b,d) is uncertain; Fractogel TSK HW-40(s) could not discriminate between the two sugars. H_2SO_4 at a

concentration of 5% w/w (85°C/2 h) was more effective in hydrolyzing the husk components than 14.3% w/w H_3PO_4. This is evident by the high proportion of the $\overline{DP}$=1 component (64.3% vs. 22.2%) and the relatively low amount of gel-excluded material (8.0% vs. 38.6%, Table 2.8). Nevertheless, both acids liberated approximately equal amounts of total soluble carbohydrates (Table 2.7).

Overall, the extent of hemicellulose extraction/solubilization and chain depolymerization are dependent on the type of chemical pretreatment. The results of chemical analysis along with the GPC data indicated that 5% NaOH is the most attractive solvent, mainly because of its capacity to extract a high proportion of non-degraded husk hemicellulose. This would enable the production of relatively homogeneous products (xylose and/or arabinose) in high yields by a second hydrolysis step. In contrast, acidic pretreatments gave mixtures of oligosaccharides of various chain lengths. Moreover, under these conditions, the production of undesirable degradation products is most likely to occur.

Chapter III

ENZYMIC HYDROLYSIS OF PRETREATED HUSK TO FERMENTABLE SUGARS

3.1 INTRODUCTION

Hydrolysis of solid cellulosic substrates to fermentable sugars is a complicated and not completely understood process, mainly for two reasons: structural constraints inherent with the cellulose supramolecular structure, as elaborated in chapter two of this thesis, and the necessity for a multicomponent cellulolytic system with a high and balanced activity of individual cellulase components that act synergistically. An additional major problem for the commercial development of an enzymic process is the low specific activity of the cellulolytic enzymes. Approximately 100 times more enzyme protein is required for cellulose hydrolysis than for the corresponding hydrolysis of gelatinized starch (Reese, 1982; Lutzen et al., 1983). Consequently, the enhancement of substrate reactivity along with the increase in enzyme activity appear to be the two limiting requirements for implementing hydrolysis of cellulosic material for practical use.

There is a variety of microorganisms that can degrade cellulose, both aerobicaly and anaerobicaly, including bacteria, actinomyces and the higher fungi. However, only those organisms which are capable of synthesizing a so-called "complete cellulase complex" extracellularly, and thus being able to utilize native crystalline cellulose such as cotton fibers rather than soluble derivatives, are considered truly cellulolytic. Among them, the most thoroughly studied is Trichoderma reesei. The cellulase system of "true cellulolytic" microorganisms consists of three major components: 1,4-ß-D-glucan glucanohydrolase (EC 3.2.1.3), 1,4-ß-D-glucan cellobiohydrolase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). These components are often referred to as endoglucanase (Cx), exoglucanase (C_1), and cellobiase, respectively.

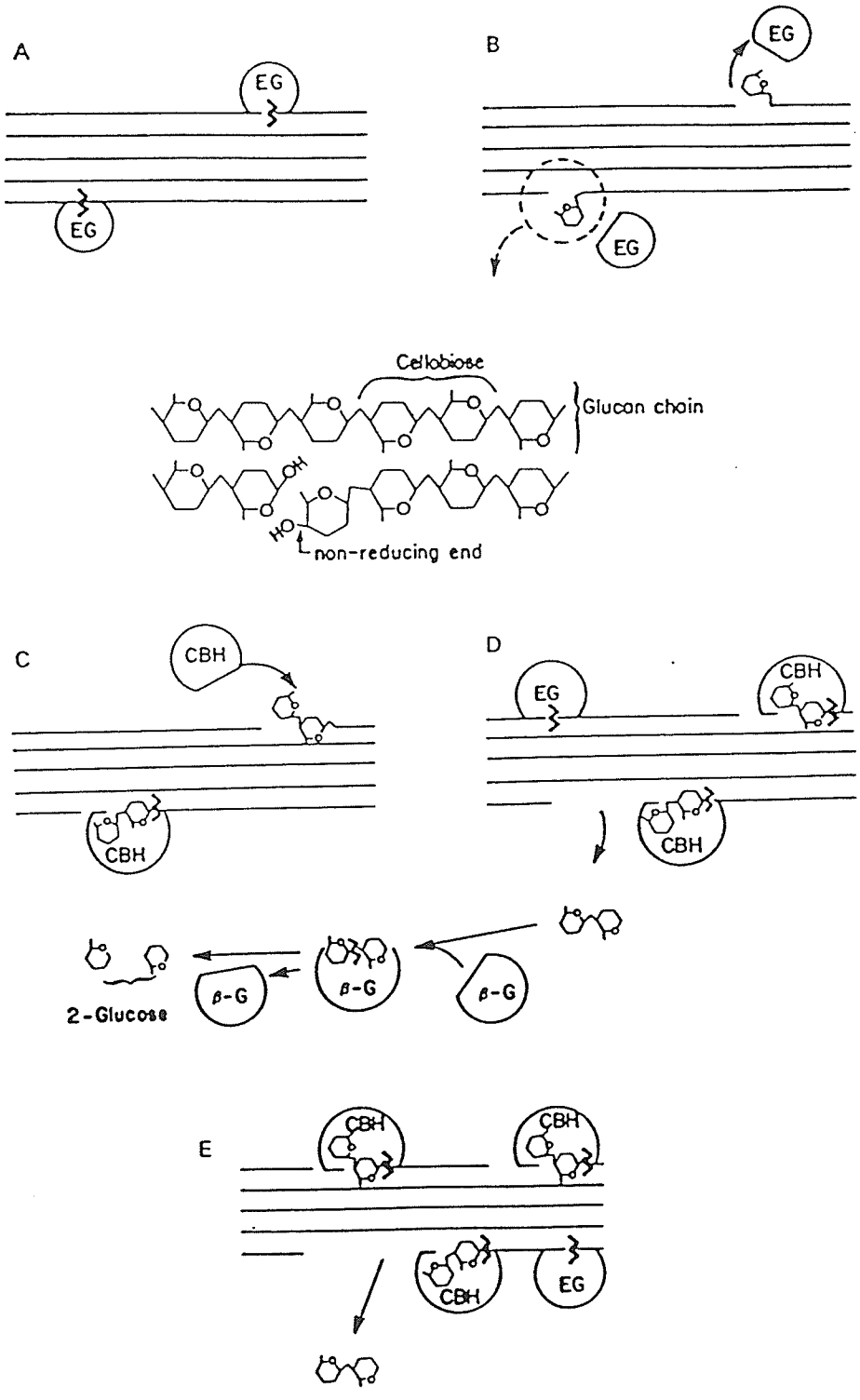
Endoglucanases attack internal cellulosic linkages by random scission of cellulose chains yielding glucose, cellobiose, and cellotriose; exoglucanases cleave cellobiosyl units from the non-reducing end of cellulose polymer chains (endwise attack); and cellobiases specifically cleave glucosyl units from the non-reducing end of cellooligosaccharides. There are indications that cellulase systems of bacteria differ substantially from those of fungi and that synergistic interaction between them may not be feasible. Wood (1980) discussed several possible explanations and suggested that the problem could be the specific substrate stereochemical requirements for each enzyme. Furthermore, apart from the different pH and temperature optima, a major distinction is the high endo/exo activity ratio in bacterial cellulases relative to the Trichoderma cellulases (Ladisch et al., 1983).

The first postulate concerning the mechanism of hydrolysis of cellulose, known as "C_1, Cx hypothesis", was advanced by Reese et al. (1950). According to this hypothesis, the C_1-enzyme (exo-1,4-ß-glucanase) was believed to initiate cellulose hydrolysis and thus make the substrate reactive toward the Cx-enzyme, endo-1,4-ß-glucanase. Extensive research aimed at fractionation, and investigation of the individual enzyme components from various sources, stimulated after introduction of the "C1, Cx hypothesis", contributed, however, to a new view regarding the mechanism of cellulolysis. This involves a sequential action by an endo-glucanase which initiates the attack by hydrolyzing a few ß-1,4-glucosidic linkages in the less crystalline regions, thus generating new nonreducing chain ends to be attacked by exo-1,4-ß-glucanases. The latter enzymes have been isolated from a number of fungi (Erikkson

and Pettersson, 1975b; Wood and McCrae, 1972; Eriksson, 1978; Berghem et al., 1975). While the exact mechanism of native cellulose hydrolysis is still a matter of controversy, there seems to be agreement that exoglucanases and endoglucanases act cooperatively and synergistically in depolymerizing cellulose to glucose and oligosaccharides, which are then converted by ß-glucosidase to glucose. Figure 3.1 illustrates a currently proposed scheme for cellulase action derived from contributions of a number of independent workers (Wood, 1975; Eriksson and Pettersson, 1975; Reese, 1975; Ghose and Bisaria, 1979; Gritzali and Brown, 1979; Klyosov et al., 1980; Sadana and Patil, 1985; Fujii and Shimizu, 1986).

Each component of the cellulase system consists of a number of distinct enzyme species. At least four different endoglucanases from commercial preparations of Trichoderma cellulase have been described, including high-molecular weight glycoprotein endoglucanases (Shoemaker and Brown, 1978a,b), as well as low-molecular weight noncarbohydrate containing endoglucanases (Hakansson et al., 1978). Each of these endoglucanases has unique substrate specificity and properties. There are two known cellobiohydrolases, I and II, each consisting of a number of isozymes that can be separated by isoelectric focusing (Fagerstam et al., 1977; Fagerstam and Pettersson, 1979). Two to six components, depending on the microbial source, have been identified in the ß-glucosidase preparations; most of them are glycoproteins and exist in multiple forms (Shewale, 1982). It has been well established by the work of Sternberg and Mandels (1980) that ß-glucosidase is limiting in standard Trichoderma cultures and that addition of exogeneous

Figure 3.1: Schematic representation of cellulase action on cellulose. EG, endoglucanase; CBH, cellobiohydrolase; ß-G, ß-glucosidase. (A) EG binds randomly to the surface of the cellulose microfibril and breaks a glucosyl bond within a glucan chain, (B) EG leaves the microfibril surface, thus exposing a reducing and a non-reducing end, (C) CBH cleaves a cellobiose unit from the non-reducing chain end, (D) cellobiose is released into solution, where split into glucose by ß-G, (E) action of EG continues in creating sites at which CBH may act (adopted from White, 1982).


A
EG
EG
B
EG
EG
Cellobiose
Glucan chain
OH
HO
non-reducing end
C
CBH
CBH
D
EG
CBH
CBH
2-Glucose
β-G
β-G
β-G
E
CBH
CBH
CBH
EG

ß-glucosidase e.g., from Aspergillus sp. greatly improves the rate at which cellulose is converted to glucose. Investigations of the total protein profile of Celluclast, a crude cellulolytic preparation from T. reesei, indeed confirmed this finding. Exoglucanase accounted for 65% of the protein, endoglucanase for 20%, non-enzymic inactive protein for 15% and cellobiase for only 1% (Lutzen et al., 1983).

Owing to the low specific activity of cellulases, considerable efforts have been focused over the years on obtaining high cellulase producing organisms and/or mutants (Montenecourt and Eveleigh, 1977; Nevalainen et al., 1980; Ghosh et al., 1982). Mandels (1982) reported on several T. reesei cellulase mutants, obtained at the Natick laboratory and at Rutgers University, which produced three to ten times higher levels of cellulase as compared with the wild strain. Furthermore, the relative proportions of endo- and exo-glucanases as well as the properties of the enzyme complex showed no marked changes. Cellulase synthesis in Trichoderma is known to be under multiple regulatory control of induction and repression. On the other hand, cellulaseless mutants commonly produce cellobiase, thus indicating that this enzyme is under different genetic control (Mandels, 1982; Stutzenberger, 1985).

End-product inhibition has been recognized as an important controlling factor in the hydrolysis of cellulose. The soluble products, cellobiose and glucose, have been reported to be inhibitors of the cellulase complex (Ghose, 1977), and of the individual enzyme components: endoglucanase (Halliwell and Griffin, 1973), exoglucanase (Berghem et al., 1975), and ß-glucosidase (Wood and McCrae, 1975; Gong et al., 1977: Maguire, 1977a). The exo-glucanase is known to be strongly and competitively inhibited by cellobiose as indicated by its

low inhibitor constant (Ki=0.06-1.1 mM), whereas glucose appears to be a less effective inhibitor (Hsu et al., 1980; Maguire, 1977b). The former inhibition can be alleviated by addition of ß-glucosidase from Aspergillus sp., either in the soluble or immobilized form (Sundstrom et al., 1981; Mandels, 1982) during saccharification. The latter type of inhibition can be overcome by glucose removal immediately after its formation using a membrane type of reactor or by simultaneous ethanol fermentation (Hong et al., 1981; Mandels, 1982; Saddler et al., 1982a). The data for ß-glucosidase show that it is subject to substrate inhibition, as well as non-competitive product inhibition. Glucose accumulation during cellulolysis supresses the hydrolysis of cellobiose and thus diverts ß-glucosidase action to hydrolysis of higher dextrins (Shewale, 1982). In view of the complexity of the kinetic behaviour of the multicomponent cellulase system along with the structural complexity of the substrate it is not surprising that kinetics of cellulose hydrolysis have not been yet established with certainty. Some modelling has been attempted on pure cellulose, rather than native cellulosic materials (Okazaki and Moo-Young, 1978; Ladisch et al., 1981; Gusakov and Sinitsyn, 1985), but this has not been fully successful.

Commonly observed in cellulose hydrolysis is the decrease in hydrolysis rate at increased levels of cellulose conversion regardless of the source of cellulase complex. Increasing resistance of the residual substrate, product inhibition, and enzyme inactivation have been suggested to account for the limited cellulose biodegradation (Henrissat et al., 1985; Mandels, 1982). For instance, Herr (1980) explained the low extent of hydrolysis of almost pure cellulose using a

ß-glucosidase-enriched enzyme preparation of T. viride ITCC-1433 mainly by end product inhibition. If glucose was continuously removed from the reaction mixture, via an ultrafiltration device, the degree of saccharification increased up to 90% in 48 h without any accumulation of cellobiose. According to Lutzen et al. (1983), who investigated numerous microbial species for cellulase production at NOVO's laboratories, the highest possible degree of degradation of cellulose that could be achieved under optimal conditions of batch hydrolysis, at high enzyme concentration and a reaction time of 48 h, was about 50%. The authors thus concluded that an industrial process of cellulose hydrolysis must include a pretreatment of native lignocellulosics to make cellulose more susceptible to enzymic degradation, a point of view that is now shared by most biotechnologists.

The hemicellulose component, which in conversion processes of lignocellulosic residues into metabolizable sugars becomes a part of the solubilized fraction, has been recognized as a valuable potential by-product deserving more research interest. The dissolved hemicellulose can be converted into monomer constituents either by acid or enzymic hydrolysis prior to fermentation to different chemicals or assimilation into microbial protein. Hydrolysis with dilute acids at elevated temperatures readily liberates pentose sugars which, however, become rapidly degraded to products that act as fermentation inhibitors; i.e., furfural derivatives (Lee et al., 1978; Gong et al., 1982). Hydrolysis by enzymes is viewed as an advantageous and more specific processing route for biomass derived hemicelluloses, but has not been yet thoroughly investigated.

Analogous to cellulases, at least three types of xylanases have been described: ß-xylosidases (EC 3.2.1.37), exo-xylanases and endo-xylanases (EC 3.2.1.18) (Reilly, 1981; Dekker, 1985). Xylanases are produced by many organisms, often together with cellulases, and their synthesis is repressed by xylose (Dekker, 1983). Commercially important producers of xylanases are molds of the genera Aspergillus and Trichoderma (Sinner and Dietrichs, 1975; Gorbacheva and Radionova, 1977; Baker et al., 1977). Studies on bacterial xylanases are confined to the genera of Bacillus and Streptomyces, while yeasts have only recently been recognized to synthesize xylanases (Dekker, 1985). These enzymes are inducible and are produced when microorganisms are grown on xylans. There are exceptions, however, in which xylanases have also been reported to be produced when cellulose was used as a microbial substrate (Dekker and Richards, 1976).

Since xylans are mainly heteropolysaccharides total hydrolysis is affected by the synergistic actions of all xylanase components and various exo-glycosidases (e.g. α-L-arabinosidase and α-D-glucuronidase). The end-products arising from complete degradation of heteroxylans are therefore D-xylose, L-arabinose, and D-glucuronic acid (or its 4-O-methyl derivative). Studies on hydrolysis of steamed birchwood hemicellulose, containing a substantial amount of acetylated xylan and some methylglucurono-substituted xylan, demonstrated the significance of acetyl-xylan esterase and α-1,2-glucuronidase in the overall xylose yield (Biely et al., 1986; Poutanen et al., 1986). Dekker (1983) reported on a relatively low extent (30-40%) of hydrolysis of hemicellulose from 6% bagasse within 24 h using a Trichoderma reesei mutant. He suggested that the low content of ß-xylosidase could be responsible for

the low overall conversion and stressed the important role of this enzyme that is analogous to that of ß-glucosidase in saccharification of cellulose by cellulases. Immobilization of ß-xylosidase on alkylamine porous glass and various cellulose derivatives was also attempted (Puls et al., 1977; Oguntimein and Reilly, 1980), but the activity of the preparations was not high enough to be industrially feasible.

The objective of the present study was, therefore, to examine chemically pretreated husk lignocellulosic residues and the chemically solubilized fractions (CSF) obtained therewith as potential substrates for the production of fermentable sugars using crude commercial preparations of cellulases and hemicellulases. The release of reducing sugars during hydrolysis along with the corresponding final yield, taken as indices of the susceptibility of husk lignocellulosics to enzymic attack, were followed to verify the beneficial effects of a particular chemical pretreatment on husk relative to its native counterpart. Comparative investigations of three commercial cellulolytic and two hemicellulolytic preparations were performed. Moreover, an attempt was made to follow the alterations in fine structure of husk cellulose upon cellulase action by monitoring the temperature of its pyrolytic degradation. In view of the data reported in chapter two, the possible relationships between certain structural parameters of husk and its overall susceptibility to cellulase were further explored.

3.2 MATERIALS AND METHODS

3.2.1 Substrates

Native corn husk, dried at 65°C for 48 h and then ground in a Wiley mill (particle size<1 mm; i.e., <16 mesh), served as control in hydrolysis experiments using cellulolytic enzymes. The main substrates were husk samples pretreated with one of the following solvents: 5% w/v NaOH, 5% w/w H_2SO_4, 5% w/w H_3PO_4 and 14.3% w/w H_3PO_4 at 85°C for 2 h in a thermostated water bath under continuous agitation, as described in section 2.2.1. Husk pretreated with 5% w/v NaOH at room temperature for 2 h, by applying mild stirring with a magnetic bar, was also employed. The husk residues, after a specified pretreatment, were recovered by filtration through Whatman #4 filter paper, and then washed thoroughly with distilled water until alkali- or acid-free (pH 6.0-7.0). Additional washing with 0.05 M acetate buffer, pH 4.8, was also applied. The residue was not allowed to dry, but in the wet state it was subjected to hydrolysis in order to avoid cellulose recrystallization on drying. Whatman No.1 filter paper and α-cellulose (Sigma Chem. Corp., St. Louis, MO) were also used in enzymic assays and in studying the effect of enzyme concentration on hydrolysis rate and yield.

The chemically solubilized material (CSF), following husk pretreatments, was adjusted to pH 4.8, and lyophilized prior to enzymic hydrolysis. The pH of CSF from NaOH treatments was adjusted with concentrated CH_3COOH. Part of the CSF was desalted by ultrafiltration (Diaflo YM2 membrane, having a MW cut-off 1000; AMICON, Danvers, MA). A concentrated NaOH solution (ca. 10% w/w) was employed for pH adjustments of the 5% w/w H_2SO_4 or 5% w/w and 14.3% w/w H_3PO_4-extracts. Part of the pH

adjusted-CSF of the 5% w/w H_2SO_4 pretreated sample was partially desalted by addition of $BaCO_3$ (1 g per 50 mL CSF). The precipitated $BaSO_4$ was discarded after centrifugation at 2,500 xg for 10 min, whereas the supernatant was freeze dried. Lyophilized CSFs in 0.05 M acetate buffer, pH 4.8, at 2% concentration, were used in all subsequent hydrolysis experiments.

3.2.2 Enzyme preparations and activity determination

The commercial cellulase preparation Celluclast 1.5 L, derived from Trichoderma reesei, was kindly supplied by NOVO Labs. (Bagsvaerd, Denmark). Its total cellulolytic activity was 73.4 IU mL^{-1}; one IU is defined as the amount of enzyme liberating 1 μmole of glucose equivalents per minute at 50°C and pH 4.8 (assay conditions are described below). The corresponding specific activity was 726.7 IU g^{-1} protein with the protein content being 10.1%. The other two commercial cellulolytic enzymes tested were: Rohament CT, provided by Rohm GmbH (Darmstadt, Germany) and Takamine Cellulase, a multi-enzyme complex from Aspergillus niger, from Miles Lab. Inc. (Elkhart, Indiana). The former had total cellulolytic activity of 160.4 IU g^{-1} and specific activity of 1076.5 IU g^{-1} protein (protein content 14.9%), while the latter exhibited cellulolytic activity of 16.6 IU g^{-1} and a specific activity of 111.4 IU g^{-1} protein (protein content 4.0%). A commercial preparation of ß-glucosidase from Aspergillus niger, Novozym 188, with a declared activity of 250 CBU g^{-1} was also provided by NOVO Labs; one CBU is the amount of enzyme that liberates 2 μmoles glucose per minute from a 0.2% cellobiose solution at pH 5.0 and temperature 40°C. It was used as

recommended by the supplier as a supplement to Celluclast, since this complex is deficient in ß-glucosidase.

The total cellulolytic activity, including endoglucanase, exoglucanase and ß-glucosidase, of the above three cellulase preparations was determined according to a modified method of Mandels et al. (1976). Filter paper Whatman No.1 shred into 1x1 cm pieces and suspended in 0.05 M acetate buffer, pH 4.8, at a concentration of 5%, was used as a substrate (total volume of 9.5 mL). This material was incubated at 50°C with 0.5 mL of diluted enzyme solution/suspension (Celluclast was diluted at 50 µL per mL of buffer, while Rohament CT and Takamine Cellulase were suspended at 3.0 mg and 15 mg, respectively, per mL of buffer). After 30 min of digestion under continuous agitation, the solutions were filtered, boiled for 10 min to inactivate the enzyme and centrifuged at 2,500 xg for 10 min. The reducing sugars in the supernatant were measured by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952) using glucose as standard. Assay conditions were chosen accordingly so that glucose formation was linear with respect to both time and enzyme concentration. The protein content of the cellulase preparations was determined by micro-Kjeldahl (N x 6.25) according to the AOAC (1975) standard method. All assays were performed in triplicate.

Rohament CA, a commercial hemicellulase from Aspergillus sp. cultures, was provided by Rohm GmbH with a declared activity of 1800 CU mg^{-1}. One CU corresponds to the amount of enzyme which reduced the viscosity of 1 mg carboxymethylcellulose in solution (1.5% w/v in acetate buffer, pH 4.5) in 40 min at 30°C by $\Delta 1/\eta$ sp = 0.05. It includes pentosanase and ß-glucanase components. In certain experiments this preparation was partially purified from contaminating low MW sugars

prior to its use by ultrafiltration (Diaflo PM 10 membrane, having a MW cut-off 10,000; AMICON, Danvers, MA) in 0.05 M acetate buffer, pH 4.8. Celluclast, presumably having side hemicellulolytic activity, was also tested in hydrolysis of extracts. ß-Xylosidase (Sigma X-5375) from Aspergillus niger with a declared activity of 5 U mg^{-1} protein (one unit of enzyme activity hydrolyzes 1.0 µmole of o-nitrophenyl-ß-D-xyloside to o-nitrophenol and D-xylose per minute at pH 5.0 and temperature 25°C) was applied in combination with Celluclast. Since there is no standard testing protocol for hemicellulase activity measurements, because of the chemical heterogeneity of the substrates, none of these enzymes was assayed for activity. They were used in concentration levels recommended by the manufacturer.

3.2.3 Enzyme hydrolysis of husk residues and CSF

Hydrolysis was carried out using residues of solvent pretreated husk (in the wet state) suspended in 0.05 M acetate buffer, pH 4.8. Based on separate dry-weight measurements of the residues, the appropriate amount of wet material was added to achieve a solids concentration of 2% w/v. Parallel hydrolysis experiments were conducted at 50°C using Rohament CT at a concentration of 48 IU g^{-1} substrate, Celluclast at 184 IU g^{-1} and Celluclast supplemented with 1.25 $mL.g^{-1}$ of substrate (310 CBU) of Novozym 188. Since the reaction mixture was of a heterogeneous nature, sample withdrawal from the same reaction flask at various time intervals was inappropriate. Therefore, to monitor the progress of cellulose hydrolysis, individual reaction flasks were employed for each sampling time. Following 4, 14, 24 and 48 h of hydrolysis the reaction mixture

was filtered and boiled for 10 min to inactivate the enzyme. After cooling, the reaction products were centrifuged at 2,500 x g for 10 min. The supernatants were then assayed for reducing sugars, using the Nelson-Somogyi method, and for monosaccharide composition by HPLC (conditions are described below). The amount of reducing sugars, expressed as glucose, was used to calculate the degree of residue conversion (DC) as follows:

$$\text{Degree of conversion} = \frac{(\text{weight of glucose released}) \times 0.9}{(\text{dry weight of residue})} \times 100$$

The above general procedure was applied to study the effects of enzyme concentration on hydrolysis using native husk and α-cellulose, at 3% w/v, as substrates. The total cellulase activity applied varied between 12-48 IU g^{-1} and 82-184 IU g^{-1} substrate for Rohament CT and Celluclast, respectively. To examine the effect of husk particle size on rate and extent of hydrolysis, two fractions obtained using a Wiley mill were used: particle size <1.0 mm (<16 mesh) and between 1.0 and 3.0 mm (16 mesh < sample < 6 mesh). Husk ground in a Udy cyclone mill with particle size < 0.2 mm (< 60 mesh) was also used. Celluclast and Rohament CT at 82 IU g^{-1} and 24 IU g^{-1} substrate, respectively, were employed for these experiments. Hydrolysis was performed at 3% w/v substrate concentration; other conditions as stated above.

Hydrolysis of CSF (2% w/v) in 0.05 M sodium acetate buffer, pH 4.8, was carried out at 65°C (Rohament CA) or 50°C (Celluclast or Celluclast in combination with Rohament CA). Typically, 200 mg of lyophilized CSF in 10 mL of acetate buffer were incubated with the following enzyme preparations: Celluclast, 73 IU; Celluclast, 37 IU and Rohament CA,

$14x10^4$ CU; Rohament CA, $28x10^4$ CU; Rohament CA (ultrafiltered), $56x10^4$ CU. Hydrolysis was allowed to proceed for 24 h and the hydrolyzates were treated as outlined above for the cellulase hydrolyzates of husk residues. The supernatants were assayed for monosaccharide composition by HPLC and the degree of conversion was calculated on the basis of xylose plus arabinose content relative to the pentose-containing oligosaccharides (PCO) in the hydrolyzed CSF:

$$\text{Degree of conversion} = \frac{\text{Weight of (xylose + arabinose)}}{\text{Weight of PCO}} \times 100$$

3.2.4 Chemical analysis of the hydrolyzates and husk residues

Monosaccharide analysis of the hydrolyzates was carried out by HPLC using a Waters Associates Chromatograph (Milford, MA) equipped with a M6000A solvent delivery system, a U6K injector and a model 441 refractive index detector. The system was interfaced to a VISTA data station (Varian 401) for data acquisition and peak area integration. All samples (20 μL injection volume) were run isocratically at a flow rate of 0.6 mL min^{-1} using filtered and degassed distilled water as eluent through an Aminex HPX-87P (300 x 7.8 mm) column (Bio-Rad Labs., Richmond, CA) at 85°C in conjuction with a guard column. Column calibration was carried out with standard solutions (4 $mg.mL^{-1}$) of glucose, xylose, arabinose, mannose, galactose and cellobiose, all products of Aldrich Chem. Corp. (Milwaukee, WI). Prior to sample injection a standard cleanup procedure was applied by passing the sample through a SEP-PAK C18 cartridge (Waters Associates) and filtering through a 0.45 μm cellulose acetate filter (Millipore Corp., Bedford, MA). For enzymic

CSF hydrolyzates, after filtration through the cellulose acetate filter, the samples were deionized by passing them through a mixed-bed ion exchange cartridge (Bio-Rad Labs).

DSC analysis of cellulase-treated husk residues was performed on a Du Pont 9900 thermal analysis system with a 910 cell base and a pressure DSC cell, as described in section 2.2.5.1. The residual husk solids, following hydrolysis, were filtered, washed with distilled water until acid-free and air-dried before subjected to DSC analysis.

3.3 RESULTS

3.3.1 Effects of residue particle size, enzyme source and concentration on husk hydrolysis

Studies on structural alterations of husk by chemical pretreatments along with the data on chemical characterization of CSF (sections 2.4.1 and 2.4.3) led to the selection of five pretreated residues as substrates for hydrolysis. The chemical composition and crystallinity index of these materials are given in Table 3.1. Three commercial cellulase preparations, in the form of culture filtrates of Trichoderma reesei (Celluclast), Aspergillus niger (Takamine) and of an unknown source (Rohament CT) were employed in these studies. However, after preliminary testing, Takamine was excluded from all subsequent experiments because of its relatively low specific activity and the presence of contaminating sugars in high concentration (approximately 48% w/w).

The effects of enzyme concentration on α-cellulose and husk hydrolysis using Celluclast or Rohament CT are shown in Figures 3.2 and 3.3, respectively. As illustrated in these figures, after 50 to 70 h reaction time, the susceptibility of native husk was much lower than α-cellulose for both enzyme preparations and at all levels of enzyme concentration. This result most likely reflects the presence of lignin in native husk. Although the initial reaction rates up to 6 h were similar for both substrates, marked differences emerged between 6 and 70 h. Thus, while hydrolysis of husk reached a plateau value after 6 h, α-cellulose showed a continuous increase in the production of reducing sugars. Increasing the Celluclast concentration for more than two-fold (82 to 184 IU g^{-1} substrate) resulted in very little additional improve-

TABLE 3.1

Chemical composition and crystallinity index of corn husk residues subjected to enzymic hydrolysis by cellulases.

Pretreatment (solvent/temp.-time)	Residue[1] (% wt)	Hemicellulose[1] (%)	Cellulose[1] (%)	Lignin[1] (%)	CrI (%)
Native husk	100	45±1.1	39±0.5	6.6±0.6	51
5.0% w/v NaOH/25°C - 2 h	57±2.0	20±0.8	62±1.4	3.9±0.5	60
5.0% w/v NaOH/85°C - 2 h	38±1.7	13±0.2	74±0.7	2.6±0.3	67
5.0% w/w H_2SO_4/85°C - 2 h	47±1.0	8±0.2	69±0.6	6.1±0.7	61
5.0% w/w H_3PO_4/85°C - 2 h	71±1.7	29±0.9	46±1.0	6.3±0.4	57
14.3% w/w H_3PO_4/85°C - 2 h	60±1.1	23±0.7	52±0.7	6.5±0.8	59

[1] Data presented are means ± standard deviation (n=3).

Figure 3.2: The time course of hydrolysis of α-cellulose and husk by Celluclast at various enzyme concentrations: substrate concentration 3% w/v, acetate buffer 0.05 M, pH 4.8, temperature 50°C.

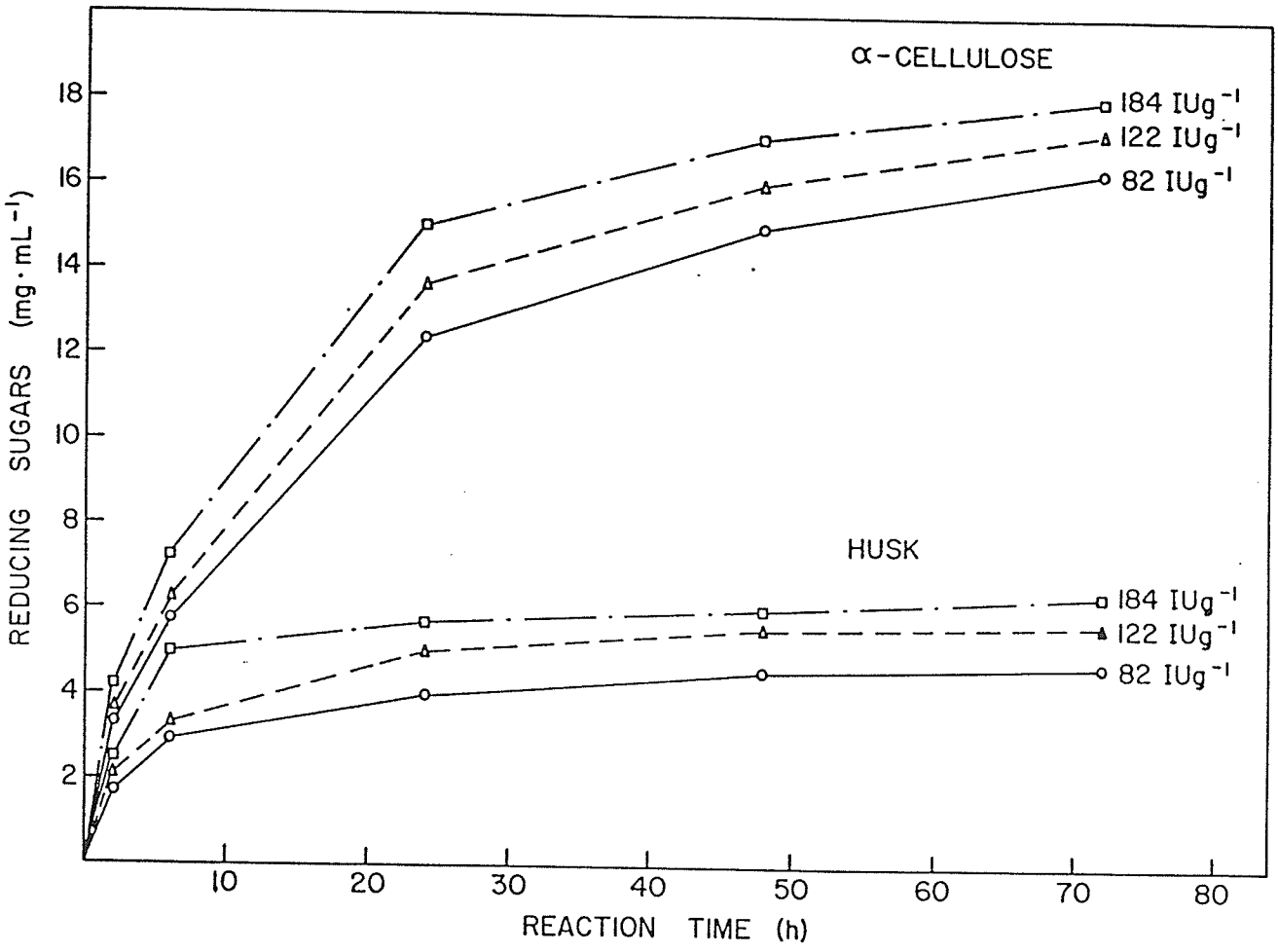
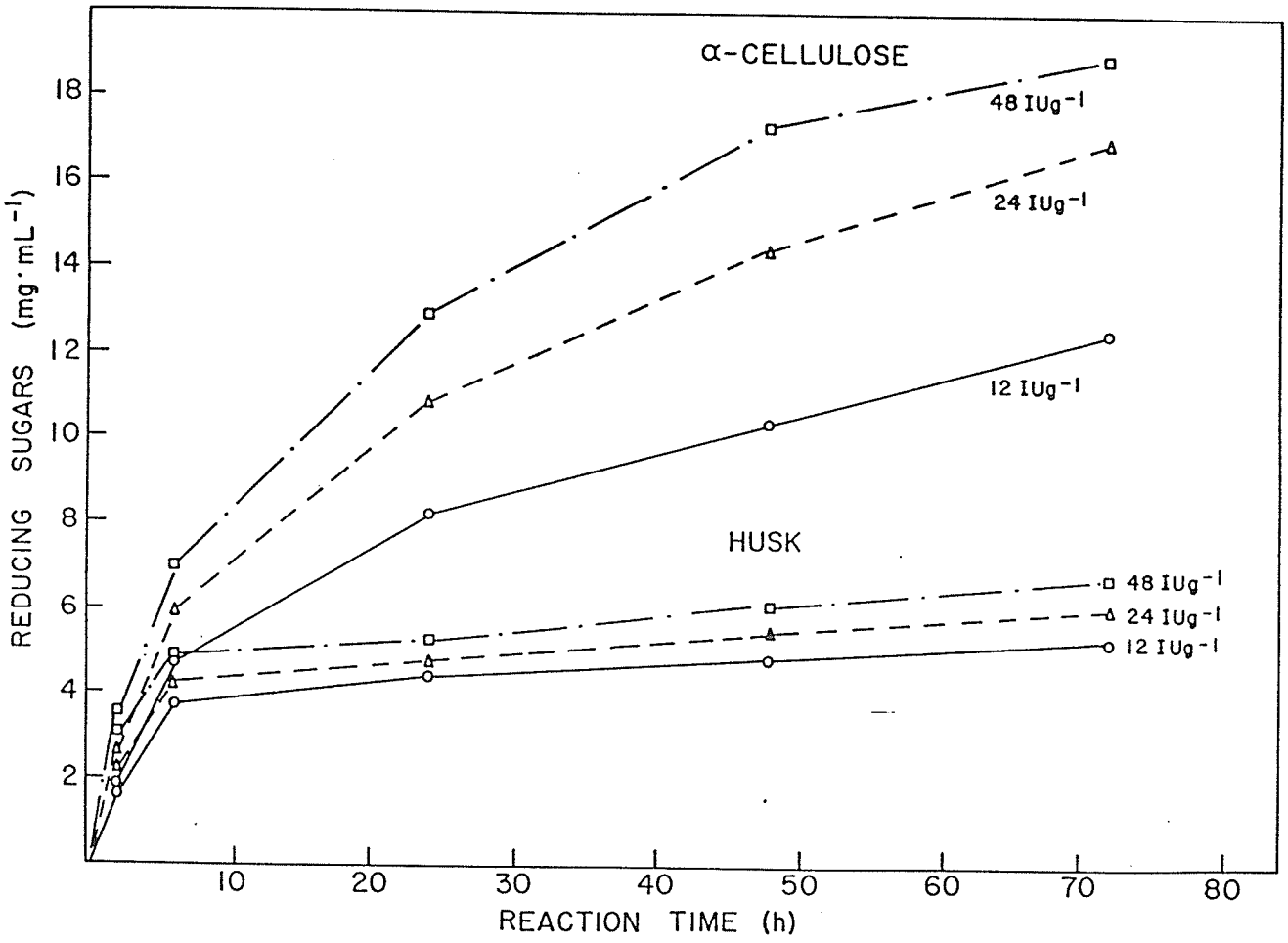

α-CELLULOSE
184 IUg⁻¹
122 IUg⁻¹
82 IUg⁻¹
HUSK
184 IUg⁻¹
122 IUg⁻¹
82 IUg⁻¹
REDUCING SUGARS (mg·mL⁻¹)
REACTION TIME (h)
2
4
6
8
10
12
14
16
18
10
20
30
40
50
60
70
80

Figure 3.3: The time course of hydrolysis of α-cellulose and husk by Rohament CT at various enzyme concentrations: substrate concentration 3% w/v, acetate buffer 0.05 M, pH 4.8, temperature 50°C.


α-CELLULOSE
48 IUg⁻¹
24 IUg⁻¹
12 IUg⁻¹
HUSK
48 IUg⁻¹
24 IUg⁻¹
12 IUg⁻¹
REDUCING SUGARS (mg·mL⁻¹)
REACTION TIME (h)
2
4
6
8
10
12
14
16
18
10
20
30
40
50
60
70
80

ment in the degree of hydrolysis for both substrates (Figure 3.2). Similar data have been reported recently by Beldman et al. (1987). This is consistent with the view that the extent of cellulose hydrolysis is highly dependent on enzyme concentration only over a certain range of enzyme concentration (Ferchak et al., 1980). Furthermore, it is well known that reaction rates in heterogeneous systems are governed by adsorption processes; i.e., after saturation of a solid substrate by enzyme, no further adsorption and thus rate enhancement would occur. Similar trends in hydrolysis profiles of husk were also observed with Rohament CT (Figure 3.3). In contrast, the yield and rate of α-cellulose degradation by this enzyme were somewhat dependent on the amount of enzyme activity present (within 12-48 IU g^{-1} substrate). Furthermore, it was interesting to note that a much lower cellulase activity was required with Rohament CT (48 IU g^{-1}) to achieve approximately the same degree of hydrolysis obtained with 184 IU g^{-1} of Celluclast. These findings may reflect differences in composition of the cellulase complex and thus affinities for the substrate between the two preparations.

The results on hydrolysis yields of α-cellulose, filter paper and husk of various particle sizes are shown in Table 3.2. Decreasing the particle size of husk from > 16 mesh to between 16 and 60 mesh resulted in increased yields for the samples analysed at 2, 6 and 24 h intervals. Particle size thus has a definite effect on the extent of hydrolysis which is typical of heterogeneous reactions involving insoluble substrates. Consequently, husk of particle size <16 mesh was chosen as the experimental material for all subsequent chemical and enzymic treatments of husk residues. It was of interest also to note that very finely

TABLE 3.2

Hydrolysis yield of α-cellulose, filter paper and husk of various particle sizes[1].

Enzyme	Substrate (particle size, p.s.)	Reaction time 2h R.S.[2] (mg.mL^{-1})	2h D.C.[3] (%)	6 h R.S. (mg.mL^{-1})	6 h D.C. (%)	24 h R.S. (mg.mL^{-1})	24 h D.C. (%)
Celluclast (82 IU g^{-1})							
	Husk, 16 mesh<p.s.<6 mesh	1.6±0.1	5.0	2.9±0.1	9.2	3.7±0.2	11.7
	" p.s.<16 mesh	2.1±0.3	6.6	3.5±0.2	11.1	4.8±0.1	15.2
	" p.s.<60 mesh	1.9±0.1	5.8	3.2±0.2	10.1	4.5±0.4	14.1
	α-cellulose	2.9±0.1	9.1	5.7±0.4	18.0	13.0±0.3	41.1
	Filter paper	3.7±0.2	11.7	7.3±0.3	23.0	14.4±0.5	45.8
Rohament CT (24 IU g^{-1})							
	Husk, 16 mesh<p.s.<6 mesh	1.8±0.3	5.7	3.1±0.2	9.6	4.0±0.4	12.5
	" p.s.<16 mesh	2.9±0.1	9.3	3.7±0.2	11.7	5.2±0.1	16.5
	" p.s.<60 mesh	2.2±0.2	6.9	3.4±0.2	10.8	4.7±0.2	14.8
	α-cellulose	2.4±0.2	7.5	6.4±0.1	20.3	11.9±0.2	37.6
	Filter paper	3.8±0.1	11.8	7.2±0.4	22.6	13.4±0.3	42.5

[1] Hydrolysis conditions as described in section 3.2.3.

[2] Reducing sugars; data are expressed as concentration (mg.mL^{-1}±SD, n=3).

[3] Degree of substrate conversion (section 3.2.3).

ground husk (< 60 mesh) in a Udy mill gave intermediate hydrolysis levels under the experimental conditions of these studies (Table 3.2). This may reflect the fact that constant dispersion of husk particles throughout the aqueous medium was not possible under the agitation conditions employed. In fact, the particles formed agglomerates within the liquid phase during incubation with the enzymes. Therefore, the above discrepancy regarding the relatively low hydrolysis yield of husk with particle size < 60 mesh may arise from diffusional constraints (removal of end products) and decreased substrate accessibility to cellulases.

Both pure cellulose substrates tested, filter paper and α-cellulose, exhibited similar, but higher susceptibilities to hydrolysis, as compared to the husk samples (Table 3.2). In effect, filter paper cellulose was found slightly more reactive than α-cellulose which could reflect differences in structure, degree of polymerization and accessible surface area of cellulose.

3.3.2 Effect of chemical pretreatments on yield and composition of enzymic hydrolyzates of husk

Hydrolysis data of both native and chemically pretreated husk residues using Celluclast (184 IU g^{-1}), Rohament CT (48 IU g^{-1}) and Celluclast supplemented with cellobiase (Novozym 188; 310 CBU g^{-1}) are presented in Figures 3.4, 3.5 and 3.6. In all cases, the reactive and/or available cellulose portion was very rapidly hydrolyzed, followed by a much slower hydrolysis of the more resistant/inaccessible parts. The levelling-off of the reaction beyond 10 h was also evident with enzyme digests containing cellobiase. This suggests that end-product

Figure 3.4: Enzymic hydrolysis using commercial cellulases of native husk (a) and husk residues pretreated with 5% w/w H_2SO_4 at 85°C for 2 h (b) (husk solids 2% w/v in 0.05 M acetate buffer, pH 4.8, temperature 50°C).

Celluclast, 184 IU g^{-1}; Rohament CT, 48 IU g^{-1}; Celluclast, 184 IU g^{-1} and cellobiase (Novozym 188), 310 CBU g^{-1}; bars indicate standard deviations (n=3).

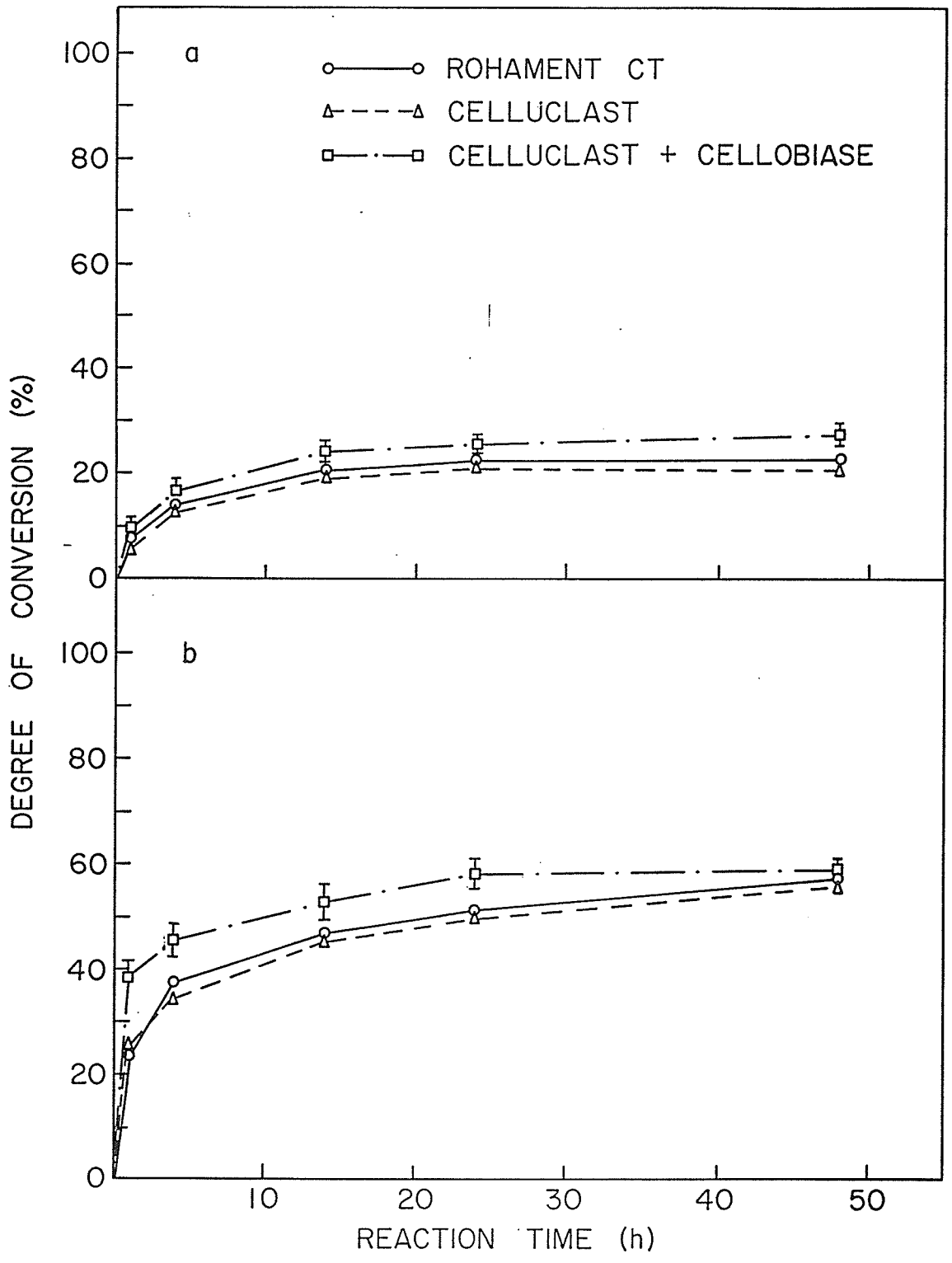

a
b
ROHAMENT CT
CELLUCLAST
CELLUCLAST + CELLOBIASE
DEGREE OF CONVERSION (%)
REACTION TIME (h)
0
20
40
60
80
100
10
20
30
40
50

Figure 3.5: Enzymic hydrolysis using commercial cellulases of husk residues (2% w/v in 0.05 M acetate buffer, pH 4.8, temperature 50°C) pretreated with 5% w/v NaOH at room temperature for 2 h (a) and at 85°C for 2 h (b).

Celluclast, 184 IU g^{-1}; Rohament CT, 48 IU g^{-1}; Celluclast, 184 IU g^{-1} and cellobiase (Novozym 188), 310 CBU g^{-1}; bars indicate standard deviations (n=3).

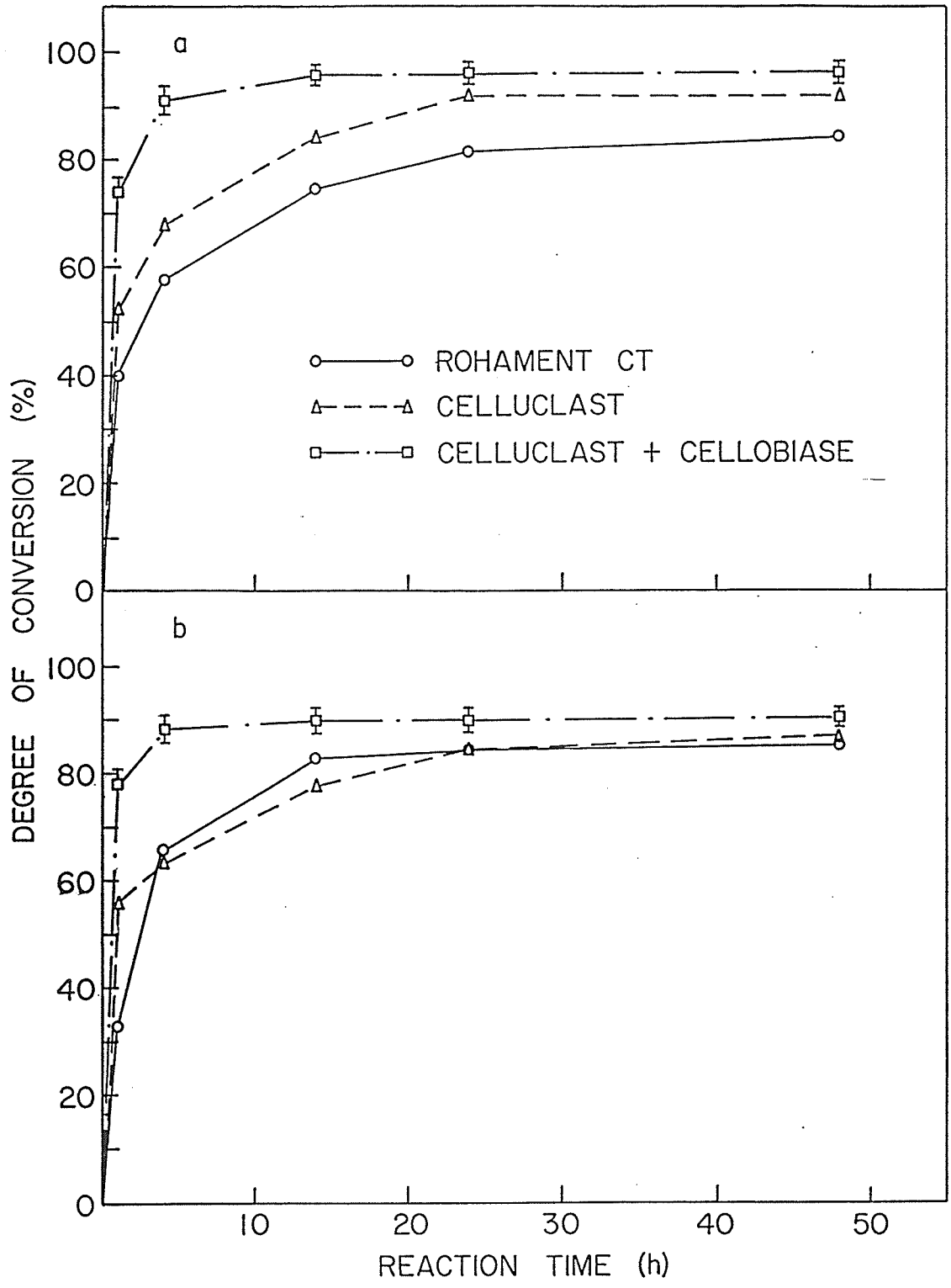
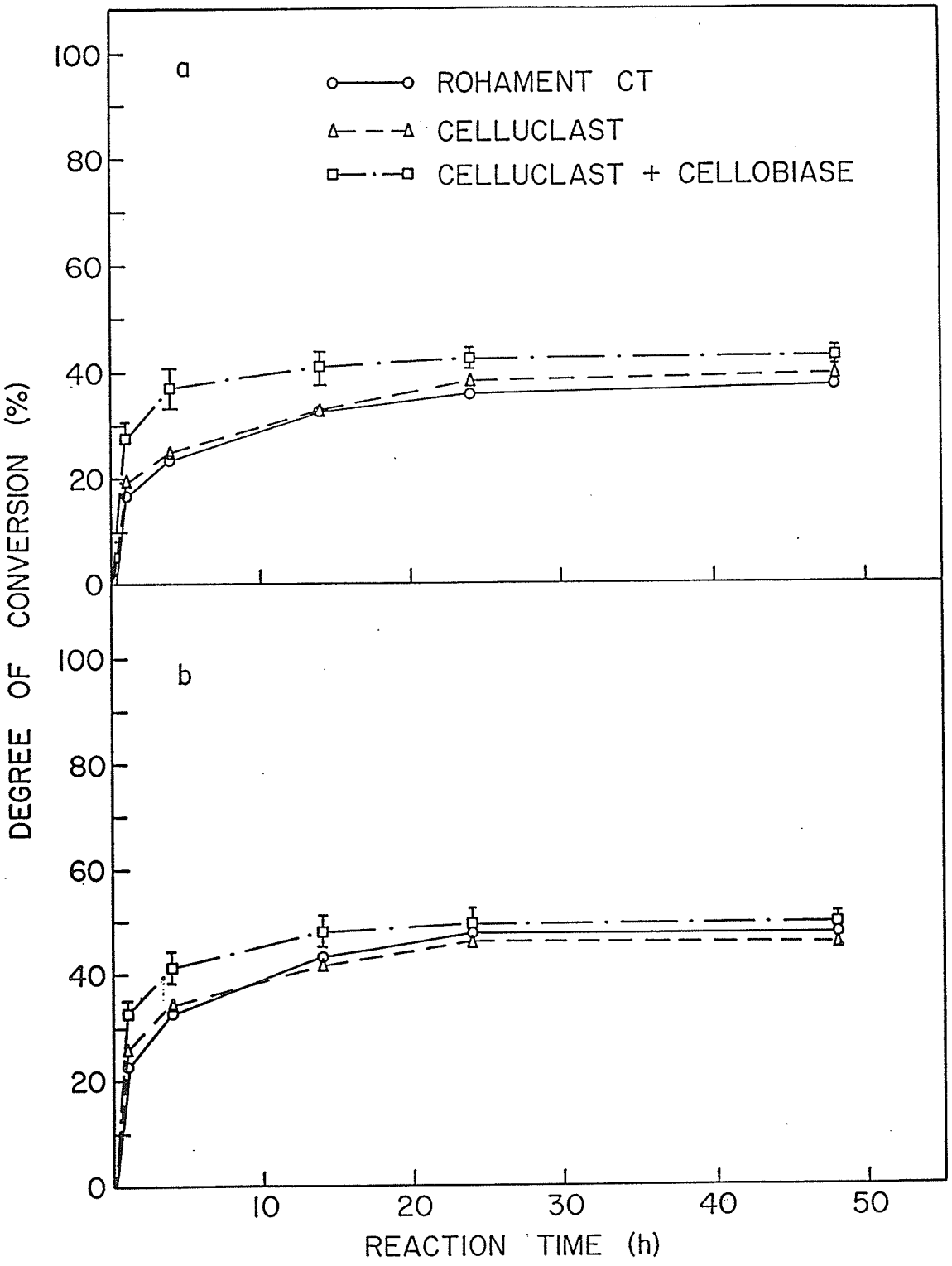

a
b
100
80
60
40
20
0
DEGREE OF CONVERSION (%)
ROHAMENT CT
CELLUCLAST
CELLUCLAST + CELLOBIASE
10
20
30
40
50
REACTION TIME (h)

Figure 3.6: Enzymic hydrolysis using commercial cellulases of husk residues (2% w/v in 0.05 M acetate buffer, pH 4.8, temperature 50°C) pretreated with 5% w/w H_3PO_4, 85°C - 2 h (a) and 14.3% w/w H_3PO_4, 85°C-2 h (b).

Celluclast, 184 IU g^{-1}; Rohament CT, 48 IU g^{-1}; Celluclast, 184 IU g^{-1} and cellobiase (Novozym 188), 310 CBU g^{-1}; bars indicate standard deviations (n=3).


a
b
ROHAMENT CT
CELLUCLAST
CELLUCLAST + CELLOBIASE
DEGREE OF CONVERSION (%)
REACTION TIME (h)
0
20
40
60
80
100
10
20
30
40
50

inhibition by cellobiose did not significantly influence the above hydrolytic pattern and that reaction kinetics are mainly governed by the structural constraints inherent with the solid substrate. Nevertheless, addition of cellobiase did accelerate hydrolysis at the early stages of the process. These observations are in agreement with several reports on bioconversion of various types of cellulosic agricultural residues (Ghose and Bisaria, 1979; Ferchak et al., 1980; Herr, 1980; Buchholz et al., 1981).

Among the various solid residues examined, untreated husk was a poor substrate for hydrolysis, giving at most, a 28% yield in reducing sugars after 48 h (Figure 3.4a). These data clearly demonstrated the limited susceptibility of native husk to cellulases and the necessity to treat this material in some way prior to the use of enzymes. In fact, hydrolysis of all chemically pretreated husk residues showed improved yields in reducing sugars (Figures 3.4b, 3.5 and 3.6). The highest degree of residue conversion (96%±0.8) was accomplished using husk residues pretreated first with 5% w/v NaOH at 25°C for 2 h and then hydrolyzed with Celluclast plus cellobiase (Figure 3.5a). A similar yield value (90%±0.6) was achieved for the 5% w/v NaOH/85°C - 2 h pretreated sample (Figure 3.5b). These findings most likely reflect the effects of alkali in swelling the lignocellulose structure and extracting hemicellulose and lignin from the complex (Table 3.1); i.e., making cellulose more susceptible to cellulase attack. In contrast, all acid pretreatments, although causing substantial hydrolysis of the hemicellulose component, brought about very little delignification (Table 3.1). As a result, they were less effective in increasing the degree of hydrolysis of husk residues (Figure 3.4b and 3.6). The corresponding yields for the 5% w/w

H_2SO_4, 5% w/w H_3PO_4 and 14.5% w/w H_3PO_4 (85°C for 2 h) pretreatment were 59%, 42% and 49%, respectively.

On the basis of the above experimental evidence, demonstrating the superiority of alkali pretreatments in enhancing the hydrolysis yield, the effect of NaOH concentration was studied to optimize this pretreatment (Table 3.3). Increasing the NaOH concentration, up to 4% w/v, resulted in a progressive increase of both amount of solubilized fraction and yield in reducing sugars. Further increase in alkali concentration (e.g. 5% w/v), however, caused no additional enhancement in the hydrolysis yield. It must be emphasized here that these samples were air dried after chemical pretreatment and thus they exhibited greater resistance to hydrolysis when compared to wet residues of all other studies. As such, the data reported in Table 3.3 have only relative value.

Analysis of the 24 h enzymic digests for oligosaccharide composition (by HPLC) revealed mainly the presence of monosaccharides (Table 3.4). A typical chromatogram of a hydrolyzate is illustrated in Figure 3.7b. Major products identified included glucose, xylose, arabinose, and mannose; cellobiose and galactose were present in trace amounts in all hydrolyzates. An examination of the chromatographic profiles of Celluclast hydrolyzates with and without added cellobiase showed very little differences in the amounts of reducing sugars and monosaccharide composition. These findings are thus in agreement with the hydrolysis data presented in Figures 3.4, 3.5 and 3.6 and further suggest that end-product inhibition, due to cellobiose, is practically negligible under the hydrolysis conditions employed in these experiments.

TABLE 3.3

Effect of NaOH concentration used in chemical pretreatment on the degree of solubilization and yield of reducing sugars upon subsequent hydrolysis of husk residues by Rohament CT (49 IU g^{-1})[1].

Pretreatment[2] (solvent/temp-time)	Degree of solubilization (%)	Reaction time 2 h R.S.[3] ($mg.mL^{-1}$)	2 h D.C.[4] (%)	24 h R.S. ($mg.mL^{-1}$)	24 h D.C. (%)
Distilled H_2O/25°C-2 h	8.0±0.5	2.5±0.1	11.2	5.5±0.3	24.8
1% w/v NaOH/25°C-2 h	19.6±0.4	7.4±0.3	33.2	11.4±0.2	51.1
2% w/v NaOH/ " "	25.7±1.0	8.5±0.4	38.4	12.3±0.4	55.2
3% w/v NaOH/ " "	30.9±0.7	8.9±0.1	40.1	13.3±0.4	59.8
4% w/v NaOH/ " "	33.4±1.2	8.9±0.5	40.1	14.2±0.3	63.9
5% w/v NaOH/ " "	37.3±0.8	8.9±0.2	40.1	14.2±0.3	64.0

[1] Hydrolysis conditions as described in section 3.2.3.

[2] Pretreatments were carried out under stirring with a magnetic bar and the residual solids after neutralization and washing were air dried before enzymic hydrolysis.

[3] Reducing sugars expressed as concentration ($mg.mL^{-1}$±SD, n=3).

[4] Degree of residue conversion (section 3.2.3) to reducing sugars.

TABLE 3.4

Reducing sugars[1] and monosaccharide composition[1] of enzymic hydrolyzates (24 h) of various husk residues[2]

Pretreatment (solvent/temp-time)	Enzyme	R.S.[3] ($mg.mL^{-1}$)	Glucose ($mg.mL^{-1}$)	Xylose ($mg.mL^{-1}$)	Arabinose ($mg.mL^{-1}$)	Mannose ($mg.mL^{-1}$)
None (control)						
	Rohament CT	4.70±0.25	2.35±0.02	0.70±0.03	-	0.19±0.05
	Celluclast	4.29±0.14	2.43±0.01	0.63±0.03	-	0.11±0.09
	Cellu+Novoz[4]	5.84±0.17	2.26±0.02	0.76±0.02	-	trace
5% w/v NaOH/25°C - 2 h						
	Rohament CT	17.92±0.23	10.34±0.01	2.83±0.02	0.38±0.01	0.10±0.08
	Celluclast	19.43±0.15	10.21±0.03	3.32±0.05	0.49±0.02	0.23±0.02
	Cellu+Novoz	20.27±0.29	9.73±0.05	3.09±0.04	0.66±0.02	0.14±0.05
5% w/v NaOH/85°C - 2 h						
	Rohament CT	18.00±0.14	12.42±0.04	2.61±0.07	0.16±0.05	0.11±0.04
	Celluclast	18.32±0.23	13.41±0.02	2.84±0.01	0.55±0.01	0.10±0.08
	Cellu+Novoz	19.10±0.36	12.22±0.04	2.68±0.04	0.51±0.03	0.26±0.07
5% w/w H_2SO_4/85°C - 2 h						
	Rohament CT	12.23±0.07	7.22±0.03	1.33±0.01	0.12±0.04	0.20±0.04
	Celluclast	12.00±0.23	7.93±0.01	1.47±0.06	0.41±0.01	0.08±0.09
	Cellu+Novoz	12.41±0.15	7.27±0.04	1.29±0.09	0.47±0.01	0.11±0.03
5% w/w H_3PO_4/85°C - 2 h						
	Rohament CT	8.13±0.16	3.75±0.02	1.18±0.04	-	-
	Celluclast	8.43±0.21	3.44±0.05	0.97±0.03	-	-
	Cellu+Novoz	8.78±0.34	3.27±0.07	0.81±0.04	-	-
14.3% w/w H_3PO_4/85°C - 2 h						
	Rohament CT	10.19±0.14	5.11±0.02	0.99±0.03	-	-
	Celluclast	10.13±0.15	4.85±0.02	0.91±0.02	-	-
	Cellu+Novoz	10.40±0.23	4.55±0.05	0.85±0.06	-	-

[1] Data are expressed as concentration ($mg.mL^{-1}$±SD, n=3).

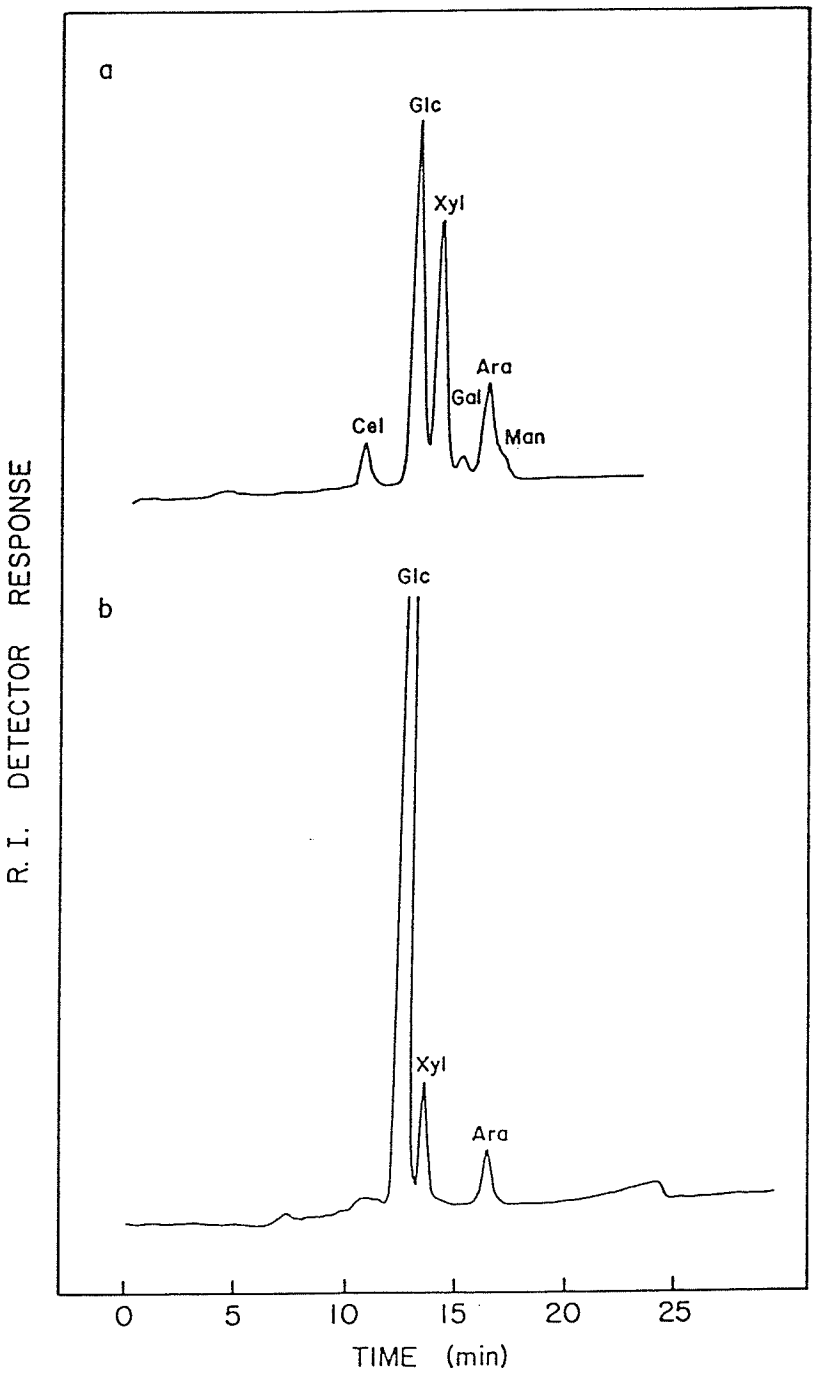
[2] Hydrolysis conditions and HPLC analysis of enzymic hydrolyzates as described in sections 3.2.3 and 3.2.4, respectively.

[3] Reducing sugars.

[4] Celluclast + Novozym 188.

Figure 3.7: Typical HPLC chromatograms: standard carbohydrate mixture (a) and an enzymic hydrolyzate (Celluclast) of 5% w/w H_2SO_4, 85°C/2 h-pretreated husk residues (b).

The Bio Rad HPX-87P (300 x 7.8 mm) column was eluted with water at 85°C and a flow rate of 0.6 mL min^{-1}; injection volume 20 uL. The molar ratio of sugars in standard mixture (a) was: Glucose (Glc): Xylose (Xyl): Arabinose (Ara): Mannose (Man): Galactose (Gal): Cellobiose (Cel) = 1.3 : 1.0 : 0.2 : 0.1 : 0.1 : 0.1.


a
Glc
Xyl
Ara
Gal
Cel
Man
b
Glc
Xyl
Ara
R. I. DETECTOR RESPONSE
0
5
10
15
20
25
TIME (min)

The hydrolysis data of chemically solubilized husk material (CSF) by Rohament CA and Celluclast are summarized in Table 3.5. Small amounts of glucose, not reported in this table, were also detected in the hydrolyzates, particularly with the acidic CSF samples. Since the objective of this study was to maximize xylose and arabinose yields, the hydrolyzates were analyzed by HPLC after 24 h of reaction, when total reducing sugars reached a constant value. The CSF samples derived from NaOH pretreatments were the substrates of interest since they consist primarily of carbohydrate constituents with $\overline{DP}$>12 (Table 2.8); i.e, these materials cannot be used, as such, for fermentation without further depolymerization. Moreover, since NaOH significantly improved the susceptibility of the residual husk solids toward cellulase action (Figure 3.5), the respective CSF deserves more attention if the overall potential of husk bioconversion is considered.

As indicated in Table 3.5, greater yields of xylose and arabinose were achieved when the chemically solubilized fractions were desalted prior to hydrolysis. However, even with the desalted samples, the degree of conversion did not reach the theoretical maximum yield; i.e., the values ranged between 73.4-79.7% and 66.2-90.2% for the 5% w/v NaOH and 5% w/w H_2SO_4 treatments, respectively. The corresponding yields for the rest of the samples (non-desalted acidic and alkaline CSF) were even lower; the values varied between 12.1-48.0% (NaOH), 44.3-57.4% (H_2SO_4) and 38.6-40.0% (H_3PO_4). Incomplete hydrolysis of hemicellulose-derived CSF samples may have been due to the fine-structure of the xylo-oligosaccharides present in CSF, in relation to substrate specificity of the enzyme components constituting Rohament CA and Celluclast, and/or end-product inhibition. For the former, it is known that the degree,

TABLE 3.5

Enzymic hydrolysis (24 h) of various chemically solubilized husk materials (CSF) by commercial enzyme preparations.

Pretreatment (solvent/temp-time)	Enzyme	PCO[1] ($mg.mL^{-1}$)	Xylose[1] ($mg.mL^{-1}$)	Arabinose[1] ($mg.mL^{-1}$)	D.C.[2] (%)
5% w/v NaOH/25°C - 2 h (desalted)[4]	Rohament CA (UF)[3]	2.03±0.02	1.46±0.04	0.16±0.02	79.7
	Celluclast[3]	"	1.19±0.01	0.30±0.01	73.4
	Celluc.+Roham.CA[3]	"	1.24±0.01	0.25±0.02	73.4
5% w/v NaOH/25°C - 2 h	Celluclast	1.04±0.01	0.50±0.02	-	48.0
5% w/v NaOH/85°C - 2 h (desalted)	Celluclast	2.10±0.01	1.37±0.03	0.21±0.04	75.2
5% w/v NaOH/85°C - 2 h	Rohament CA[3]	0.99±0.03	0.12±0.03	-	12.1
	Celluclast	"	0.40±0.04	-	40.4
5% w/w H_2SO_4/85°C - 2 h (desalted)[4]	Rohament CA	2.22±0.02	1.03±0.02	0.46±0.04	68.7
	Rohament CA (UF)	"	1.41±0.01	0.59±0.06	90.2
	Celluclast	"	1.38±0.01	0.40±0.02	80.2
	Celluc.+Roham.CA	"	1.20±0.05	0.27±0.01	66.2
5% w/w H_2SO_4/85°C - 2 h	Rohament CA	1.83±0.05	0.69±0.04	0.12±0.03	44.3
	Rohament CA (UF)	"	0.87±0.06	0.15±0.06	55.7
	Celluclast	"	0.94±0.03	0.11±0.03	57.4
5% w/w H_3PO_4/85°C - 2 h	Celluclast	0.65±0.04	0.26±0.02	-	40.0
14.3% w/w H_3PO_4/85°C - 2 h	Celluclast	1.40±0.02	0.45±0.04	0.09±0.04	38.6

[1] PCO: Pentose containing oligosaccharides, determined by the Bial reagent, as described in section 2.2.7.; data are expressed as means ± S.D. (n=3).

[2] D.C.: Degree of conversion, as defined in section 3.2.3.

[3] Enzymic hydrolysis conditions as described in section 3.2.3: Rohament CA (UF) was ultrafiltered ($56x10^4$ CU); Celluclast (73 IU); Celluclast (37 IU)+Rohament CA ($14x10^4$ CU); Rohament CA ($28x10^4$ CU).

[4] Chemically solubilized material was desalted as described in section 3.2.1.

frequency and type of side chain substitution along the (1->4)-ß-D-xylan backbone are important determinants of its susceptibility to hydrolysis (McCleary and Matheson, 1986). This is particularly relevant where hemicellulolytic enzyme preparations are deficient in α-L-arabinosidases and α-D-glucuronidases. Experiments where Celluclast was supplemented with a partially purified preparation of ß-xylosidase showed no further improvement in the overall hydrolysis yield (data not shown). The marked differences in the extent of hydrolysis between the desalted and non-desalted CSF samples (Table 3.5) also suggested that salts, present in the neutralized CSF solutions, exhibit a strong inhibitory effect on both enzyme preparations.

3.3.3 Thermal degradation (DSC) studies of husk residues after enzymic hydrolysis

The effects of hydrolysis of husk residues on their pyrolytic behavior, as revealed by DSC, were investigated using a selected number of chemically pretreated husk samples. The chemical composition of these materials, prior to enzymic degradation, is reported in Table 3.1. Representative DSC thermal decomposition profiles of several enzymatically hydrolyzed husk residues are shown in Figures 3.8 and 3.9.

In agreement with the data reported in section 2.3.4, the DSC curves of the chemically pretreated husk residues (Figures 3.8 2a, 3a and 3.9 2a, 3a) were characterized by an exotherm peaking between 320-340°C (cellulose pyrolysis) and, depending on the severity of the pretreatment conditions, by an exotherm at much lower temperatures (235-250°C) representing the degradation of residual hemicellulose components. The tran-

Figure 3.8: DSC thermal decomposition curves of husk residues. Native husk (1); 5% w/w H_2SO_4/85°C-2h (2a) and 5% w/w H_3PO_4/85°C-2h (3a) pretreated residues were further subjected to hydrolysis with Rohament CT (experimental conditions as described in section 3.2.3) for 1 h (2b, 3b) and 24 h (2c, 3c); heating rate 10°C min^{-1}. Sample weights from top to bottom (mg): 3.52, 3.40, 3.58, 3.62, 3.36, 3.55 and 3.47.

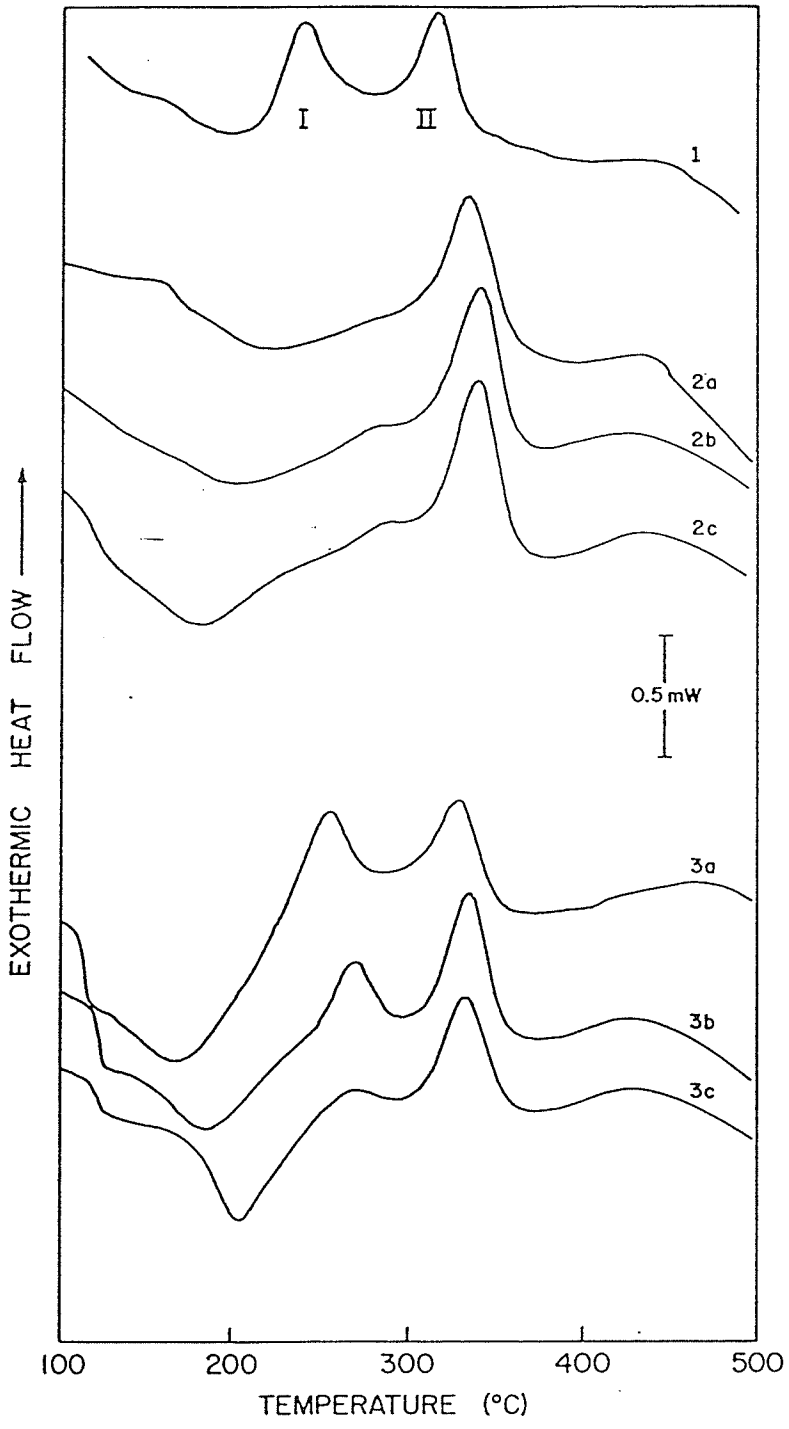
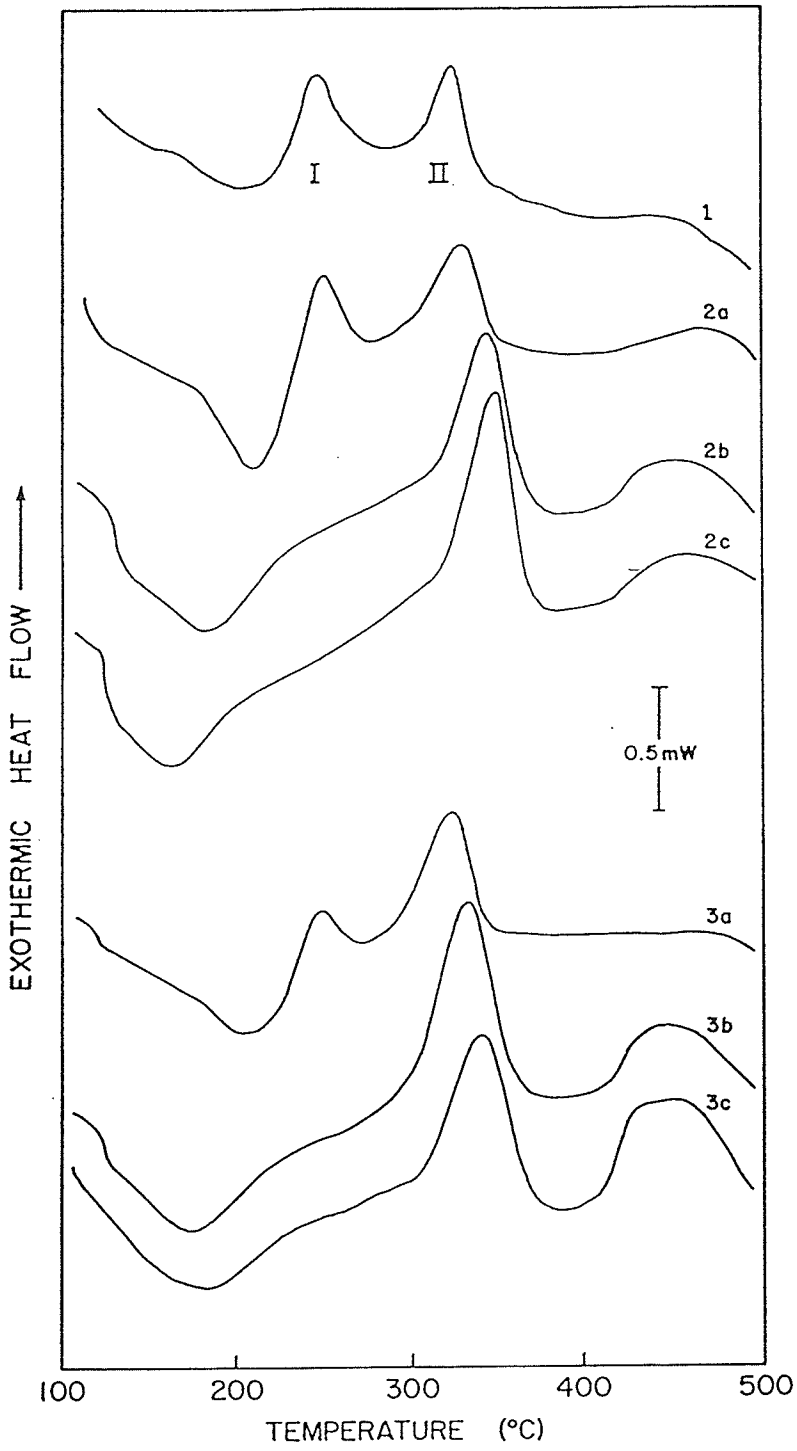

I
II
1
2a
2b
2c
0.5 mW
3a
3b
3c
EXOTHERMIC HEAT FLOW
100
200
300
400
500
TEMPERATURE (°C)

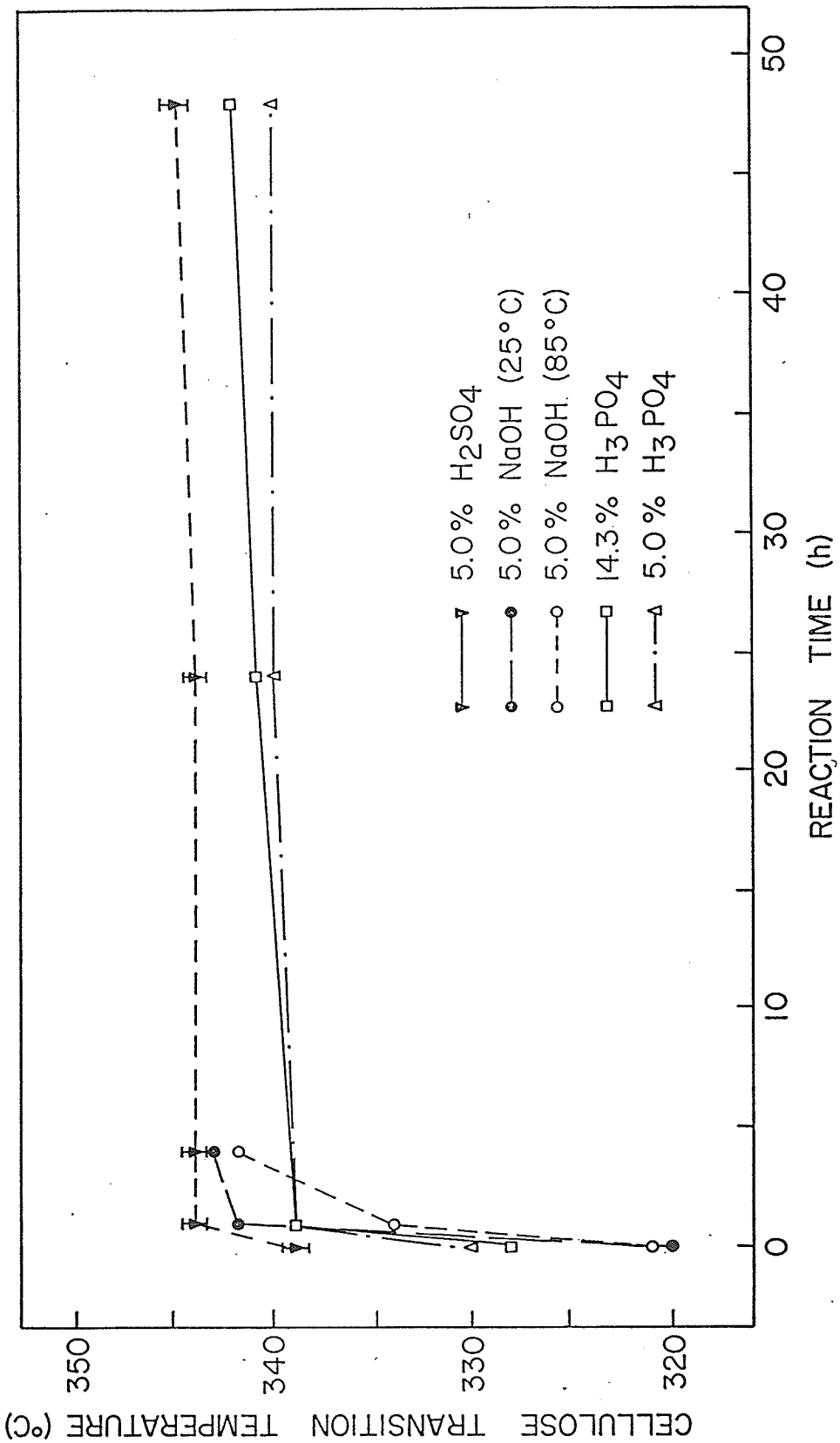
Figure 3.9: DSC thermal decomposition curves of husk residues. Native husk (1); 5% w/v NaOH/25°C-2h (2a) and 5% w/v NaOH/85°C-2h (3a) pretreated residues were further subjected to hydrolysis with Rohament CT (experimental conditions as described in section 3.2.3) for 1 h (2b, 3b) and 4 h (2c, 3c); heating rate 10°C min^{-1}. Sample weights from top to bottom (mg): 3.52, 3.61, 3.55, 3.46, 3.30, 3.45 and 3.63.


I
II
1
2a
2b
2c
0.5mW
3a
3b
3c
EXOTHERMIC HEAT FLOW
100
200
300
400
500
TEMPERATURE (°C)

sition temperatures of cellulose, for the three types of pretreatments employed (85°C) were of the order H_2SO_4 (5% w/w; 338°C)>H_3PO_4 (5% w/w; 330°C)> NaOH (5% w/v; 320°C), which indicates the influence of solvent on the stability of the residual cellulosic material. Hydrolysis of these residues with Rohament CT resulted in elimination of the hemicellulose transition (235°C-250°C) during the early stages of the process. These observations are indicative of the presence of hemicellulolytic activity in this preparation. From the DSC thermal profiles of Figures 3.8 and 3.9, it is also evident that there was an increase in temperature of cellulose pyrolysis, which can be explained analogously to the pyrolytic stability of the chemically pretreated husk residues discussed in section 2.3.4. Hydrolysis of cellulose is expected to occur preferentially at the amorphous regions of the molecules, which undergo thermal degradation at a much lower temperature than cellulose crystallites (Basch and Lewin, 1973a). As hydrolysis proceeds and hemicellulose plus the amorphous junction zones of cellulose are removed the sample becomes enriched in crystallites and thus exhibits higher stability toward hydrolysis. Figure 3.10 summarizes the dependence of cellulose peak temperature on hydrolysis time. As indicated in the figure, all samples showed a monotonic increase in pyrolysis temperature within the first hour of reaction. Beyond this point, however, the temperatures remained constant over the entire reaction time-span. Interestingly, the overall reaction time dependence of the DSC transition temperatures for these samples resembled the respective patterns of their hydrolysis kinetics (Figures 3.4, 3.5 and 3.6). Following the initial rapid phase of hydrolysis, levelling-off of the hydrolysis rate and the constant temperature of cellulose pyrolysis are thus indicative

Figure 3.10: DSC transition peak temperature (cellulose) as a function of reaction time, means ± S.D. (n=3).

Chemically pretreated samples (5% w/w H_2SO_4/85°C-2h, 5% w/w H_3PO_4/85°C-2h, 14.3% w/w H_3PO_4/85°C-2h, 5% w/v NaOH/25°C-2h, 5% w/v NaOH/85°C-2h) were hydrolyzed with Rohament CT, as described in section 3.2.3, and the residues were analyzed by DSC at various time intervals.


CELLULOSE TRANSITION TEMPERATURE (°C)
350
340
330
320
0
10
20
30
40
50
REACTION TIME (h)
5.0% H2SO4
5.0% NaOH (25°C)
5.0% NaOH (85°C)
14.3% H3PO4
5.0% H3PO4

of structural homogeneity of the residual biomass solids; i.e., they consist of crystallites of uniform size and organization.

3.4 DISCUSSION

The evaluation of the efficiency of pretreatments, in the context of overall hydrolytic conversion of lignocellulosic materials to sugars, is limited by variations in the essential characteristics of hydrolytic processes, including the saccharification conditions (temperature, pH), the concentration of substrate and enzyme as well as the assays used for the determination of enzyme activities and reaction products. Variations in these parameters do not allow a direct comparative analysis of the numerous studies published in this research area. However, some generalized comments could be made. Most importantly, higher degrees of conversion (DC) for lignocellulosic materials or their respective hydrolyzable carbohydrate constituents have been reported whenever chemical or physical pretreatments preceeded hydrolysis. Reported values for typical factors of increase are between 1.3 to 3.0 (Millett et al., 1976; Fan et al., 1981; Macdonald et al., 1983; Puri, 1984; Tanaka et al., 1985; Grethlein, 1985; Carr and Doane, 1984). The experimental findings of this study are in general agreement with such reports; i.e., the increase in the DC ranged between 1.6 (44 vs. 28%, Figure 3.6a) for 5% w/w H_3PO_4, 85°C,2 h-treated husk and 3.4 (96 vs. 28%, Figure 3.5a) for 5% w/v NaOH, 25°C, 2 h-treated husk. Consequently, these values clearly indicate the crucial role of pretreatment in enhancing the substrate reactivity and susceptibility to cellulase action.

It must be emphasized that under the most optimum conditions established for husk saccharification (pretreatment with 5% w/v NaOH, 25°C-2 h, followed by hydrolysis using Celluclast supplemented with cellobiase; section 3.3.2), around 87% of the initial husk dry matter was converted

to reducing sugars. This corresponds to almost quantitative conversion of the originally available carbohydrate material in husk. In fact, the cellulose- enriched solid fraction (57% of husk dry matter) and the corresponding hemicellulose-enriched solubilized fraction (43% of husk dry matter) were hydrolyzed into sugars by 96% and 80%, respectively. Comparable results regarding hydrolysis of residual husk following the pretreatment with alkaline hydrogen peroxide has been recently reported by Gould (1984). Furthermore, DC values reported for chemically pretreated softwoods and hardwoods are within 55-83% (Bungay et al., 1983; Grethlein et al., 1984; Grethlein, 1985; Holtzapple and Humphrey, 1984), while those for corn stover, wheat straw and bagasse are 67-77%, 70-85% and 66-80%, respectively (Macdonald et al., 1983; Tanaka et al., 1985; Fan et al., 1981; Detroy et al., 1980; Carr and Doane, 1984; Cunningham and Carr, 1984). In view of the above findings, this study has proven that husk is equally competitive as a lignocellulosic substrate for fermentable sugars production, when compared to other related and more intensively investigated agricultural residues. Moreover, the high cellulose/lignin ratio for this material, as discussed in chapter one, appears to be a highly justifiable criterion for its selection as a substrate.

Since the hydrolysis conditions were virtually identical throughout this study, differences in the cellulolysis rate and extent among the various pretreated husk samples could be solely attributed to the compositional/structural features of the respective substrates. The modifications in the structure of husk cellulosics imposed by alkali treatments were evidently superior to those imposed by the acidic

solvents, as far as the yield in reducing sugars. While for the former, the DC ranged between 83-96% (Figure 3.5), for the latter it varied from 44-59% (Figure 3.6, 3.4b). This is in general agreement with the trends of hydrolytic degradation of corn stover (Tanaka et al., 1985), wheat straw, bagasse and sunflower stalk (Farid et al., 1983) pretreated with NaOH and H_2SO_4 under comparable treatment regimes.

The beneficial influence of NaOH pretreatment may be viewed as a destabilizing effect on the hydrogen bond network, responsible for the inter- and intra-association of ß-glucosidic chains within the cellulose structure. This destabilization is believed to occur through hydration of the cellulose structure causing pronounced swelling of the cellulose fiber bundles along with disintegration of the fibers (Figures 2.6, 2.7; section 2.3.3). Improved accessibility of cellulose surfaces to enzyme adsorption, which is an important determinant in heterogeneous hydrolysis of cellulose can be thus established. In this respect, several authors have indicated that the available surface area for cellulase adsorption determines the rate and overall extent of lignocellulosics (Grethlein, 1985; Puri, 1984; Stone et al., 1969) and cellulose (Weimer and Weston, 1985) hydrolysis. The unrestricted swelling of cellulose in NaOH- pretreated husk could also be facilitated, at least in part, by the concomitant removal of lignin with alkali. Despite the observed compositional and macro-structural changes following NaOH-pretreatments, there seemed to be no significant alterations in the microstructure of cellulose chains, as revealed by the unchanged thermal resistance of the cellulose component (Table 2.6).

On the other hand, acid-pretreated husk samples did not exhibit pronounced morphological alterations (except surface pitting of the cellulose fibers). Furthermore, their compositional analysis (Table 3.1) suggested that the lignin-cellulose interassociation remained almost unaffected. A marked increase in the thermal resistance of cellulose to pyrolytic degradation was shown, however, presumably due to chain depolymerization in the amorphous phase of the cellulose structure (Table 2.6, Figures 2.9 and 3.8). Extensive hemicellulose removal upon acid pretreatment (5% w/w H_2SO_4/85°C-2 h; Table 3.1) did not seem to improve the reactivity of the residual cellulosics toward further hydrolysis. These findings lead to the conclusion that the extent of heterogeneous hydrolysis of husk solids is mainly related to the surface area available for enzyme adsorption, the level of lignification and the microstructure of the cellulose component itself. They are thus in agreement with other literature reports (Stone et al., 1969; Puri, 1984; Grethlein, 1985) which demonstrated the interdependence between biodegradability and distinctive features of the lignocellulose structure/composition, as proposed by Gharpuray et al. (1983).

Lately, attempts were made to establish relationships among certain structural features of cellulose and its relative hydrolysis rate. In this respect, the following empirical expression, which emphasized an over-riding influence of cellulose crystallinity on relative hydrolysis rate (RHR) was proposed (Fan et al., 1980):

$$RHR = 0.0262\ (SSA)^{0.195}\ (100 - CrI)^{1.04}$$

where SSA refers to specific surface area. Furthermore, Gharpuray et al., (1983) have rated the specific surface area as the most influential

structural feature, followed by the crystallinity of cellulose, while the lignin content was found to be inversely related to the relative enzymic hydrolysis (REH):

$$REH = 2.044\ (SSA)^{0.988}(100 - CrI)^{0.257}(lignin)^{-0.388}$$

Despite the fact that the above empirical equations seem useful in predicting the biodegradability of any lignocellulosic material based on its structural attributes, their application on a broad range of substrates is highly questionable. This is mainly due to the heterogeneity and structural complexity of each substrate, which, in turn, do not permit a clear distinction between hydrolytic effects caused by alterations in individual structural features. For example, changes in surface properties as a result of a chemical pretreatment may mask or overshadow the concomitant effect due to crystallinity changes (Millett et al., 1979).

In a practical saccharification process pretreated residues should not be subjected to air drying, but kept in a wet state instead, since drying was observed to reduce the yield of reducing sugars. For example, drying of 5% w/v NaOH-treated husk at 25°C lowered its DC by 17 % (64 vs. 81%; Table 3.3 and Figure 3.5a). Similar responses for pretreated wheat straw and corn stover were also published (Fan et al., 1980; Puri and Pearce, 1986). It could be postulated that water present in the fiber capillaries, acting as a plasticizer, maintains dilation of the fiber wall, which further reduces the interchain associations through hydrogen bonding. Removal of water, however, results in collapse of cell wall capillaries, and thus diminishes the surface accessibility for interaction with enzymes. The observation that available surface

area in pure cellulose could be reduced perhaps as much as 80% on drying (Stone and Scallan, 1968) further supports this postulate. In addition, Saddler et al. (1982a) found that air drying had a greater effect on pretreated samples than on the native substrate which is consistent with the experimental data of this study. Besides detrimentally affecting the cellulose surface area, drying promotes partial recrystallization of amorphous cellulose as pointed out by Watherwax (1977) and Zeronian (1985). Consequently, the reduced surface area in conjunction with the enhanced crystallinity of cellulose are considered to be the major contributing factors in lowering DC of pretreated husk residues subjected to air drying prior to hydrolysis.

The effect of particle size of cellulosic substrates on hydrolysis efficiency may be interpreted analogously to the influence of surface area to hydrolysis. Therefore, one would expect that the number of enzyme-substrate interactions (number of adsorption sites per gram of cellulose) would be a direct function of substrate particle size. This theory was indeed confirmed by Mandels et al., (1971) who observed that cellulase adsorption for Solca Floc cellulose increased as particle size decreased from 60 to 6.7 μm average diameter. The data presented in section 3.3.1 of this study are in accord with this principle. The husk with 1 mm particle size was more rapidly and extensively hydrolyzed relative to that of 2 mm diameter, which is also consistent with the results of Tanaka et al. (1985) regarding corn stover hydrolysis. However, finely ground husk having particles below 0.2 mm (< 60 mesh) in diameter exhibited lower hydrolytic potential as compared to those of 1 mm under the experimental conditions of this study. This finding could

presumably be related to diffusional constraints governing enzyme-substrate interactions as affected by agglomeration of husk particle with small diameter and could be likely overcome by more intensive mixing within the reaction system; i.e., appropriate mixing would be expected to enhance enzyme-substrate contacts at the level of every individual particle and most likely alleviate end-product inhibitory effects caused by localized concentration gradients of reaction products. Interestingly, Macdonald et al. (1983) found that the total amount of sugar produced after hydrolysis of corn stover was independent of particle size over the range of very small diameters, 40-220 nm (50-325 mesh).

An important consideration regarding hydrolysis of lignocellulosic materials is the enzyme consumption, already recognized to be high due to the low specific activity of the cellulolytic enzymes in general. In this regard, significant variations in the enzyme consumption per unit weight of husk (48 vs. 184 IU/g) required to attain similar hydrolysis levels were detected between Rohament CT and Celluclast (section 3.3.1). Differences in the balance of endo/exo-gluncanase activities in conjuction with a higher adsorption capacity of Rohament CT cellulase enzyme components could likely explain these observations. The unbalanced enzyme profile of Celluclast was first demonstrated by Lutzen et al. (1983), who reported that exo-glucanase is by far the prevailing component accounting for 65% of the Celluclast protein, while 20% was ascribed to endo-glucanase. There are no comparable results, however, available for the Rohament CT in terms of enzyme composition. Moreover, differences in adsorbability of individual cellulase components, as

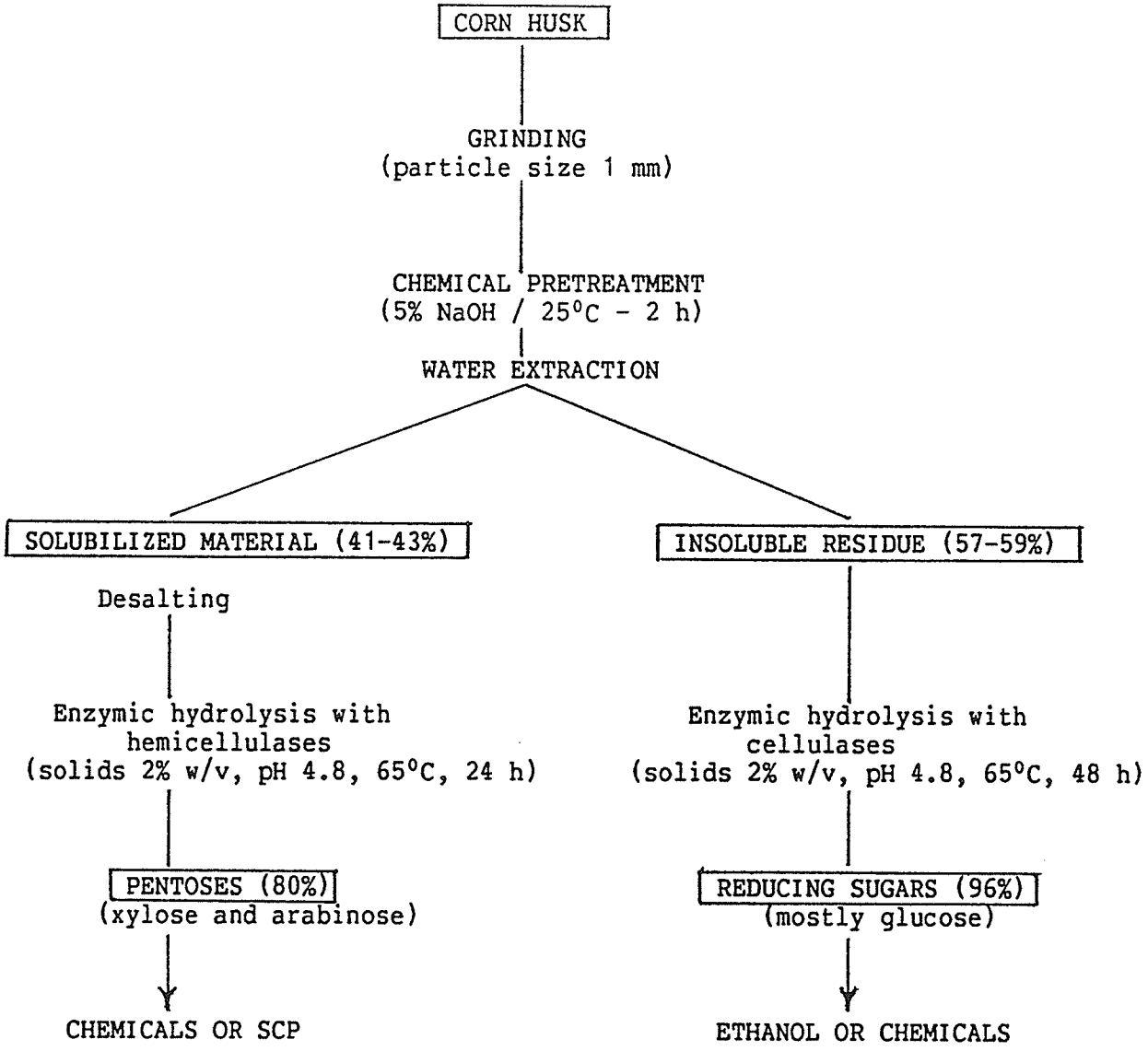
reported in several studies (Mandels et al., 1971; Ghose and Bisaria, 1979; Tanaka et al., 1986), are likely to be relevant in explaining the overall performance of the Rohament CT and Celluclast cellulase complexes. Similarly, variations in the adsorbability of the same enzyme component (e.g. exo-glucanase), depending on its microbial source, have also been reported. Ghose and Bisaria (1979) found a preferential adsorption of endo-glucanase as compared to exo-glucanase from T. reesei QM 9414 to bagasse (adsorption activation energies were found to be 3.37 vs. 4.77 $kJg^{-1}mol^{-1}$, respectively), and based on this finding they suggested a dominant role of endo-glucanase in initiating the attack on cellulose. Further support to this viewpoint comes from the observation of Klyosov et al. (1986) that the extent of hydrolysis of the crystalline part of microcrystalline cellulose mainly depends on the endo-glucanase activity and on the adsorption capacity of endo-glucanase on insoluble substrates.

The role of ß-glucosidase activity in the synergistic breakdown of cellulose by cellulase complex has been demonstrated by the hydrolysis progress curves of Figures 3.4, 3.5, and 3.6. Application of Celluclast in conjunction with Novozym 188 (cellobiase) as compared to Celluclast alone resulted in accelerated hydrolysis rates particularly during the initial stages of the reaction where the most rapid accumulation of cellobiose is expected in the vicinity of substrate-enzyme complex. These results most likely reflect the relief of end-product (cellobiose) inhibition of endo- and exo-glucanases. The observation that the influence of ß-glucosidase supplementation increased from native to acid- to alkali-pretreated husk, which is the order of increasing substrate susceptibility to hydrolysis, further supports the above notion.

Furthermore, the absence of cellobiose in all final hydrolyzates suggested that the combined action of exo- and endo-glucanases in later stages of hydrolysis, or the presence of some other route could be responsible for cellobiose breakdown to glucose. In this respect, Herr (1980) and Ladisch et al. (1980) reported that endo-glucanase from T. reesei exhibits activity toward cellobiose, while Hsu et al. (1981) documented that exo-glucanase does not hydrolyze cellobiose. In any event, the experimental results of the present study clearly indicated that the duration of hydrolysis could be appreciably decreased (14-16 h vs. 48 h) if a well-balanced cellulase complex along with a highly reactive cellulosic substrate are employed. This would have a direct bearing on the economic aspects of the overall conversion process. In summary, a relatively simple chemical and enzymic procedure has been developed to hydrolyze cellulose and hemicellulose constituents from corn husk as shown in Figure 3.11. Consideration of the lignin component and its potentially applicable degradation products has been omitted from this processing scheme because of the very low content of lignin in husk dry matter and the complex procedures required for its recovery.

Figure 3.11: Proposed scheme of chemical and enzymic processing of corn husk.

Figures in parentheses refer to the yield percentage relative to the preceding fraction in the scheme.


CORN HUSK
GRINDING
(particle size 1 mm)
CHEMICAL PRETREATMENT
(5% NaOH / 25°C - 2 h)
WATER EXTRACTION
SOLUBILIZED MATERIAL (41-43%)
INSOLUBLE RESIDUE (57-59%)
Desalting
Enzymic hydrolysis with
hemicellulases
(solids 2% w/v, pH 4.8, 65°C, 24 h)
Enzymic hydrolysis with
cellulases
(solids 2% w/v, pH 4.8, 65°C, 48 h)
PENTOSES (80%)
(xylose and arabinose)
REDUCING SUGARS (96%)
(mostly glucose)
CHEMICALS OR SCP
ETHANOL OR CHEMICALS

CONCLUSIONS AND RECOMMENDATIONS

The objective of this study was to investigate the effect of chemical pretreatments on enzymic hydrolysis of corn lignocellulosic residues into fermentable sugars and to further determine the role of structural features of lignocellulosics in its degradability. Since husk exhibited the highest cellulose to lignin ratio among all other corn residues it was selected as the experimental material of this study. The anatomical features of husk, distribution of the constituents within the tissue as well as the recognition of possible structural barriers (e.g. lignin) toward cellulolysis of lignocellulosic fibers were shown. The presence of cellulose and hemicellulose, found by chemical analysis to comprise 82.7% of husk, were confirmed by complementary histochemical tests. Additional histological evidence for the lignin component is, however, needed since staining with toluidine blue O could not verify the presence of lignified cell walls with certainty.

Preliminary investigations of a variety of chemical pretreatments, under various solvent concentration-temperature- time regimes, indicated that conditions of high temperature and pressure (121°C, 105 kPa), as suggested by several studies in the literature, were not necessary to bring about disruption of the lignin-hemicellulose-cellulose complex. Severe treatments could be effectively compensated by milder operating conditions under extended reaction times (120 min vs. 15 min).

Furthermore, such treatments are attractive because of their simplicity and low energy intensiveness. Studies undertaken to optimize pretreatments with respect to solvent type (NaOH, H_2SO_4, H_3PO_4), concentration (0.2-5.0%) and reaction temperature (25-85°C) have revealed that at concentrations <1.0%, the degree of husk solubilization remained relatively constant (10-14%), while at concentrations >1.0% it became strongly dependent on temperature and solvent type. The limiting temperature values of 25, 55 and 75°C for NaOH-, H_2SO_4- and H_3PO_4-pretreatments, respectively, indicated the superiority of alkali, as compared to acidic solvents, in dissolving husk lignocellulosics. Chemical analysis of the residues and the corresponding solubilized material after alkali treatments revealed that substantial amounts of lignin and hemicellulose components were extracted; 1.6 vs. 6.6% (lignin) and 13.0 vs. 45.0% (hemicellulose) for treated and native husk, respectively. Moreover, the simultaneous action of alkali and strong mixing by applying various shearing forces at room temperature further enhanced husk solubilization. These data provide the basis for a more extensive study of combined mechanical and chemical treatments prior to enzymic hydrolysis of husk lignocellulosics.

The effect of NaOH on native husk was also recognized morphologically by the pronounced swelling and fragmentation of cellulose fibers, which in turn increases the available surface area for adsorption of cellulolytic enzymes. However, the crystalline regions of husk cellulosics remained birefringent. In fact, cadoxen (cadmium ethylenediamine hydroxide) was the only solvent examined that had the capacity to penetrate cellulose crystallites and cause intracrystalline swelling.

Thermoanalytical studies of husk residues by differential scanning calorimetry (DSC) have shown that NaOH pretreatments caused no major changes in the thermal resistance of the cellulose component, as evidenced by the unaltered pyrolytic transition temperature (317°C). This could be explained by considering the destabilizing influence of amorphous regions of cellulose which remained relatively intact under alkali conditions. On the other hand, acidic pretreatments extensively depolymerized the hemicellulose component, but did not seem to affect the lignin-cellulose complex; lignin content decreased from 6.6% in native husk to 6.1%. Furthermore, a marked increase in the thermal resistance of cellulose to pyrolytic degradation was detected, presumably due to an increased proportion and/or perfection of cellulose crystallites following chain depolymerization of the amorphous regions.

Based on X-ray diffraction analysis data, all chemical pretreatments resulted in an increase in crystallinity of the solid husk residues; this effect was more pronounced with alkali- than acid-treated materials. The higher degree of crystalline order might be a direct consequence of enriching the residues in cellulose or it could also reflect partial conversion of amorphous into crystalline cellulose (recrystallization). Further work is needed to reveal the nature of these phenomena. For example, it would be worthwhile to examine the impact of moisture content on the recrystallization process as well as the influence of various sample drying regimes on crystallinity of the residues.

The modifications in structure of husk cellulosics imposed by alkali treatments were evidently superior to those of the acidic solvents, as

reflected by a much higher degree of conversion (DC) of husk into reducing sugars upon subsequent enzymic hydrolysis. The DC was within 83-96% for the former (5% w/v NaOH, 25°C/2 h- treated husk hydrolyzed with various cellulolytic preparations) and only between 42-59% for the latter (5% w/w H_3PO_4, 85°C/2 h- and 5% w/w H_2SO_4,85°C/2 h-treated husk hydrolyzed by Rohament CT). Further improvements in the overall efficiency and economics of alkali pretreatments could be accomplished by decreasing the alkali consumption, since there is an indication that 3% w/v NaOH could be as equally effective as 5% w/v NaOH in solubilizing native husk. Based on the above experimental findings regarding the effects of pretreatments on husk solubilization, composition, morphological characteristics, physico-chemical properties and enzymic susceptibility it was suggested that the overall enhancement in the extent of enzymic hydrolysis is governed by the surface area available for enzyme adsorption, degree of delignification and microstructure of the cellulose itself. Further investigations of the influence of crystallinity on cellulose reactivity using complementary techniques to probe crystallinity alterations (e.g. solid state C^{13}-NMR) are recommended to clarify the relationship between these two parameters.

The pyrolytic behavior of native and chemically treated husk lignocellulosics, as examined by DSC (differential scanning calorimetry) and TGA (thermogravimetric analysis), were found to be indicative of compositional and structural modifications caused by the chemical pretreatments. Consequently, the feasibility of using thermal analysis techniques as probes of structural order of lignocellulosic materials was established. Analysis of TGA data for cellulose thermal degradation

demonstrated that pyrolysis obeys first-order reaction kinetics. The corresponding apparent activation energy values were in the range of 95-166 kJ/mol and were related to the degree of crystallinity of the sample. The X-ray crystallinity values were also found to exhibit positive relationships with other TGA thermal parameters. The observations that the two polymeric families, cellulose and hemicellulose, undergo thermal decomposition at distinct temperature ranges (238 and 317°C, respectively) led to the suggestion that DSC could be used to determine the relative proportion of these constituents by measuring the reaction enthalpies associated with their transitions. This analysis would also be more rapid than any other existing wet chemistry method for determination of these polymeric constituents. However, additional development of the thermal methodologies is required to fully assess the analytical potential of DSC.

Enzymic breakdown of husk residues using two commercial cellulolytic preparations proceeded by a rapid hydrolysis of the most reactive/accessible cellulose regions, followed by a much slower hydrolysis of the more resistant parts. An increase in the transition temperature of cellulose on pyrolysis of residual husk solids after 1 h of enzymic hydrolysis further confirmed the view of a preferential degradation of amorphous cellulose in the initial stages of the reaction. Moreover, leveling off of the cellulolysis beyond 10 h was found even with enzyme digests containing cellobiase. These results, therefore, indicated that cellulolysis kinetics are governed mainly by the structural constraints inherent with the cellulosic substrate and not by end-product (cellobiose) inhibition as suggested in some literature reports. Nevertheless,

it was shown that the hydrolysis time could be substantially reduced (14-16 h vs. 48 h) if a well-balanced cellulase complex along with a highly reactive substrate (e.g. 5% w/w NaOH, 25°C/2 h-pretreated residues) are employed. In this respect, optimization of reaction conditions would also reduce the cost of the overall conversion process. Future work should be directed to characterize cellulolytic preparations with regard to endo/exo-glucanase ratio and cellobiase activity in order to relate the performance of crude cellulolytic enzymes to their composition. These studies must be further extended to investigations of the kinetic parameters and adsorptive properties of individual enzyme components to provide further insights into the mechanism of cellulose biodegradation in model and natural substrate systems.

Overall, all chemical pretreatments employed improved the yield of husk lignocellulose hydrolysis, as evidenced by the DC of 42- 96% for the pretreated husk residues, relative to 28% for their native counterpart. Under the most optimum conditions established for saccharification (pretreatment with 5% w/v NaOH, 25°C-2 h, followed by hydrolysis using Celluclast supplemented with cellobiase) almost quantitative conversion of the originally available carbohydrate material in the husk has been achieved yielding glucose and xylose as the major sugar components. Consequently, this study proved that husk is equally competitive as a lignocellulosic substrate for fermentable sugar production, when compared to other related and more intensively studied agricultural residues. Furthermore, it was also shown that crude commercial cellulase preparations are effective in hydrolyzing pretreated husk residues without the need of any purification.

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Appendix A

CALCULATION OF THE KINETIC PARAMETERS FROM THE TGA DATA FOR A REPRESENTATIVE SAMPLE.

Sample: residual husk after pretreatment with 5% (w/w) H_2SO_4/85°C/2 h

1) Application of Broido's equation on the raw data presented in Table A.1.

Wo = 14.87 mg

Wx = 0.1487 mg (equal to ash content)

Wo-Wx = 14.7213 mg

$$y = \frac{Wt - Wx}{Wo - Wx}$$

The amount of sample not yet decomposed (Wt) at time t was obtained by applying the Du Pont software analysis program.

By applying linear regression analysis [ln(ln 1/y) vs. $1/Tx10^3$], within the temperature range where the main pyrolytic events take place (302.5-334.0°C), the data were fitted according to the following equation:

$y = -16.2168x + 27.3285$ (r=-0.99).

From the slope of the straight line, an apparent activation energy of 135 kJ per mole was calculated (R=8.31 J mol^{-1} $degree^{-1}$).

TABLE A.1

T(K)	$1/Tx10^3(K^{-1})$	Wt	y	ln[ln(1/y)]
565.0	1.7594	11.442	0.7671	-1.3277
569.5	1.7555	10.858	0.7275	-1.1451
572.5	1.7463	10.400	0.6964	-1.0164
575.5	1.7372	9.867	0.6602	-0.8788
580.0	1.7237	8.893	0.5940	-0.6522
584.5	1.7104	7.755	0.5167	-0.4150
587.5	1.7017	6.928	0.4605	-0.2543
592.0	1.6888	5.647	0.3735	-0.0153
595.0	1.6802	4.826	0.3177	+0.1368
598.0	1.6718	4.133	0.2706	+0.2677
602.5	1.6593	3.331	0.2162	+0.4264
607.0	1.6470	2.889	0.1861	+0.5195
616.0	1.6230	2.606	0.1669	+0.5823

2) Application of Freeman and Carroll's equation on the raw data presented in Table A.2.

The amount of material not yet reacted (a-x) at temperature T(K) and rate of weight loss (dx/dtime) were obtained by applying the Du Pont software analysis program. Rate of weight loss over temperature (dx/dT) was calculated from the corresponding dx/dtime and the constant heating rate ($1.5^{o}C\ min^{-1}$).

TABLE A.2

t(°C)	1/Tx10³ (K⁻¹)	Δ(1/T)x10³	a-x	log(a-x)	Δlog(a-x)	dx/dtime	dx/dT	log(dx/dT)	Δlog(dx/dT)	[Δlog(dx/dT)/Δlog(a-x)]	[Δ(1/T)x10³/Δlog(a-x)]
263.5	1.8634	-	13.186	1.1201	-	0.04	0.0267	-1.5740	-	-	-
274.0	1.8277	0.0357	12.788	1.1068	0.0133	0.08	0.0533	-1.2730	-0.3010	-22.6316	2.6842
278.5	1.8127	0.0507	12.546	1.0985	0.0216	0.10	0.0667	-1.1761	-0.3979	-18.4213	2.3472
286.0	1.7884	0.0750	12.016	1.0798	0.0403	0.14	0.0933	-1.0300	-0.5440	-13.4988	1.8610
292.0	1.7694	0.0940	11.442	1.0585	0.0616	0.18	0.1200	-0.9208	-0.6532	-10.6040	1.5260
296.5	1.7555	0.1079	10.858	1.0357	0.0844	0.21	0.1400	-0.8539	-0.7201	-8.5320	1.2784
299.5	1.7463	0.1171	10.400	1.0170	0.1031	0.25	0.1666	-0.7782	-0.7958	|-7.7187|	|1.1358|
302.5	1.7372	0.1262	9.867	0.9942	0.1259	0.32	0.2133	-0.6709	-0.9031	|-7.1732|	|1.0024|
307.0	1.7237	0.1397	8.893	0.9490	0.1711	0.36	0.2400	-0.6198	-0.9542	|-5.5769|	|0.8165|
311.5	1.7104	0.1530	7.755	0.8896	0.2305	0.41	0.2733	-0.5633	-1.0107	|-4.3848|	|0.6638|
314.5	1.7017	0.1617	6.928	0.8406	0.2795	0.44	0.2933	-0.5326	-1.0414	|-3.7259|	|0.5785|
319.0	1.6888	0.1746	5.647	0.7518	0.3683	0.43	0.2867	-0.5426	-1.0314	|-2.8004|	|0.4741|
322.0	1.6802	0.1832	4.826	0.6836	0.4365	0.38	0.2533	-0.5963	-0.9777	|-2.2399|	|0.4197|
325.0	1.6718	0.1916	4.133	0.6163	0.5038	0.34	0.2267	-0.6446	-0.9294	|-1.8448|	|0.3803|
329.5	1.6593	0.2041	3.331	0.5226	0.5975	0.22	0.1467	-0.8337	-0.7403	-1.2390	0.3416
334.0	1.5470	0.2164	2.889	0.4607	0.6594	0.10	0.067	-1.1761	-0.3979	-0.6034	0.3282

When linear regression analysis was applied on the data [$\Delta\log (dx/dT)/\Delta\log (a-x)$ vs. $\Delta (1/T)x10^3/\Delta\log (a-x)$], within the temperature range (299.0-325.0)^{0}C, the following straight line equation was obtained:

$$y = -7.9381x + 0.9957 \qquad (r=-0.99).$$

From the slope of this line, an apparent activation energy of 152 kJ per mole was calculated. The intercept of this line at the ordinate is close to unity (-0.9957) and represents the order of the reaction.

Appendix B

SCHEMATIC REPRESENTATION OF THE CHROMATOGRAPHIC SYSTEM USED IN GEL PERMEATION CHROMATOGRAPHY STUDIES OF CHEMICALLY SOLUBILIZED HUSK.