

## Hydrolysis of Cellulose and Hemicellulose

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### I. INTRODUCTION

Cellulosic biomass includes agricultural (e.g., corn stover and sugarcane bagasse) and forestry (e.g., sawdust, thinnings, and mill wastes) residues, portions of municipal solid waste (e.g., waste paper), and herbaceous (e.g., switchgrass) and woody (e.g., poplar trees) crops. Such materials are abundant and competitive in price with petroleum, and cellulosic biomass can provide a sustainable resource that is truly unique for making organic products [1]. Furthermore, cellulosic biomass can be produced in many regions of the world that do not have much petroleum, opening up a new route to manufacturing organic fuels and chemicals.

The structural portion of cellulosic biomass is a composite of cellulose chains joined together by hydrogen bonding. These long cellulose fibers are in turn held together with hemicellulose and lignin, allowing growth to large aerial plants that can withstand weather and attack by microorganisms and insects. Traditionally, humans have employed cellulosic biomass for such uses as construction, soil stabilization, animal feed, and paper manufacture. However, the cellulose and hemicellulose portions of biomass, representing about 40–50% and 20–30% by dry weight of plants, respectively, are polysaccharides that can be broken down into sugars and fermented or chem-

ically altered to valuable fuels and chemicals. Sugars from such sources have been used to make ethanol and other products during periods of war by approaches such as the Scholler process during the 1940s and in controlled economies such as the former Soviet Union. Research over the last two decades has advanced biological conversion of cellulosic biomass to the point of becoming economically competitive for production of fuels and chemicals that offer important strategic, environmental, and economic advantages [2]. The challenge is to overcome the risk of commercializing first-of-a-kind technology and to continue to advance hydrolysis processes so that fuels and chemicals from cellulosic resources are competitive without subsidies.

This chapter presents a comprehensive overview of the technology and economic status for cellulose and hemicellulose hydrolysis, including a description of important structural features of cellulosic materials, applications, process steps, and stoichiometry for hydrolysis reactions. Included is an overview of the basic chemistry and processes followed by consideration of both chemical and enzymatic approaches to these reactions. The structure and functions of enzymes are described along with pretreatment of biomass to achieve high yields for these systems. The chapter continues with an overview of acid

hydrolysis and kinetics, followed by a similar treatment for hemicellulose hydrolysis to give insight into opportunities and limitations for acid-based processes. Enzymatic hydrolysis follows with a short description of different sources and types of cellulase enzyme activities involved in breaking down cellulose to glucose, as well as features of these components that influence cellulase action. The chapter then examines biomass structural characteristics that influence cellulose hydrolysis by enzymes, types of cellulose hydrolysis processes, experimental results for enzymatic conversion of cellulose, and summarizes some of the factors influencing hydrolysis kinetics. Since pretreatment is currently necessary to achieve high enzymatic yields of glucose from cellulosic biomass, options for preparing biomass for enzymatic processing are discussed.

Many promising pretreatment approaches do not fully hydrolyze hemicellulose to sugar monomers, and hemicellulase enzymes provide an attractive route to making them fermentable. Therefore sources of such enzymes, how they act, features impacting their action, experimental results with hemicellulases, and modeling approaches to predict their performance are described. The economics of hydrolysis-based processes are then summarized including a description of current cost-reduction advancements, identification of opportunities to reduce costs further, and synergies for production of multiple products from cellulosic materials. The chapter concludes with a brief description of the benefits of generating sugars through biomass hydrolysis for conversion to ethanol and other products.

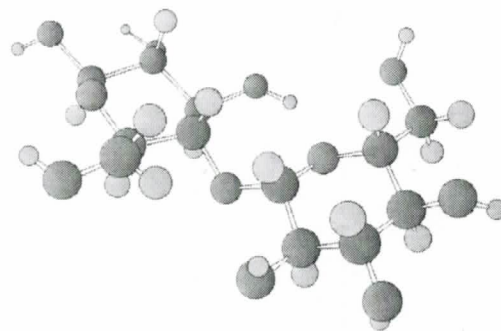
## II. OVERVIEW OF THE RELEVANT STRUCTURAL FEATURES OF CELLULOSIC MATERIALS

### A. Cellulose

As a regular, linear homopolymer, cellulose might reasonably be thought to be structurally uncomplicated. This perception would not be entirely correct, however, since cellulose chains are organized together into progressively more complex assemblies at increasing size scales. As a result of this emergent structural complexity and heterogeneity, the structure of cellulose has been remarkably difficult to unravel for such a nominally simple substance, and it is only relatively recently that a consensus picture of its organization has begun to emerge. Several recent reviews have surveyed cellulose structures, but this question is still an active topic of study [3–5].

#### 1. Chemical Composition

The chemical structure of cellulose, which is a linear polymer of  $\beta$ -(1 $\rightarrow$ 4)-linked D-glucopyranose monomer units, is in fact quite simple. Typically, cellulose chains in primary plant cell walls have degrees of polymerization (DPs) in the range from 5000 to 7500 glucose monomer units, with the DP of cellulose from wood being around 10,000 and around 15,000 for cellulose from cotton [5]. The



**Figure 1** Cellobiose, the basic repeating unit of cellulose.

basic repeating unit of cellulose is cellobiose (Fig. 1), the  $\beta$ -(1 $\rightarrow$ 4)-linked disaccharide of D-glucopyranose. At ambient temperatures, the relatively rigid glucose rings are all found in their lowest energy,  ${}^4C_1$  puckered chair conformation (Fig. 1), and do not easily make transitions to the other chair conformer or to the various possible twist-boat forms. With the rings in this conformation, all of the hydrogen-bonding hydroxyl and hydroxymethyl substituents of the pyranose rings are equatorial, directed around the periphery of the ring, while all of the hydrophobic aliphatic protons are in axial positions, pointing either up or down relative to the average plane of the rings.

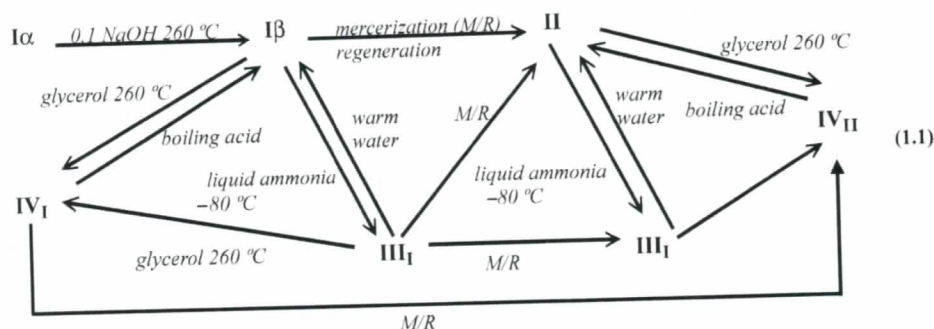
#### 2. Structure and Morphology of Cellulose Fibers

Bulk cellulose has a substantial degree of crystallinity, and its structure has long been the subject of intense study. A crystalline structure for cellulose was first described by Mark and Meyer [6] in 1928. Unfortunately, disorder and polydispersity in chain lengths prevent the formation of single crystals, and x-ray diffraction studies of the crystal structure of cellulose have been limited to fiber diffraction experiments. For this reason, the details of the crystal structure of native cellulose are still a matter of debate more than 70 years later. However, combined with modeling calculations, such experiments can be used to deduce plausible conformations consistent with the limited data. All available evidence indicates that crystalline cellulose chains are in an extended, flat, twofold helical conformation, but small variations in this conformation or in the packing of the cellulose chains within the crystal give rise to a number of crystalline polymorphs, many of which can be interconverted by various processing treatments [5,7]. Under normal conditions, cellulose is extremely insoluble in water, which is of course necessary for it to function properly as the structural framework in plant cell walls.

Seven crystal polymorphs have been identified for cellulose, which are designated as I $\alpha$ , I $\beta$ , II, III $_I$ , III $_{II}$ , IV $_I$ , and IV $_{II}$ , as indicated in the following diagram 1.1 [5].



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These polymorphs differ in physical and chemical characteristics such as solubility, density, melting point, crystal shape, and optical and electrical properties [4,6]. In nature, cellulose I $\alpha$  and I $\beta$  are the most abundant crystal forms and hence are referred to as native cellulose. Initially, x-ray fiber diffraction experiments of different native cellulose sources led to two models of native cellulose differing in the number and orientation of glucose units in the unit cell [8]. Later experiments by Atalla and VanderHart [9] in 1984 using  $^1\text{H-NMR}$  indicated that native cellulose contained two allomorphs which were designated I $\alpha$  and I $\beta$ . This picture has been confirmed by Wada et al. [10] using electron diffraction experiments. Cellulose I consists of chains arranged in a parallel fashion such that the (1 $\rightarrow$ 4) glycosidic bonds point in the same direction along the microfibril. The nature of cellulose biosynthesis requires this parallel packing. Although cellulose I is the enzymatically synthesized crystal form for this polymer, it is not the lowest-energy form; the individual fibers are kinetically trapped in this arrangement by the crystal lattice after synthesis.

Cellulose I $\alpha$  is the major allomorph produced by bacterial and fungal sources and is a triclinic  $P_1$  crystal with one cellobiose residue per unit cell [11]. The cellulose chains are oriented in a parallel manner as would be expected for a unit cell with one chain. Cellulose I $\alpha$  is converted to the more stable I $\beta$  form through an annealing process at 270°C in various media [5]. Cellulose I $\beta$  is the major crystal form in higher plant species and is monoclinic in nature with two cellobiose moieties per unit cell. Cellulose I $\alpha$  and I $\beta$  are found within the same microfibril, and hence parallel packing of the cellulose chains in cellulose I $\beta$  is the logical conclusion and is consistent with x-ray diffraction data, analysis with silver staining, and cellobiohydrolase digestion [12–14].

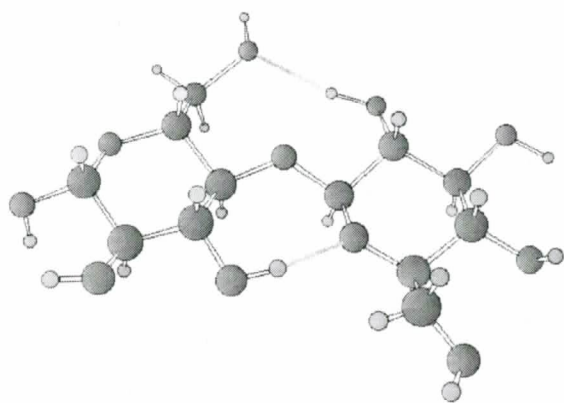
Cellulose II is the major polymorph in industrially processed cellulose. Cellulose II can be formed upon regeneration or mercerization of cellulose I and is also the most thermodynamically stable allomorph. The nature of the cellulose II structure remains to be definitely agreed upon. Most recently, Langan et al. [15] produced a cellulose II structure from neutron fiber diffraction analysis. The neutron diffraction data extended to a 1.2-Å resolu-

tion from two highly crystalline fiber samples of mercerized flax, and these workers constructed two models of cellulose II in an antiparallel arrangement with the primary alcohols in the GT conformation\* for the origin chain and in the TG conformation for the center chain. It is important to note that fiber diffraction studies cannot directly determine the primary alcohol rotamer conformation, which must be deduced from models consistent with the limited diffraction data. Apparently, no workers have attempted to fit cellulose II fiber diffraction data using a GG model for either parallel or antiparallel chains, and the question of the structure of cellulose II must be regarded as still unresolved.

The problem of describing the overall conformation of polysaccharide chains can be reduced to the problem of specifying the rotational torsion angles  $\phi$  and  $\psi$  around each successive glycosidic linkage [17]. For single crystals of cellobiose, the values of these angles are (42°, -18°) [18]. Because the glycosidic linkage is of the equatorial-equatorial type, a broad range of values of  $\phi$  and  $\psi$  are possible which involve no clashes between two linked rings. For cellulose chains, the linkage torsion angles are approximately (28°, -30°), with small variations in different polymorphs but with all cellulose structures having essentially the same extended flat ribbonlike conformation in crystals. Fig. 2 illustrates the conformation of the cellobiose repeat unit in cellulose, and Fig. 3 shows the conformation of a short hexaose oligomer in this conformation.

There are several very important qualitative features of the crystal conformation of the individual cellulose chains that should be noted. As can be seen from Fig. 3, in the very extended crystalline conformation, each glucose unit is "flipped" by 180° with respect to the previous and subsequent rings, so that the exocyclic primary alcohol groups alternately point to the right and left of the chain direction. This feature is important for the binding grooves

\*GT conformation refers to the rotamer of the primary alcohol group, where the first T (trans) or G (gauche) refers to the orientation of the C4-C5-C6-O6 torsion with respect to O5 and the second letter designates the orientation with respect to C4 [16].



**Figure 2** Flat, ribbonlike conformation of cellobiose as found in the linear cellulose chain.

and pockets of cellulase enzymes that must accommodate cellulose chains. Secondly, the chain is stabilized by strong hydrogen bonds along the direction of the chain, from the exocyclic O6 primary alcohol hydroxyl group to the O2 secondary alcohol hydroxyl group of the subsequent residue, and from the O3 hydroxyl group to the O5 ring oxygen of the next sugar residue. These hydrogen bonds help to maintain and reinforce the flat, linear conformation of the chain beyond the rigidity that might be expected for this type of linkage. Importantly, in most models for crystalline cellulose, there are no hydrogen bonds between chains in different crystal layers. With all of the aliphatic hydrogen atoms in axial positions and all of the polar hydroxyl groups in equatorial positions, the top and bottom of the cellulose chains are essentially completely hydrophobic, while the sides of the chains are hydrophilic and capable of hydrogen bonding. This topology is extremely important for the packing of chains into crystals. In all of the proposed crystal packing schemes, the chains are stacked with a pairing of hydrophobic faces, and these hydrophobic regions must make some contribution to the insolubility of cellulose under normal conditions. Furthermore, binding sites for cellulose segments in proteins must contain

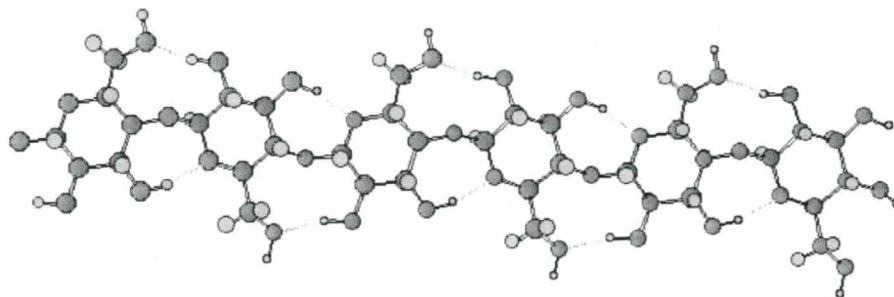
hydrophobic surfaces such as tryptophan or phenylalanine side chains to pair up with these nonpolar faces.

In native plant cellulose, the chains have extended segments of regular, repeating crystal conformation and a number of independent chains packed together into bundles or microfibrils which typically consist of from around 30 to 200 independent chains. In these microfibrils, long sequences of the individual chains are found in the extended conformation and similar segments from adjacent chains pack together into highly regular microcrystalline regions. The microfibrils are not entirely crystalline, however, even in so-called bacterial microcrystalline cellulose samples, as the regular segments of the individual chain conformations alternate with segments which are irregular in conformation. This alternation of regular microcrystalline regions with more flexible disordered regions is almost certainly the result of evolutionary selection since in order to serve effectively as the principal structural component of plant cell walls, both great strength and flexibility are needed.

## B. Hemicelluloses

The majority of the polysaccharides found in plant cell walls belong to the cellulose, hemicellulose, or pectin classes. Cellulose, the dominant structural polysaccharide of plant cell walls, is a linear  $\beta$ -(1 $\rightarrow$ 4)-D-glucopyranoside polymer. Although cellulose functions as the rigid, load-bearing component of the cell wall, the rigidity of the cellulose microfibril is strengthened within a matrix of hemicelluloses and pectins. Pectins are noncellulosic acidic cell wall polysaccharides and are divided into three classes: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II. Pectins function as a sol-like matrix, providing water and ion retention, support and facilitation of cell wall modifying enzymes, cell wall porosity, cell-to-cell adhesion, cell expansion, cell signaling, developmental regulation, and defense.

Hemicelluloses are generally classified according to the main sugar residue in the backbone, e.g., xylans, mannans, and glucans, with xylans and mannans being the most prevalent. Depending on the plant species, developmental stage, and tissue type, various subclasses of



**Figure 3** Cellohexaose showing the planar conformation and 180° flipping of the cellobiose residues in linear cellulose.



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hemicellulose may be found, including glucuronoxylans, arabinoxylans, linear mannans, glucomannans, galactomannans, galactoglucomannans,  $\beta$ -glucans, and xyloglucans. These different subtypes can be grouped into two general categories based on the hydration of the fibers. Low hydration polysaccharides include the arabinoxylans, glucuronoxylans, xyloglucans, and linear mannans. With the exception of the linear mannans, which serve mainly as a seed storage compound, this class of hemicellulose functions primarily to stabilize the cell wall through hydrogen-bonding interactions with cellulose and covalent interaction with lignin and are water-soluble in their native state generally due to their branched construction. Standard extraction protocols using alkali de-esterifies them of some of their side chains, disrupting the entropy of the water of solubilization enough to cause the partially debranched chains to aggregate through interchain hydrogen bonding and fall out of solution. Other hemicelluloses, composed mainly of hydrocolloids, are used primarily as an extracellular energy and raw materials storage system and as a water retention mechanism in seeds. Galactoglucomannans, glucomannans, galactomannans, and  $\beta$ -glucans tend to be heavily hydrated and have fewer, if any, ester-linked side chains. Alkaline extraction does not usually render them insoluble.

### 1. Structural Functionality of Hemicelluloses

Also referred to as cross-linking glycans, hemicelluloses are loosely defined as noncellulose, nonpectin cell wall heteropolysaccharides. Hemicelluloses are believed to be involved in regulation of wall elongation and modification, and, unlike lignin, are thought to have a strong effect on the interactions between cellulose microfibrils. Their chemical composition and structural features vary widely across species, subcellular location, and developmental stages. They have often been reported as chemically associated or cross-linked to other polysaccharides, proteins, or lignin. Studies of bacterial cellulose produced by *Acetobacter xylinum* in the presence of various hemicellulose fibers have shown that these hemicelluloses readily become complexed into the interior and along the surface of the cellulose microfibril [19–21]. The results of this interaction are dependent on the type of hemicellulose involved. These results have been supported by Fourier Transform Infrared (FT-IR) work on intact wood fiber [22]. Probably no chemical bonds exist between cellulose and hemicellulose, but mutual adhesion is provided by hydrogen bonds and van der Waals forces.

The side groups of xylan may have an important role in the bonding of lignin to hemicellulose. Both ester linkages between lignin and methylglucuronic acid residues and ether bonds from lignin to arabinosyl groups have been reported [23]. Low molecular weight phenolic lignin components, such as ferulic acid and *p*-coumaric acid, are covalently bound to hemicelluloses [24].

Side groups, especially the acetyl substituents, affect the physicochemical properties and biodegradability of soluble or matrix-bound hemicelluloses. Acetylation has

been shown to increase the solubility of polysaccharides in water, probably by hindering the aggregation of molecules [25,26]. On the other hand, the presence of ester-linked noncarbohydrate residues impedes the ability of individual glycanases to liberate uniform degradation products and therefore decreases the enzymatic degradability of polysaccharides. The effect of acetyl side groups on the enzymatic hydrolysis of hemicelluloses was first demonstrated by Biely et al. [27]. Chemical deacetylation of aspen wood and wheat straw has been shown to increase enzymatic degradation. Xylan became five- to sevenfold more digestible which in turn improved significantly the accessibility of cellulose to enzymatic hydrolysis [28].

### 2. Chemical Composition and Sources of Hemicellulose Subtypes

#### Xylans

The term "xylan" is a catchall for polysaccharides that have a  $\beta$ -(1 $\rightarrow$ 4)-D-xylopyranose backbone with a variety of side chains. Xylan is the predominant hemicellulose in most plant cell walls, generally comprising about 1/3 of the total plant biomass [29]. Xylan is found primarily in the secondary cell wall and seems to accumulate on the surface of the cellulose microfibril as the cellulose synthase complex extrudes the microfibril [30]. This deposition continues as the additional cell wall material is laid down, resulting in heavier xylan deposition on the cellulose microfibrils in the outermost layers of the secondary cell wall. Xylans form cross-links between cellulose, lignin, other hemicelluloses, and pectin by hydrogen bonding to the other polysaccharides and by covalent linkages through the arabinofuranosyl side chains to the ferulic and coumaric acids found in lignin.

The composition and linkages of the side chains determine the specific variety of xylan. Removal of these side chains generally enhances the rate of degradation by endoxylanase enzymes [31]. Xylans from grasses and annuals having an elevated level of  $\alpha$ -L-arabinofuranoside substituents are referred to as arabinoxylans. Arabinoxylan side chains are mainly arabinofuranose and acetyl groups and are even more heterogeneous than those from woody tissues. The L-arabinofuranoside branches are linked  $\alpha$ -(1 $\rightarrow$ 2,3) to the xylopyranose backbone. The ester-linked acetyl groups are also attached to the C2 or C3 hydroxyl. Hardwood xylans, highly substituted with acetyl and 4-*O*-methyl glucuronic acid, are termed glucuronoxylans. 4-*O*-Methylglucuronic acid is linked to the xylan backbone by  $\alpha$ -(1 $\rightarrow$ 2) glycosidic bonds, and the acetic acid is esterified at the 2 and/or 3 carbon hydroxyl group. The molar ratio of xylose/glucuronic acid/acetyl residues is about 10:1:7 [32].

In contrast to hardwoods and herbaceous hemicellulose, softwood hemicellulose is dominated by galactoglucomannan (15–20% dry weight), with xylans comprising only 7–10% of the biomass dry weight. The galactoglucomannans are divided into two subtypes of low and high galactose content with galactose/glucose/mannose ratios of 0.1:1:4 and 1:1:3, respectively [32]. Softwood xylans, arabino-4-*O*-methylglucuronoxylans, are not acetylated,



but the xylan backbone is substituted at carbon 2 and 3 with 4-*O*-methyl- $\alpha$ -D-glucuronic acid and  $\alpha$ -L-arabinofuranosyl residues, respectively. Softwood xylan has a xylose/glucuronic acid/arabinose ratio of approximately 8:1.6:1 [33] to 10:2:1 [32]. Softwood xylan also has a lower DP of about 100 compared to hardwood xylylans with DP around 200 [34].

#### Mannans and Mannan Derivatives

The term mannan indicates a linear polymer of  $\beta$ -(1 $\rightarrow$ 4)-linked mannopyranosyl residues. The structure, and hence the degradation, of mannan is very analogous to cellulose, both being linear  $\beta$ -(1 $\rightarrow$ 4)-linked monosaccharide polymers [35]. Mannan, however, is found in only a few particular plants, notably in the endosperm of the ivory nut from the Tagua Palm (*Phytelephas macrocarpa*) and a few other plants [35]. The mannan polymer can be branched, however, with various combinations of mainly glucose and galactose residues, giving rise to much more common glucomannans, galactomannans, and galactoglucomannans.

Galactomannan, believed to be an extracellular carbohydrate storage, is found predominantly, if not exclusively, in seeds of legumes [36] and is composed of a polymeric  $\beta$ -(1 $\rightarrow$ 4) mannopyranosyl backbone highly substituted with  $\beta$ -(1 $\rightarrow$ 6)-linked galactopyranose residues [37,38]. The degree of substitution varies with source. Areas of galactose-free mannosyl residues can form junction zones through interchain hydrogen bonding where there are six or more unsubstituted mannose residues. The number of free mannose regions and degree of overall substitution determine properties such as viscosity and solubility. More highly substituted galactomannans tend to have high degrees of solubility and form more viscous solutions. Locust bean gum (LBG), derived from the carob tree (*Ceratonia siliqua*), contains an average of 2000 sugar residues, with a galactose about every 3.5-mannosyl residues. Guar gum, from the seed of the leguminous shrub *Cyamopsis tetragonoloba*, contains more galactose residues than LBG, having a galactose every 1.5–2 mannose units, and is longer than LBG, with residue counts of around 10,000.

Glucomannan is a storage polysaccharide found mainly in the root of the Konjac plant (*Amorphophallous konjac*). It consists of a  $\beta$ -(1 $\rightarrow$ 4)-linked mannopyranose and glucopyranose backbone in a ratio of 1.6:1 [39]. The backbone residues are substituted in a  $\beta$ -(1 $\rightarrow$ 3) linkage with several sugars and short oligosaccharides, as well as with *O*-linked acetyl groups about every 15 residues [40,41]. Hardwoods contain low levels of glucomannan, with a glucose-to-mannan ratio of 1:1 to 1:2 [32]. Although most grasses do not contain glucomannan in the mature tissue, ramie has been shown to contain approximately 7.5% glucomannan in the whole plant [42].

Galactoglucomannan is found mainly in softwoods and is composed of  $\beta$ -(1 $\rightarrow$ 4)-linked  $\beta$ -D-glucopyranosyl and  $\beta$ -D-mannopyranosyl units which are partially substituted by  $\alpha$ -D-galactopyranosyl and acetyl groups [34]. Two types of galactoglucomannans can be separated, water and alkali-soluble fractions, with ratios of mannose/glucose/

galactose/acetyl residues of 3:1:1:0.24 for the water-soluble fraction and 3:1:0.1:0.24 for the alkali-soluble fraction [43].

#### Glucans

In addition to cellulose, the dominant polysaccharide in plant biomass, two other  $\beta$ -glucans play important roles in plant cell wall structure and function.  $\beta$ -Glucan and xyloglucan are structurally similar to cellulose, being based on a  $\beta$ -linked glucose backbone.  $\beta$ -Glucan consists of mixtures of  $\beta$ -(1 $\rightarrow$ 3)- and  $\beta$ -(1 $\rightarrow$ 4)-linked glucose residues, while xyloglucan is a straight  $\beta$ -(1 $\rightarrow$ 4) glucopyranose polymer with varying degrees of  $\alpha$ -(1 $\rightarrow$ 6)-linked xylose residues. Both structures are involved in support and cross-linking of the cellulose matrix through hydrogen bonding interactions with cellulose, other hemicelluloses, and pectins. Xyloglucan is believed to be extensively involved in support of the cell wall through hydrogen bonding interactions with the cellulose [44,45]. These hydrogen bonds are extended to multiple cellulose microfibrils, giving strong support between these cellulose bundles and forming what is referred to as the cellulose/xyloglucan network [46]. Rapid freezing, deep-etching electron microscopy evidence indicates that the cellulose-xyloglucan interactions extend beyond surface interactions to include embedding of the xyloglucan into the cellulose microfibril [45]. Inclusion of xyloglucan chains in the media during cellulose formation by *Acetobacter aceti* spp. *xylinum* has also shown that the interaction of xyloglucan with cellulose extends into the microfibril [47]. This interaction is believed to play a part in regulating cell wall expansion, mainly through weakening of the rigid structure of the cellulose microfibrils, allowing flexibility, and modification of these cross-links by specific enzymes [44,48].

Xyloglucan is found predominantly in dicotyledons [48]. In mature wood from hybrid poplar, xyloglucan comprises approximately 3% of the cell wall dry weight, reduced from the 6% found in the early primary cell wall [49]. Xyloglucans are polysaccharide polymers composed of a linear backbone of  $\beta$ -(1 $\rightarrow$ 4)-linked glucopyranose. In this aspect, they are very similar to cellulose. The difference arises due to the substitution of the glucoses with xylopyranose in an  $\alpha$ -(1 $\rightarrow$ 6) linkage. The two predominant patterns that occur are based on a repeated tetramer, either three sequential substituted glucoses followed by a single unsubstituted glucose (XXXG) or a pair of substituted glucoses followed by a pair of unsubstituted glucoses (XXGG) [50]. The xylose side chains can in turn be substituted with one or more of the following disaccharides:  $\alpha$ -(1 $\rightarrow$ 2)-L-fucosylpyranose- $\beta$ -(1 $\rightarrow$ 2)-D-galactopyranose or  $\alpha$ -(1 $\rightarrow$ 2)-L-galactopyranose- $\beta$ -(1 $\rightarrow$ 2)-D-galactopyranose, with the fucose residues being found mainly in primary cell wall [46,51–55]. As the linkage goes through a galactose  $\alpha$ -linked to the glucose, these are designated by an L in the pattern designation, such as XXLGL, where glucoses 1 and 2 are substituted with xylose, glucose 3 is linked to a galactose, and glucose 4 is unsubstituted [56].  $\alpha$ -(1 $\rightarrow$ 2)-L-Arabinofuranose has also been shown to be substituted onto either the main glucose chain or onto the galactose or xylose side groups [46,56,57]. In solanaceous plants, such



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as tobacco, tomato, and potato, the xyloglucan has a high degree of arabinosyl residues attached to up to 60% of the xylose side chains in an O-2 linkage [46]. These polymers are referred to as arabinoxyloglucans. It has also been shown that xyloglucans are acetylated through O-linkages to the arabinose or galactosyl side chains [46,54,57,58]. Despite this side-chain substitution, a specific acetylxyloglucan esterase has not been discovered.

$\beta$ -Glucan is a glucopyranose polymer containing either  $\beta$ -(1 $\rightarrow$ 3) or mixed  $\beta$ -(1 $\rightarrow$ 3),  $\beta$ -(1 $\rightarrow$ 4) linkages. The ratio of (1 $\rightarrow$ 4) to (1 $\rightarrow$ 3) linkages varies by species and gives specific properties to individual  $\beta$ -glucan polymers. In maize coleoptiles, the mixed  $\beta$ -(1 $\rightarrow$ 3),  $\beta$ -(1 $\rightarrow$ 4) glucan is deposited early in cell development, comprising greater than 70% of the cell wall material in the endosperm, and decreases rapidly after day 5 [59]. The second type of plant  $\beta$ -glucan consists of callose, a  $\beta$ -(1 $\rightarrow$ 3)-linked glucose polymer found primarily in rapidly growing structures such as pollen tubes and developing seeds [60–64].

### III. HEMICELLULOSE AND CELLULOSE HYDROLYSIS FOR BIOMASS CONVERSION

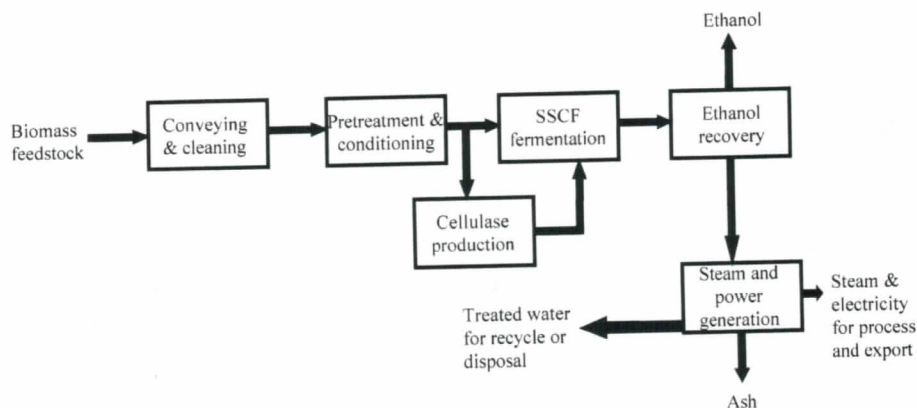
#### A. Overview of Conversion Chemistry

Cellulose and hemicellulose can be hydrolyzed to sugars and microbially fermented into various products such as ethanol or chemically converted into other products [2,65–73]. The primary challenge is that the glucose in cellulose is joined by beta bonds in a crystalline structure that is far more difficult to depolymerize than the alpha bonds in amorphous starch. Although the amorphous structure of hemicellulose makes it more easily hydrolyzed than cellulose,

hemicellulose is typically made up of five different sugars—arabinose, galactose, glucose, mannose, and xylose—as well as other components such as acetic, gluconic, and ferulic acids, and native organisms do not efficiently ferment this range of sugars to products. The remaining 20–30% or so of cellulosic biomass is often mostly lignin, a phenyl-propene aromatic compound that cannot be fermented but can be used as a high-energy content boiler fuel. In addition, cellulose contains protein, minerals, oils, and other components with the amount varying with species. Ideally, each of these fractions can be utilized to make fuels, chemicals, food, and feed [73].

#### B. Typical Process Steps

Of the many possible routes for cellulose and hemicellulose hydrolysis, our example will focus on a coupled acid and enzymatic hydrolysis approach considered by many to be most ready for near-term applications [2,66,68,70–74]. Fig. 4 presents a simplified process flow diagram for making ethanol from hemicellulose and cellulose sugars from pretreatment and enzymatic hydrolysis, respectively, based on this approach. The process starts with feedstock transportation to the process facility for storage or immediate processing coupled with cleaning operations to remove foreign objects such as rocks and dirt. Next, the material is pressurized for feeding to pretreatment reactors at high solids concentrations to minimize energy (steam) use and maximize sugar concentrations, and acid and steam are added to promote hemicellulose hydrolysis. Although actual conditions are determined by economic tradeoffs and some variability among feedstocks, about 0.5–1% sulfuric acid is applied for about 10–20 min at 140–190 °C to recover up to about 80–90% of the hemicellulose sugars [75].



**Figure 4** A simplified process flow diagram for hydrolysis of hemicellulose and cellulose to sugars for fermentation to ethanol based on dilute acid pretreatment, enzymatic hydrolysis of cellulose, and fermentation of both cellulose and hemicellulose to ethanol in the same vessel as for cellulose hydrolysis by the SSCF operation.

Following pretreatment, the reactor contents are usually rapidly cooled by flashing to a lower pressure to stop sugar degradation reactions. The solids containing most of the cellulose and lignin from the feed are washed and combined with the liquid hydrolyzate stream that is conditioned with lime to remove inhibitors. Ion exchange is applied, if necessary, to remove acetic acid and other components that can retard downstream biological operations. About 4–9% of the washed solids and possibly some conditioned hydrolyzate can be used to produce cellulase by an organism such as *Trichoderma reesei*. These enzymes can be added to the bulk of the pretreated solids to hydrolyze cellulose to glucose. Many operations are currently based on adding the cellulase to the pretreated solids along with the conditioned hemicellulose sugar stream and fermenting the resultant sugar stream with an organism such as genetically engineered *Escherichia coli* that converts all of five sugars in hemicellulose and cellulose to ethanol with the high yields vital to commercial success. In this simultaneous saccharification and cofermentation (SSCF) configuration, the glucose released by enzymatic hydrolysis of cellulose, as well as the free sugars from hemicellulose hydrolysis, are converted into ethanol as rapidly as they are released. Ethanol is recovered from the fermentation broth by distillation and dehydration. The bottoms from the distillation column contain unreacted cellulose, lignin, cellulase, fermentative organisms, and other residual ingredients and are recovered and burned to generate all the heat and electricity needed for the conversion process with excess electricity sold for extra revenue. A portion of the water in the bottoms is recycled to the process, while the rest passes to wastewater treatment for proper disposal. Methane gas produced during anaerobic digestion of dissolved components is also fed to the boiler to generate heat and electricity.

The SSCF configuration is favored to reduce equipment costs and the powerful inhibition of cellulase activity by glucose and, particularly, cellobiose. Adding cellulase, pretreated cellulose, and fermentative organisms in the SSCF configuration has been clearly demonstrated to reduce ethanol production costs [66]. The low concentration of free glucose and presence of ethanol also make it more difficult for invading microorganisms to take over the fermentation reaction and form undesirable products. Although SSCF has similar potential to improve fermentation of cellulosic sugars to products other than ethanol, most of these products are less volatile than water, and their separation from the complex fermentation broth at the completion of fermentation is more challenging. In addition, other combinations of pretreatment and hydrolysis reactions can be applied to release sugars from cellulose and hemicellulose fractions, as described in other sections of this chapter.

### C. Cellulose and Hemicellulose Hydrolysis Reactions

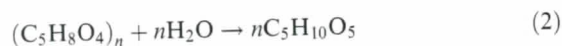
Acids or enzymes known as cellulases catalyze the reaction of water with glucan molecules in these chains to

release single glucose molecules—monomers—by the following reaction:



Thus, each glucose unit in the long chain combines with a water molecule, and 180 mass units of glucose are released from 162 mass units of glucan and 18 mass units of water, an 11.1% mass gain. Oligomers made up of several glucose molecules may also be released as intermediates in cellulose hydrolysis and often contain only 2 (cellobiose) to perhaps 3 (cellotriose) glucose units. Cellulase enzymes are very specific in only catalyzing addition of water to glucan chains, and the optimum temperature needed for reaction (1) is only about 50°C, virtually eliminating degradation reactions. Thus, only glucose is formed via enzymatically driven hydrolysis of cellulose, and yields can approach 100%. On the other hand, use of dilute acids (e.g., 1.0% sulfuric acid) to drive reaction (1) requires much higher temperatures of about 220°C that trigger the breakdown of glucose to degradation products such as hydroxymethyl furfural. Concentrated acids (e.g., 75% sulfuric acid) can be used at moderate temperatures to achieve high yields similar to those for cellulase enzymes.

Hemicellulose can also be hydrolyzed by the addition of water to release individual sugar chains contained in the longer hemicellulose molecule. The stoichiometry for the reaction of the hexose sugars galactose, glucose, and mannose that are in hemicellulose is the same as shown in Eq. (1), and an 11.1% mass gain results. On the other hand, water addition to the five-carbon sugar molecules arabinan and xylan in the hemicellulose molecule proceeds by the following stoichiometry:



with  $(C_5H_8O_4)_n$  being a chain made up of  $n$  arabinose or xylose (pentose) molecules that can be termed arabinan or xylan, respectively, and  $C_5H_{10}O_5$  being one of the corresponding pentose sugars formed by hemicellulose hydrolysis. We can see that the molecular weight of the sugar molecule released increases from 132 mass units before hydrolysis to 150 mass units of pentose sugars formed, a gain of over 13.6%. Hemicellulose is open to attack at intermediate positions along its long backbone with the release of oligomers made up of many sugar molecules that can be successively broken down to shorter-chained oligomers before single sugar molecules are formed [76]. A suitable cocktail of enzymes known collectively as hemicellulase can catalyze addition of water to hemicellulose with high specificity at modest temperatures, thus avoiding sugar degradation and resulting in high sugar yields. Dilute acids (e.g., sulfuric) can also catalyze hemicellulose hydrolysis to sugars at temperatures of about 100–200°C, but furfural and other degradation products are formed from the sugars at these temperatures if one targets good yields of hemicellulose sugars in solution. Nonetheless, degradation of the sugars released can be modest enough to recover about 80–90% of the maximum possible sugars [75]. On the other hand, operation



## Hydrolysis of Cellulose and Hemicellulose

without adding acid limits recovery of hemicellulose sugars to about 65% or less, with most in oligomeric form [77].

### IV. ACID HYDROLYSIS OF CELLULOSE

#### A. System Description and Performance

When heated to high temperatures with dilute sulfuric acid, long cellulose chains break down to shorter groups of molecules that release glucose that can degrade to hydroxymethyl furfural [78–80]. Generally, most cellulose is crystalline, and harsh conditions (high temperatures, high acid concentrations) are needed to liberate glucose from these tightly associated chains. Furthermore, yields increase with temperature and acid concentration, reaching about 70% at 260°C [80]. However, pyrolysis and other side reactions become very important above about 220°C, and the amount of tars and other difficult to handle by-products increases as the temperature is raised above these levels [81]. In addition, controlling reaction times for maximum glucose yields of only about 6 sec at about 250°C with 1% sulfuric acid presents severe commercial challenges [81].

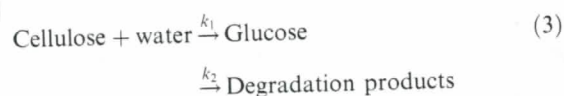
Various reactors have been tested over the years to release glucose from cellulose with dilute acid. Older reactors, such as employed during the 1940s, tended to be large vessels that could be heated through jackets. More recently, direct steam injection has been applied to rapidly heat cellulose-containing biomass to the high temperatures required. Some of these reactors have been tubular flow systems with steam addition to quickly heat biomass slurries, but rapid build up of tars limited their use [81]. Others have used twin screw extruders to promote mixing and control vessel residence times [82], but such equipment is very expensive and not easily scaled to the high throughputs needed for commercial operations. Overall, yields of about 50–60% of the glucose in cellulose can be recovered with temperatures of around 220°C in typical cocurrent or batch reactors [80,83]. However, recent results with flow of liquid through solids that are compressed throughout the reaction to minimize void volume show close to theoretical yields of glucose from acid hydrolysis of cellulose in pre-treated yellow poplar wood [84]. Unfortunately, no commercially viable equipment has yet been demonstrated to duplicate this performance.

#### B. Kinetic Models

The dilute acid hydrolysis of cellulose is typically pictured as addition of a hydrogen ion to form a conjugated acid, leading to cleavage of a glycosidic bond as a water molecule is added to release both glucose or short chains of glucose molecules (glucose oligomers) and an H<sup>+</sup> ion. In this sense, the hydrogen ion acts as a catalyst that facilitates hydrolysis. The oligomers can further hydrolyze to glucose. Amorphous portions of cellulose and oligomers break down very rapidly to form glucose monomers while crystalline regions of cellulose react much more slowly. Unfortunately, the severe conditions needed to hydrolyze crystalline cellulose also degrade glucose to products such

as hydroxymethyl furfural (HMF), levulinic acid, and formic acid. Thus recovery of glucose requires trading off conditions to maximize cellulose hydrolysis against degradation of glucose [80].

Most cellulose hydrolysis models are based on those developed by Saeman [79] for reaction of Douglas fir in a batch reactor with 0.4% acid where cellulose is hydrolyzed to glucose, which in turn breaks down to degradation products according to the following series reaction:



Assuming homogeneous first-order reactions with excess water, the rates for Eq. (3) can be described as:

$$r_C = -k_1 C \quad (4)$$

$$r_G = k_1 C - k_2 G \quad (5)$$

in which  $r_C$  represents the reaction rate of cellulose,  $r_G$  is the reaction rate of glucose,  $C$  is the mass of cellulose,  $G$  is the mass of glucose, and  $k_1$  and  $k_2$  are reaction rate constants for reaction of cellulose and glucose, respectively. The rate constants in Eqs. (4) and (5) are typically assumed to follow an Arrhenius temperature dependence of the form:

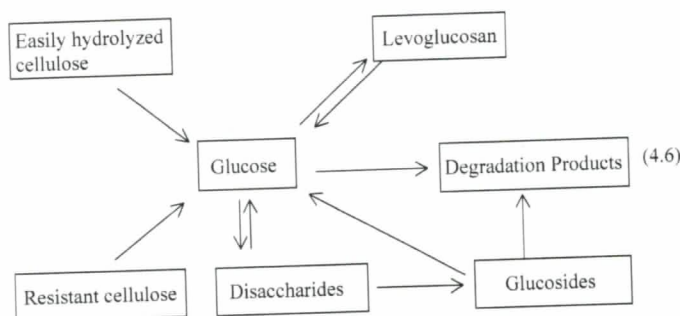
$$k_i = k_{i0} A^{m_i} e^{-E_i/RT} \quad (6)$$

in which  $k_{i0}$  is a constant,  $A$  is the acid concentration in weight percent,  $m_i$  has a value close to 1.0,  $E_i$  is the activation energy,  $R$  is the universal gas constant, and  $T$  is the absolute temperature. For batch systems, the yield of glucose is determined by incorporating Eqs. (4) and (5) into time-dependent mass balances and integrating the expressions to arrive at the following relationship:

$$G = C_0 \left[ \frac{k_1}{k_1 - k_2} \right] (e^{-k_2 t} - e^{-k_1 t}) + G_0 e^{-k_2 t} \quad (7)$$

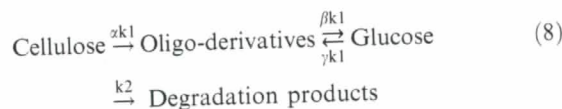
in which  $G$  is the mass of glucose at any time  $t$  and  $C_0$  and  $G_0$  are the initial quantities of cellulose (expressed as equivalent mass units of glucose) and glucose, respectively. The rate constants for these equations are usually fit to experimental data using a least squares method, and numerous studies were reported between 1945 and 1990 that applied Saeman kinetics to follow cellulose hydrolysis data of various cellulosic materials in plug flow or batch reactors (e.g., Refs. 79–81). All models predict that the glucose yield increases with acid concentration and temperature, but no model predicts yields greater than 70% of theoretical for the range of conditions examined.

Although these models provide a useful indication of the tradeoffs in converting cellulose to glucose and help define optimum conditions and operating strategies, various researchers have tried to enhance the models to more fully describe hydrolysis. For example, Conner et al. [85] incorporated reaction of two fractions of cellulose, one more easily hydrolyzed than the other, as well as reaction of glucose to an array of products, some of which can reversibly form glucose, according to the following scheme:



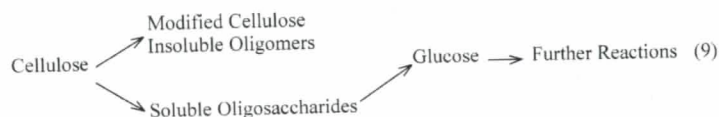
This approach improved the fit of experimental data to the model curves.

Because the conversion of oligomers to glucose is two to three times faster than hydrolysis of cellulose to soluble oligomers, oligomer formation was not initially recognized, but Abatzoglou et al. [86] found oligo-derivatives during hydrolysis of alpha cellulose in a cascade reactor with 0.2–1.0 wt.%  $H_2SO_4$ , with particularly significant amounts in the early stages of hydrolysis. To include this result, the Saeman model [79] was extended to:



Based on this mechanism, consideration was given to three possibilities: (1) the oligo-derivatives to glucose reaction is in equilibrium; (2) the oligo-derivatives to glucose reaction is not in equilibrium; and (3) there are no repolymerization reactions ( $\gamma = 0$ ). The third model proved to best represent experimental data. These results led Abatzoglou et al. to suggest using a two-step process for optimal glucose yield in which the reaction of cellulose to oligomers is catalyzed in a first stage followed by the oligomer to glucose reaction in a second stage under milder conditions. This approach would reduce formation of degradation products.

Bouchard et al. [87] applied thermogravimetric analysis, differential scanning calorimetry, and diffuse reflectance infrared to show that the chemical structure of cellulose remaining after hydrolysis in a plug flow reactor changed significantly. Using a semibatch flow-through system, Mok and Antal [88] concluded that an acid-catalyzed parasitic pathway competes with acid-catalyzed hydrolysis to produce cellulose or nonhydrolyzable oligomers that cannot be hydrolyzed to glucose, but that there is no significant chemical change in the cellulose itself. Based on these studies, the path for reaction of cellulose to nonreactive modified cellulose and insoluble oligomers that parallels production of reactive soluble oligosaccharides along with degradation of glucose limit glucose yields to less than 70%, as suggested by the following reaction pathway:



In addition, this model suggests that high severity processes induce structural rearrangements that affect the kinetic properties of the cellulose.

The oligomer formation model suggests that cellulose forms shorter chain length species prior to forming monomeric glucose, as one would expect based on depolymerization models. It is interesting to note that prior to Saeman reporting his first-order kinetic model, Simha [89] in 1941 presented depolymerization models based on random and nonrandom scission of the long polymer chains to progressively shorter oligomers and monomers. For the former, the number and weight average molecular weight both drop rapidly and then tended to slow, with the average degree of polymerization  $\alpha$  given by:

$$\alpha = 1 - \exp(-kt) \quad (10)$$

On the other hand, for preferred breaking of the ends of the chain, the weight average molecular weight decreases much more slowly than the number average value because the rapid formation of monomers contributes far less to the former. Cellulose hydrolysis was best described by assuming a reaction behavior intermediate between these extremes with bonds for molecules at the ends of a chain reacting twice as fast as those in the interior. Unfortunately, little work has been reported on the application of such depolymerization models to cellulose hydrolysis subsequent to these studies.

More recently, nearly theoretical yields of glucose from acid hydrolysis of cellulose in pretreated yellow poplar wood have been reported for flow of liquid through solids that are compressed by a spring to minimize their volume throughout the reaction in a “shrinking bed percolation” device [84]. The release of glucose varied depending on whether a batch, percolation, or shrinking bed percolation reactor configuration was employed, with the latter obtaining the highest yields. While biphasic kinetics were observed for the batch system at lower hydrolysis severities, the initial hydrolysis rate constant was about five times greater for the percolation reactor than for batch operation using 0.07% sulfuric acid at 225°C. Overall, the



## Hydrolysis of Cellulose and Hemicellulose

data suggest that an intermediate step occurs between cellulose hydrolysis and glucose release to the bulk fluid that is affected by the flow velocity. They also observed a flattening of the glucose yield curves with increasing acid concentration. Diffusion resistance due to boundary layer resistances resulting from structured water, viscosity, and rehydrogen bonding of released glucose was postulated to cause these changes in performance with flow.

## V. ACID HYDROLYSIS OF HEMICELLULOSE

### A. System Description and Performance

In simple terms, acid hydrolysis of hemicellulose is similar to that for cellulose: acid catalyzes the breakdown of long hemicellulose chains to form shorter chain oligomers and then to sugar monomers that the acid can degrade. However, because hemicellulose is amorphous, less severe conditions are required to release hemicellulose sugars. Steam is often directly injected into the biomass to rapidly heat it up, and the acetic acid contained in the hemicellulose chains can provide hydrogen ions to promote hydrolysis. Yet, in this case, hemicellulose sugar yields are limited to about 65% of the maximum possible [77]. On the other hand, adding dilute sulfuric or other acids results in much higher hemicellulose sugar yields and more digestible cellulose at a relatively low cost [75,90]. Steady progress has been made in refining the technology to remove hemicellulose with better yields and improved cellulose digestibility, and the process has been demonstrated to be effective on a variety of biomass feedstocks [75]. For example, yields of about 85–90% of the sugars can be recovered from the hemicellulose fraction with temperatures around 160°C, reaction times of about 10 min, and acid levels of about 0.7%. In addition, about 85% to over 90% of the remaining solid cellulose can be enzymatically digested to produce glucose. These high yields are vital to low costs [2,66,68,71] and have led many to favor dilute sulfuric acid hydrolysis for near-term applications [68,71].

Despite often being considered a front-runner, dilute sulfuric acid hydrolysis is expensive, representing about one-third of the total cost of making ethanol in one study [2]. Its corrosive environment mandates use of expensive materials of construction [66,68,71]. In addition, reaction degradation products such as furfural and lignin fragments and solubilized biomass compounds such as acetic acid, all of which can be inhibitory to fermentative microbes, must be removed by overliming, steam stripping, or other processes before fermentation [91,92]. Although lime is least expensive for acid neutralization and hydrolysate conditioning, the gypsum formed has reverse solubility characteristics that cause downstream difficulties. Furthermore, even low-cost sulfuric acid and lime impact costs that mount when disposal is included. Additionally, about a 7-day reaction time with expensive cellulase loadings of up to 20 IFPU/g cellulose is needed to realize good yields in subsequent enzymatic hydrolysis. Finally, dilute acid pretreatment can consume considerable process power for size reduction [66,68,71]. Thus, it is

vital to optimize dilute acid pretreatment in conjunction with downstream processing to minimize these costs as much as possible. In line with this, flow-through and countercurrent flow reactors have shown many desirable features for pretreatment by hemicellulose hydrolysis including high yields of hemicellulose sugars and enhanced cellulose digestion with low or no acid addition [93–95]. However, the large volumes of water needed and difficult-to-implement equipment configurations present important challenges that must be addressed if the technology is to be applied commercially.

### B. Kinetic Models

Overend and Chornet [96] combined treatment temperature and time in a single severity parameter  $R_0$  for hemicellulose depolymerization by steam or aqueous pretreatment without acid addition, with  $R_0$  defined as:

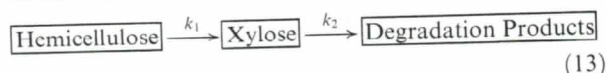
$$R_0 = t \times \exp \left[ \frac{T - 100}{14.75} \right] \quad (11)$$

in which the time  $t$  is usually expressed in minutes and  $T$  is the temperature in °C. Xylan removal from hemicellulose solids follows a consistent pattern when plotted against  $R_0$  or  $\log R_0$  for a variety of feedstocks and temperatures, allowing one to readily estimate tradeoffs among time and temperature. Although Eq. (11) would only be expected to describe solids decomposition for first-order kinetics, xylose recovery somewhat coincidentally also tracks well when plotted against  $R_0$  for a wide range of conditions, and it has been shown that  $\log R_0$  is equal to about 3.8 at the point of maximum hemicellulose sugar yield for a variety of feedstocks and process systems. The severity parameter was subsequently extended to include the effect of acid addition on hemicellulose solubilization and sugar recovery in a combined severity parameter for dilute acid hydrolysis defined as [86]:

$$C_0 = [H^+]t \times \exp \left[ \frac{T - 100}{14.75} \right] \quad (12)$$

with  $[H^+]$  being the hydrogen ion concentration and all other parameters being as defined for Eq. (11). The severity parameter is similar to an 'H' factor relationship used in the context of Kraft pulping processes and is a valuable tool for both process control and predicting yields.

Stepwise kinetic models have also been developed to describe hemicellulose hydrolysis, and most can trace their origins to those applied by Saeman [79] to describe cellulose hydrolysis in 1945. Thus they assume a homogeneous first-order series reaction mechanism. Furthermore, many of the models incorporate the further assumption that oligomers react so fast to monomers that oligomers can be omitted from consideration [97]. For this two-step reaction, hemicellulose is hydrolyzed to xylose that in turn breaks down to degradation products in a second reaction as follows:

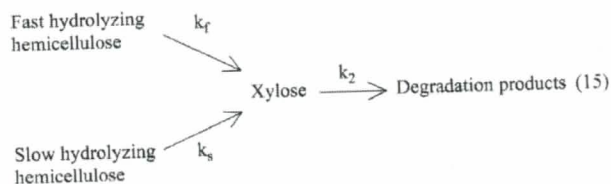


The same rate expressions and integrated forms for batch hydrolysis apply as used to describe cellulose hydrolysis in Section IV. As before, the rate constants  $k_i$  are assumed to follow an Arrhenius temperature relationship of the form

$$k_i = k_{i0} A^{m_i} e^{-E_i/RT} \quad (14)$$

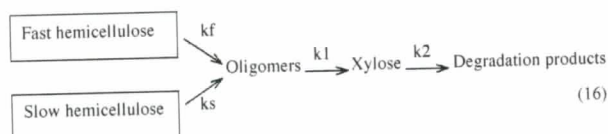
in which  $k_{i0}$  is the preexponential factor,  $A$  is the concentration of acid by weight,  $m_i$  is a power close to 1.0, and  $E_i$  is the activation energy. The maximum yield predicted by this type of model varies from 83% to 95%.

In 1955, Kobayashi and Sakai divided hemicellulose into two arbitrary fractions with one hydrolyzing more quickly than the other according to two distinct kinetic constants. This approach was used to account for the decrease in reaction rate with conversion, and most subsequent models of hemicellulose hydrolysis have been based on this reaction scheme, as follows [97]:



The proportion of fast and slow fractions is determined by fitting to kinetic data and are typically about 65% and 35% respectively, with only slight variations among materials studied.

Although ignored in the models above, oligomer intermediates are frequently observed experimentally. For example, Kim and Lee [98] measured increases in monomer yields if they subjected the hydrolysate solution from batch hemicellulose hydrolysis to secondary acid treatment, showing that dissolved oligomers are present. Oligomers are particularly significant in flow systems such as hydrothermal pretreatment in which oligomers are removed before they have time to form monomers, and the concentration of solubilized material could be more than tripled by recirculation [93,94]. In a reverse-flow, two temperature reactor configuration, the fraction of dissolved xylose as monomers can be as low as 31% [95]. Recently, Li et al. [76] applied liquid chromatography techniques to measure a distribution of chain lengths for uncatalyzed hydrolysis of xylan that clearly shows formation of long-chained species followed by their decomposition to shorter-chained oligomers and then monomers. A few models include formation of oligomers as intermediates in the following type of reaction sequence [99]:



Others have included two groups of oligomers in the pathway for hydrolysis of hemicellulose without addition of acid to recognize that the initial oligomers are of longer chain length than those formed later [100]. In this case, an intermediate oligomer formation step is added to the sequence in Eq. (16) as follows:



in which  $O_H$  represents oligomers with a higher degree of polymerization and  $O_L$  represents those with a lower degree of polymerization. In a subsequent paper, a better fit to data was reported when a step was added for direct degradation of low degree of polymerization oligomers to furfural [101].

In addition to considering the nature of the reacting species, hemicellulose models have been modified in an attempt to more accurately reflect reaction catalysis with several focused on including the effect of neutralizing capacity of the substrate. In one, minerals in the substrate were reported to neutralize up to 70% of the acid added [99]. Procedures were developed to calculate the molal hydrogen ion concentration,  $[H^+]$ , based on acid addition and the neutralizing capacity of the substrate, and these values were incorporated into the rate equation instead of the weight percent acid concentration typically used with all other terms defined as previously [102]:

$$k_i = k_{i0} \times [H^+]^{m_i} \times e^{-E_i/RT} \quad (18)$$

In a similar manner, the effective acid concentration was determined from the neutralizing capacity of the substrate and the amount and concentration of the applied acid assuming that all substrate cations were immediately available and effective to give [85]:

$$[H^+] = \text{molality of added acid} - \text{molality of the cations} \quad (19)$$

Another paper concluded that the weight percent acid concentration does not account for the nonlinearity of the hydrogen ion concentration with increasing acid addition or consider the effect of substrate neutralizing capacity and used pH in the rate constants as follows [103]:

$$k_i = k_{i0} \times e^{(-E_i/RT - 2.303m_i(\text{pH}))} \quad (20)$$

Such an approach could explain the range of yield curves observed when comparing models using only the applied acid concentration.

Although significant effort has been devoted to describing the kinetics of hemicellulose hydrolysis, the models do not predict consistent results. For example, the kinetic constants fit to describe overall hemicellulose dissolution and sugar recovery differ from those determined for breakdown of pure xylose by acid, and models from different studies project different hemicellulose removal rates and yields [97]. In addition, yields of monomers and oligomers increase when more water is added for hydrolysis with just water, a finding that is inconsistent with first-



## Hydrolysis of Cellulose and Hemicellulose

order kinetics or the assumption that acetic acid released during hydrolysis catalyzes the reaction [104]. Pushing liquid through biomass accelerates removal of hemicellulose with flow rate, again inconsistent with conventional first-order kinetic models that only show an effect of acid concentration or temperature [93,94,105]. Furthermore, treating the hydrolysis of hemicellulose solids to dissolved sugars as a homogeneous reaction is counter to the basic nature of this solid/liquid system, and many questions arise about the models used to date. For example, could the biphasic kinetic really be attributed to mass transfer limitations that affect release of sugars into solution? Oligomers appear to be important in distinguishing the performance of various process configurations for breakdown of hemicellulose to sugars and also could help explain the deviations in model predictions. Could oligomer solubility in the particulate biomass affect hydrolysis rates? Is the binding of xylan to lignin important? Answering these questions and clarification of hemicellulose hydrolysis should provide insight that can be valuable to more confident applications of the technology and also to defining advanced pretreatment approaches.

## VI. CELLULASE SYSTEMS FOR CELLULASE HYDROLYSIS

### A. Sources of Cellulase Enzymes

#### 1. Fungal Enzyme Systems

More than 100 cellulolytic fungi have been reported to date and this number is continually increasing [106–108]. Aerobic fungi (hyphomycetes, ascomycetes, and basidiomycetes) are represented in this grouping. Some members of these groups can also modify and degrade lignin in wood samples. The fungal cellulase system was initially interpreted largely in terms of substantial biochemical and molecular biological developments for the *T. reesei* system. In many ways, this system was the developmental archetype cellulase system. Many reviews have adequately described the 20-plus years of systematic research conducted at the Army Natick Laboratory on this subject [109]. Much of the subsequent research in this field has focused on mutation/selection of better *T. reesei* strains for enzyme commercialization, including biomass conversion.

It is also clear that anaerobic as well as aerobic environments produce important and functionally intriguing fungal cellulase producers [110]. Common to the rumen are anaerobic fungi and bacteria that produce enzymes capable of degrading the cell walls of plants [111]. The best studied anaerobic cellulase producers to date are probably *Orpinomyces* sp. [112], *Piromyces* sp. [113], and *Neocallimastix* sp. [114]. The molecular arrangement of the fungal cellulosome, however, remains unknown, and some predict that it may differ from bacterial cellulosome in many aspects [115–118].

Today, many researchers are working to better understand the role fungal hydrolytic enzymes play in the natural process of plant cell wall degradation [119]. Aiding in this

process is the application of new tools for direct analysis of biomass, including improved methods for using electron microscopy [120] and near-field scanning optical microscopy (NSOM) as probes of enzyme action.

#### 2. Bacterial Enzyme Systems

Bacterial cellulase systems include those produced by aerobic and anaerobic bacteria and actinomycetes and are the focus of considerable study. It is probable that more diversity in protein folds and structure/function relationships will eventually be found in the bacterial paradigm [108]. Coughlan and Ljungdahl [121] have extensively reviewed bacterial cellulases, identifying 46 unique bacterial producers of cellulases. More recently, Lynd et al. [122] have reviewed the concept of exploiting *Clostridium thermocellum* and related microorganisms for “consolidated bioprocessing” (CBP), in which the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of resulting sugars to desired products occur in one step. Perhaps the most studied actinomycete cellulase producer, *Thermobifida fusca*, has been shown to harbor cellulases from many glycosyl hydrolase families, including families 1, 5, 6, 9, 48, and 74 [123–125]. It is noteworthy that to date, no bacterial cellulase system includes a cellobiohydrolase from family 7. In general, the cellulase systems produced by actinomycetes are not associated with cellulosome-like structures; however, this question is still debated.

In the early 1980s, Bayer and Lamed [126–132] first described a multienzyme complex in the anaerobic, thermophilic, cellulolytic bacterium, *C. thermocellum*, that was termed the cellulosome. Using a variety of biochemical approaches, negative staining techniques, transmission, and scanning electron microscopy, the cellulosome in this bacterium was found to be packaged into protuberance-like cell-surface macromolecular complexes that are released into the medium in the latter stages of growth. The molecular mass of the complex was determined to be in the region of several MDa, and isolated cellulosome complexes were found to be approximately 18 nm in size [132]. The composition of a given cellulosome varies depending on the substrate that apparently regulates enzyme expression. Cellulosomes can be found on the cell surface and in the extracellular growth medium. The cellulosome has been described as the principle mechanism by which some anaerobic cellulolytic microorganisms achieve efficient breakdown of the recalcitrant polysaccharides present in plant cells. The cellulosome is believed to be more efficient than the free enzyme system because it “collects” and “positions” enzymes onto the substrate surface. The cellulosome has been found in numerous cellulolytic, anaerobic microorganisms including clostridia, ruminococci, and anaerobic fungi [126–131,133]. In the past two decades, molecular biological approaches (genomics) have been employed to find new cellulosomal genes, an activity that has provided information needed for our collective understanding of the structure/function relationships of the cellulosome.



Extensive review of the bacterial cellulosome can be found in the literature [134–140]. Two types of subunits have been identified from the bacterial cellulosome complex. Nuncatalytic subunits, called “scaffoldins”, serve to position and organize the other subunits and to attach the cellulosome to the cell surface and/or to the substrate. The other type of subunit is represented by the enzymes, which hydrolyze the plant cell wall polysaccharides [141–143]. Based on sequence analysis and directed biochemical evidence, at least three types of protein domains are involved in the function and assembly of the cellulosome. Catalytic domains (enzymes) carry out substrate hydrolysis. Binding domains function to provide binding capacity to the substrate or the cell surface. For example, the carbohydrate-binding module (CBM) is responsible for mediating the binding of the scaffolding to carbohydrate substrate [143]. An S-layer homologous (SLH) domain mediates the association of the cellulosome to the cell surface [141]. Recognition domains, called cohesin and dockerin, are known as receptor/adaptor pairs. The specific interaction between cohesin and dockerin forms the positional self-assembly of the cellulosome.

## B. General Classification of Cellulase Enzymes

### 1. Endoglucanases

The “endo-1,4- $\beta$ -glucanases” or 1,4- $\beta$ -D-glucan 4-glucanohydrolases (EC 3.2.1.4), which act randomly on soluble and insoluble 1,4- $\beta$ -glucan substrates, are commonly measured by detecting the decrease in viscosity or reducing groups released from carboxymethylcellulose (CMC) [144].

### 2. Exoglucanases

The “exo-1,4- $\beta$ -D-glucanases” include both the 1,4- $\beta$ -D-glucan glucohydrolases (EC 3.2.1.74), which liberate D-glucose from 1,4- $\beta$ -D-glucans and hydrolyze D-cellobiose slowly, and 1,4- $\beta$ -D-glucan cellobiohydrolases (EC 3.2.1.91), which liberate D-cellobiose from 1,4- $\beta$ -glucans. Differentiation of these enzyme classes requires analytical techniques to distinguish glucose and cellobiose and is usually carried out by HPLC or GC. These enzymes can be further distinguished by their ability to liberate free sugars from either the reducing or nonreducing end of the cellulose chain [145]. Determination of which preference a given enzyme has is usually carried out through synergy studies with enzymes of known orientation [146–148].

### 3. Beta-D-Glucosidase

The “ $\beta$ -D-glucosidases” or  $\beta$ -D-glucoside glucohydrolases (EC 3.2.1.21) act to release D-glucose units from cellobiose and soluble cellobiohydrolases, as well as an array of glycosides. Measurement of this activity is carried out either specifically on cellobiose or cello-oligomers with product analysis by HPLC or GC, or by direct spectrophotometric or fluorometric analysis of various chromogenic and fluorogenic analogs of cellobiose and cello-oligomers.

## C. Cellulase Structure and Function

### 1. Synergism

Gilligan and Reese [149] first showed that the amount of reducing sugar released from cellulose by the combined fractions of fungal culture filtrate was greater than the sum of the amounts released by the individual fractions. Since that initial report, many investigators have used a variety of fungal preparations to demonstrate a synergistic inter-action between homologous exo- and endo-acting cellulase components [109,149–152]. Cross-synergism between endo- and exo-acting enzymes from filtrates of different aerobic fungi has also been demonstrated many times [152–155].

Exo–exo synergism was first reported in 1980 by Fägerstam and Pettersson [156]. Exo–endo synergism is explained best in terms of providing new sites of attack for the exoglucanases. These enzymes normally find available cellobiohydrolase “ends” at the reducing and nonreducing termini of cellulose microfibrils. Random internal cleavage of surface cellulose chains by endoglucanases provides numerous additional sites for attack by cellobiohydrolases. Therefore, each hydrolytic event by an endoglucanase yields both a new reducing and a new nonreducing site. Thus, logical consideration of catalyst efficiency dictates the presence of exoglucanases specific for reducing termini and nonreducing termini. Indeed, recent x-ray crystallographic work reported by Teeri et al. [157] confirms that the reducing terminus of a cellobiohydrolase can be shown in proximal orientation to the active site tunnel, i.e., reducing end in first, of *T. reesei* CBH I. Claeyssens et al. [158] confirmed from kinetic data that *T. reesei* CBH II preferred the nonreducing terminus of the cellulose chain.

Baker et al. [153] recently reported a standardized, comparative study that measured glucose release and synergistic effects in the solubilization of microcrystalline cellulose by binary mixtures of 11 fungal and bacterial cellulases (8 endoglucanases and 3 exoglucanases). Evaluation of 16 endo–exo pairs revealed that bacterial/fungal hybrid pairs are very effective in solubilizing microcrystalline cellulose. Of nine bacterial/fungal hybrid pairs studied, six were ranked among the nine most synergistic combinations, and six bacterial/fungal pairs were also among the top nine pairs in terms of soluble-sugar release. One hybrid pair (*Acidothermus cellulolyticus* E1 and *T. reesei* cel7A) was ranked first in both synergism and sugar release. In exo–exo synergism experiments, the performance of *Thermomonaspora fusca* E<sub>3</sub> confirmed its mode of action as “CBH II-like” (that is, E<sub>3</sub> is synergistic with *T. reesei* CBH I, but not with *T. reesei* CBH II).

### 2. Glycosyl Hydrolase Diversity

Glycosyl hydrolases have recently been grouped by using protein sequence alignment algorithms hydrophobic cluster analysis (HCA) by Henrissat [159]. HCA relies on the basic rules underlying the folding of globular proteins and uses a bidimensional plot to display the amino acid sequence of a protein depicted as an “unrolled longitudinal



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cut" of a cylinder [159]. The "helical net" produced by this graphical display allows the full sequence environment of each amino acid to be examined. Gilkes et al. [160] originally proposed nine glycosyl hydrolase families, based on the glycosyl hydrolase sequences available at that time, and, in the ensuing 5 years, these researchers have added substantially to the original classification list [161,162]. Today, Bairoch [163] has identified 56 glycosyl hydrolase families. This classification system provides a powerful tool for glycosyl hydrolase enzyme engineering studies because many enzymes critical for industrial processes have not yet been crystallized or subjected to structure analysis. Still, for those working to improve cellulase function by design; the fact that cellulases and beta-glucosidases are distributed throughout glycosyl hydrolase families 1, 3, 5, 6, 7, 8, 9, 12, 44, 45, 48, 61, and 74 is challenging. Since tertiary structure and key residues at active sites are generally better conserved than amino acid sequence, it is no surprise that structural studies, combined with sequence comparisons directed at active site residues, have allowed many of the families to be grouped in clans having a common fold and a common catalytic apparatus [164]. Eight such clans recently proposed for the glycosyl hydrolases are GH-A (including families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 51, and 53), GH-B (families 7 and 16), GH-C (families 11 and 12), GH-D (families 27 and 36), GH-E (families 33 and 34), GE-F (families 43 and 62), GE-G (families 37 and 63), and GE-H (families 13 and 70) [163]. Among these, clans GH-A and GH-B include cellulases. These cellulase families include members from widely different fold types, i.e., the TIM-barrel,  $\beta\alpha\beta$ -barrel variant (a TIM-barrel-like structure that is imperfectly superimposable on the TIM-barrel template),  $\beta$ -sandwich, and  $\alpha$ -helix circular array. This diversity in cellulase fold structure must be taken into account when considering the transfer and application of design strategies between different cellulases.

### 3. Enzyme Adsorption and Nonproductive Binding

The process of binding to cellulose is an important first step in enzymatic cellulose degradation, and the study of cellulose binding is of fundamental importance for understanding the cellulose degradation process. Cellulases that carry either a fused cellulose binding module (CBM) or a CBM attached by a linker peptide seem highly susceptible to loss on cellulose. Although the CBM undoubtedly plays a critical role in the hydrolysis of cellulose for some cellulases, this small, yet highly reactive domain may also lead to general adsorption and nonproductive binding of many enzymes to cellulose [165]. The binding of Cel7 enzymes, such as *T. reesei* CBH I, and isolated CBMs does not follow a simple Langmuir adsorption isotherm. This has been explained by the probability that binding sites for the proteins on the cellulose surface overlap each other; however, the two-domain structure of the enzymes, where both domains bind, may also play a role [166]. Lignocellulose, which contains lignin and other polysaccharides in addition to cellulose, presents an even more complex substrate for enzyme action. Clear evidence of a relationship between

lignin content and cellulase digestion of lignocellulosic substrates has been demonstrated [167]. Sild et al. [168] recently reported a kinetic and theoretical study of cellobiohydrolase action on cellulose and proposed that in addition to being a surface peeling process, these enzymes may eventually produce a substrate surface so complex that, unassisted by other cellulases, action is greatly impeded.

### 4. Processive Cellulases

Cellulases from glycosyl hydrolase families 6, 7, and 9 (and possibly others) are thought to act in a processive manner on cellulose. That is, these enzymes do not become disengaged with the single celloextrin substrate until that chain is fully hydrolyzed or until the enzyme becomes denatured or otherwise compromised. They may, in fact, move on the cellulose surface as a consequence of the hydrolytic cycle. The *T. reesei* Cel7A was probably the first enzyme in this group to be studied in depth. Like most family 7 cellobiohydrolases, the *T. reesei* Cel7A contains a large catalytic domain connected to a smaller cellulose binding module by an amino acid linker peptide consisting of about 30 amino acids. Using small-angle x-ray scattering, Receveur et al. [169] recently reported the solution conformation of the entire two-domain Cel145 protein from *Humicola insolens* as well as the effect of the length and flexibility of the linker on the spatial arrangement of the constitutive modules. The measured dimensions of the enzyme show that the linker exhibits an extended conformation leading to a maximum extension between the two centers of mass of each module corresponding to about four cellobiose units on a cellulose chain. These results were consistent with a model proposed by Himmel et al. [170] where cellulases can move on the surface of cellulose with a "caterpillar-like" linear displacement.

## VII. ENZYMATIC HYDROLYSIS OF CELLULOSE

### A. Experimental Systems

The initial approach to enzymatically converting cellulose to ethanol involved separate operations for pretreatment of biomass to open up the structure of biomass for attack of cellulose by cellulase, production of cellulase enzyme on pretreated biomass, addition of the cellulase produced to pretreated biomass to release glucose, and glucose fermentation to ethanol or other products [171]. Over time, the title separate hydrolysis and fermentation or SHF emerged to designate this sequence of operations. Improvements in pretreatment and cellulase enzymes enhanced the performance of the SHF process by increasing the accessibility of the substrate to cellulase and improving the balance and level of activity of the enzyme, respectively, to enhance the performance of the SHF approach [65–67]. With regard to the former, dilute acid pretreatment emerged as a front-runner for preparing cellulosic biomass for enzymatic conversion with high yields at a relatively reasonable cost [75,90–92]. For the latter, classical mutation and selection



successively improved the enzymes from the fungus *T. reesei* discovered during World War II with the evolution from earlier strains such as QM9414 to improved varieties such as Rut C30 [172] and onto Genencor 150L that was more effective because of enhanced levels of  $\beta$ -D-glucosidase that convert cellobiose into glucose [173]. Unfortunately, glucose and particularly cellobiose are powerful inhibitors of cellulase action. Although yields can be improved by adding high loadings of cellulase, this strategy results in excessive enzyme costs. Otherwise, hydrolysis yields have been historically limited to lower glucose concentrations than considered desirable to recover ethanol or other fermentation products economically.

An alternative strategy known as the simultaneous saccharification and fermentation (SSF) process adds cellulase and the fermentative organism to the same vessel to rapidly convert glucose to ethanol as it is released, reducing the accumulation of this powerful inhibitor of cellulase activity [174]. Although the temperature of the SSF process has to be reduced below the optimum for cellulase alone to accommodate the lower temperature tolerance of fermentative organisms, particularly at the low sugar and high ethanol concentrations of the process, SSF performed better in terms of rates, yields, and concentrations of ethanol than hydrolysis alone at higher temperatures that are optimum for cellulose hydrolysis [66]. Nonetheless, cellulase action was still slow, with typical SSF reaction times of about 5–7 days to achieve modest ethanol concentrations.

Following identification of the SSF configuration in the mid-1970s, fermentative organisms were sought that tolerate the combined stresses of higher temperatures (to increase hydrolysis rates), low glucose levels (due to rapid sugar metabolism by the organism), and high ethanol concentrations. However, rapid conversion of cellobiose to glucose was found to be more important than the fermentation temperature, and the best results were with a cellulase such as Genencor 150L that is high in  $\beta$ -glucosidase [66]. Further research showed that coculture of a less ethanol tolerant cellobiose fermenting organism *Brettanomyces clausenii* with more ethanol tolerant *Saccharomyces cerevisiae* yeast enhanced performance, and later, a single yeast, *Brettanomyces custerii*, was identified that both ferments cellobiose directly into ethanol and is ethanol-tolerant, eliminating the need for the coculture system [175]. More recently, bacteria have been genetically engineered to ferment xylose to ethanol and also ferment cellobiose to ethanol either naturally or through additional genetic modifications, and these organisms provide similar cellobiose utilization benefits. In addition, additional genetic modifications have been made to these organisms so they can make endoglucanase in addition to fermenting cellobiose [176]. Such strains can utilize a substantial fraction of amorphous cellulose and could also reduce the need for externally added enzyme if a mixture rich in cellobiohydrolase is applied.

Ongoing research continues to search for fermentative organisms that are temperature-tolerant and possess other traits that better match operating conditions preferred by cellulase [177–185]. In addition, many have applied SSF

with advantage to a wide range of feedstocks pretreated at various conditions. Combining SSF with hemicellulose sugar fermentation has received attention to lower costs, and SSF has been used to make products other than ethanol. SSF has also been applied in fed-batch and continuous processes for conversion of paper sludge and wood to ethanol. Although other approaches are still being considered, SSF-based technology has been shown to be a leading candidate for near-term applications and will likely remain so until new cellulases are developed that work much faster, with minimal product inhibition, and at high temperatures [66,186].

Continuous and fed-batch processes offer advantages for producing commodity products from cellulose as compared to more conventional batch configurations including high cell and enzyme concentrations over the operating cycle, greatly reduced down time for vessel turnaround, and lower operating and capital costs for seed fermenters. For example, frequent refilling and emptying of batch fermenters is usually impractical at the  $\geq 1$  million gal volumes envisioned for such processes. In addition, eliminating seed fermenters lowered the ethanol selling price by about 12 cents per gallon and the overall cost of processing by about 20% in a 1996 study built from a process design used in NREL models [187]. Consistent with these observations, most larger operations in the existing corn ethanol industry are operated in the continuous mode.

While several thousands of batch laboratory enzymatic hydrolysis only and SSF studies operations have been published, experimental investigations of continuous and fed-batch fermentations of cellulosic feedstocks involve added complexity and cost as well as specialized and usually custom equipment, and only a few fed-batch or continuous experimental results have been reported [188]. None considered the high substrate concentrations or continuous cascade operations projected for commercial use [68,71]. In a broader context of an extensive review of all types of cellulosic-based systems in addition to just SSF, only 29 fed-batch and continuous studies were found [188], and only two used feed concentrations greater than 25 g/L cellulose. Thus far more effort is needed to develop and understand continuous SSF processes if such systems are to be utilized commercially.

## B. Structural Features Impacting Cellulase Action

### 1. Crystallinity and Boundary Water Layer

Molecular mechanics simulations have been used by Skopec et al. [189] to model the structuring of water adjacent to two different faces of microcrystalline monoclinic cellulose I $\beta$ . Strong localization of the adjacent water was found for molecules in the first hydration layer due to both hydrogen bonding to the hydroxyl groups of the sugar molecules and also due to hydrophobic hydration of the extensive regions of hydrophobic surface resulting from the axial aliphatic hydrogen atoms of the “tops” of the glucose monomer units. Importantly, significant structuring of the water was



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found to extend far out into the solution. Thermally induced fluctuations in the crystalline surface were observed accompanied in some cases by penetration of water molecules into the crystal lattice. It is hypothesized that the highly structured layers of water might present a substantial barrier to the approach of cellulase enzymes toward the (1,0,0) surface in enzyme-catalyzed hydrolysis and might significantly inhibit the escape of soluble products in dilute acid hydrolysis, contributing to the slow rates of hydrolysis observed experimentally. Conversely, the (1,1,0) step face induces much less structuring and thus might be more easily approached, resulting in a higher hydrolysis rate on this face.

### 2. Hemicellulose Content

Several attempts have been carried out to obtain a better understanding of the relative importance of lignin and hemicellulose in the enzymatic hydrolysis of pretreated cellulosic substrates. Grohmann et al. [190] related the improvement in enzyme digestibility of pretreated wood to the removal of hemicellulose which, according to Grethlein [191], results in an increase in both the accessible pore volume and the specific surface area. Stone and Scallan [192] showed that the median pore size is also strongly dependent on the degree of swelling. Increasing the severity of the pretreatment usually leads to increased solubilization of hemicelluloses in water and improved glucose yields in enzymatic hydrolysis [193–196].

### 3. Lignin Content

The common pretreatment methods do not remove significant amounts of lignin. Formation of small particles, brown oily substances, has been detected both inside and outside exploded cell walls by photomicrographs [197]. Tanahashi suggested that lignin is first depolymerized and then repolymerized. These results are consistent with the softening and agglomeration of lignaceous materials and extractives. Donaldson et al. [198] found that when these particles are smeared out by mild alkali, the hydrolysis is greatly reduced. Thus it appears that lignin is to a large extent separated from the cellulose, although not totally removed from the substrate. This supports that separation of lignin and cellulose, but not complete delignification, is an important requirement for an effective pretreatment [199].

The generally observed decline of the hydrolysis rate has been suggested to be due to the exhaustion of the more reactive, amorphous cellulose substrate. Another suggested reason for this fall-off might be due to enzyme adsorbed to the lignaceous substrate as the hydrolysis proceeds. It has been shown that the specific rate (rate of hydrolysis per adsorbed enzyme) decreased significantly as the hydrolysis proceeded. This shows evidence for the nonspecific adsorption of cellulases, obviously on lignin [200]. The reasons for the decline of the rate of hydrolysis is still not fully understood in spite of numerous studies during the last decades [201–204]. Obviously, lignin restricts the hydrolysis by shielding cellulose surfaces or

by adsorbing and inactivating enzymes. It has been proposed that the close association between lignin and cellulose prevents swelling of the fibers, resulting in limited enzyme accessibility to the cellulose [205].

Somewhat contradictory results have been obtained on the influence of delignification on enzymatic digestibility. Ramos et al. [206] and Schwald et al. [207] obtained little or no increase in digestibility after extraction of lignin from pretreated wood substrates. Selective removal of lignin and hemicellulose before steam treatment by extraction with sodium chlorite and potassium hydroxide, respectively, indicated that short steaming times were enough to produce *in situ* hydrolysis of the hemicellulose, resulting in the removal of most of the hemicellulose and residual lignin and thus greatly increasing accessibility to cellulose [207]. Attempts to remove some of the remaining lignin by simultaneous enzymatic delignification and cellulose hydrolysis showed that the enzymatic digestibility could be slightly improved [208]. The approach has not been fully exploited due to the lack of an efficient enzymatic delignification system.

Much of the remaining water insoluble lignin still contained in the wood structure is extractable by dilute alkali or by organic solvents. Removal of this alkali insoluble lignin resulted, however, in only a small increase in the yield of glucose on enzymatic hydrolysis or even decreased the rate and extent of hydrolysis of steam-treated softwood substrates [206,207]. It has been observed that primarily the nature and the redistribution of the guaiacyl lignins restricted the hydrolysis of cellulose from steam-pretreated softwood. When the residual substrate remaining after extensive hydrolysis of steam-pretreated aspen and eucalyptus was examined microscopically, it was apparent that this debris was mainly composed of vessel elements [206]. Since the lignin in vessel elements is known to have a higher guaiacyl-to-syringyl ratio than other cells found in hardwoods tissues, it is probable that the distribution of this lignin fraction restricts the swelling of the cellulosic residue and reduces the surface area available to the enzymes. Scanning electron microscopy examination of the ultrastructure of decayed stumps of red alder indicated that libriform fibers and ray parenchyma cells were almost totally degraded, whereas vessel elements remained relatively unmodified after extensive degradation. It appeared that high level of degradation was only observed where syringyl lignins were predominant and that the lower accessibility of plant vessels was partially the result of occurrence of the more recalcitrant guaiacyl lignin type in the vessel walls.

The adsorption capacity of the pretreated wood for the protein in crude cellulase has been estimated to increase significantly as the temperature of the pretreatment is increased up to 220°C, indicating an enhanced surface area of cellulose. However, it has also been observed that the adsorption capacity of the lignaceous residue decreases due to increased melting and agglomeration of lignin [197,198]. This shows that lignin not only shields the cellulose, but also acts as a competitive nonspecific adsorbent. The hydrophobicity of surface has generally been regarded as



an important factor: the more hydrophobic the surface, the higher the extent of adsorption. All the major *T. reesei* cellulases have hydrophobic amino acids exposed on the surface [209]. The hydrophobic residues on the surface of the enzyme may also lead to binding to the hydrophobic surface of lignin. The adsorption of purified cellulases from *T. reesei*, CBH I (Cel7A), and EG II (Cel5A) and their catalytic domains on steam-pretreated softwood (SPS) and lignin has been studied using tritium-labeled enzymes. Both CBH I and its catalytic domain exhibited a higher affinity to SPS than EG II or its catalytic domain. Removal of cellulose-binding domain decreased the binding efficiency markedly [210]. Thus the nonspecific unproductive adsorption of cellulases seems to be one important factor in the decline of hydrolysis rate [200,203,211]. The rate decline of enzymatic hydrolysis is also related to enzyme inactivation during hydrolysis. In addition to end-product inhibition, there are reports on soluble enzyme inhibitors. A variety of substances are formed during the pretreatments. It has been shown that the liquid formed after pretreatment has an inhibitory effect on the enzymes [212,213].

Cellulose hydrolysis has been reported to be improved when surfactants are present in the reaction mixture [214–216]. It has been proposed that surfactants prevent the unproductive binding of cellulases on lignin surfaces. Nonionic surfactants (such as Tween and Triton) were found to increase the hydrolysis, whereas the charged surfactants decreased hydrolysis. In the hydrolysis of SPS, the addition of Tween 20 allowed the reduction of the enzyme loading to about half [217]. The positive effect of surfactant addition on hydrolysis of SPS correlated with a decrease in CEL 7A (CBH I) adsorption. The increase in hydrolysis and decrease in CEL 7A adsorption observed by Tween 20 were not observed on pure cellulosic substrates.

### C. Kinetic Modeling of Enzymatic Hydrolysis of Cellulose

Kinetic models based on fundamental principles are valuable tools for building confidence in the ability to predict performance of large-scale systems that have never been run commercially [2]. Such models can also clarify cause and effect relationships better than viewing data alone, giving important insight that can facilitate identifying and overcoming key bottlenecks. Details of modeling approaches applied to describe the rate of enzymatic hydrolysis of cellulose would require more space than available here, but some general concepts important in the development of such models are summarized with more details available in recent reviews [188,218].

A key element of hydrolysis reaction models is the attack of cellulose by enzymes to release glucose. In this regard, endoglucanase and exoglucanase must act on an insoluble substrate, cellulose, and are generally pictured to do so by attaching to the substrate via an adsorption mechanism to form an enzyme–substrate complex. Furthermore, cellulase enzyme has also been shown to attach to lignin to form nonproductive enzyme–lignin complexes

that reduce the amount of enzyme available to act on cellulose, and cellulase bound to lignin did not desorb when water was added. On the basis of these and other observations, adsorption models have been developed to relate the concentrations of accessible substrate, cellulase enzyme, lignin, enzyme–substrate complexes, and enzyme–lignin complexes [219]. Langmuir-type adsorption has been shown to adequately represent this relationship in various studies.

Nutor and Converse [219] calculated a specific hydrolysis parameter defined as the hydrolysis rate divided by the amount of adsorbed enzyme and showed that it declined sharply with conversion even when corrected for glucose inhibition effects. Furthermore, the specific rate dropped with increasing substrate concentration possibly indicating that the effectiveness of the enzyme is diminished when it is spread to thin over the substrate surface, consistent with the synergistic action of the key components. In addition, the specific rate was almost independent of total enzyme concentration suggesting that hydrolysis rate is impacted by substrate conversion and/or deactivation of cellulase over time.

Cellulose is not totally homogeneous, and one would expect that large cellulase enzymes more readily access some portions than others. Thus particle reactivity would be expected to change with conversion for cellulosic substrates as shown in the literature [219]. On this basis, empirical models have been developed to include decreasing cellulase reactivity with conversion [220]. When these are multiplied by a weighting function to represent the probability that each particle population is still in the reactor for fed-batch and continuous fermentations, this approach has been shown to improve the ability to describe fed-batch and continuous SSF processes in which there are particle populations with different ages and, therefore, reactivity.

Various components in the liquid can also affect cellulose conversion. In particular, glucose and cellobiose are powerful inhibitors of cellulase action, and this effect must be integrated into kinetic models [221]. For SSF, fermentation of glucose and, in some cases, other carbohydrates must be modeled, and considerable literature is available on such systems. Many yeast kinetic models are unstructured and nonsegregated and based on the Monod equation for substrate-limited growth. Structured yeast kinetic models that describe the flux of individual metabolites, specifically the glycolytic pathway, have also been developed, but these are generally applied for the purposes of metabolic engineering. Additional terms have been added to the Monod model to describe growth inhibition by ethanol and high sugar concentrations. Other important factors to consider include the proportionality between growth and substrate consumption as represented by the cell yield and the decline in cell yield observed at low growth rates. Overall models were applied to describe SSF operations based on considerations of the types described above, although most were used to predict the performance of batch systems. As mentioned before, a model was also developed to integrate the change in reactivity with substrate age and consider the age distribution of particles that



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should be considered for continuous stirred tank systems. However, few of the models were applied to multiple continuous vessels operated in the cascade arrangement expected to be applied commercially. In addition, little experimental data have been produced for comparison, and no data are available at industrially relevant concentrations or for cascaded continuous fermenters [188].

### VIII. PRETREATMENT TO IMPROVE CELLULOSE HYDROLYSIS BY ENZYMES

#### A. Effect of Pretreatment on Enzymatic Hydrolysis

Cellulosic biomass must be pretreated to achieve high yields of glucose from cellulose by enzymes. However, the complex structure of biomass makes it difficult to isolate how pretreatment influences cellulose digestion, and understanding the relationship of cellulose digestibility by enzymes to pretreated biomass characteristics is challenging [91,92]. Although much research is still needed before we can predict a priori how to improve cellulose digestion by cellulase, enzymatic digestibility of pretreated cellulose has been related to removal of key constituents including lignin, hemicellulose, and hemicellulose acetylation [91,92]. Some have shown a direct relationship between cellulose digestion and hemicellulose removal, and lignin removal was reported not to be needed to achieve good cellulose conversion. Yet complete hemicellulose removal is often not sufficient if the pretreatment temperature is too low, and higher temperatures are needed for hemicellulose removal to be effective for some substrates than for others, suggesting that more than just hemicellulose removal is needed. Consistent with this idea, several studies have shown that removing lignin improved cellulose hydrolysis, but differences are reported in the degree of delignification needed. Hemicellulose is altered in many lignin removal techniques, confounding the role of the two components. Furthermore, although lignin dissolution remains at less than about 20–35% throughout batch operations, lignin and hemicellulose removal are nearly linearly related if liquid is continually swept through biomass during hemicellulose hydrolysis, with up to 75% of the lignin removed at higher flow rates, suggesting that lignin dissolves during hemicellulose hydrolysis but reacts to form insolubles if left in the system. Evidence has been presented that alteration of hemicellulose is not needed to affect cellulose digestibility. Removal of acetyl groups has also been shown to appreciably improve cellulose digestibility, with some differences reported in the degree of deacetylation needed, but deacetylation has been concluded to influence hydrolysis rates less than crystallinity or lignin content [222].

Cellulose digestibility has also been related to physical features of the pretreated substrate. In one approach, enzyme and hydrolysis water penetration were pictured to be impeded by cellulose crystallinity, but rates were found to drop with increasing crystallinity in some studies and

increase in others. In addition, even highly crystalline substrates were hydrolyzed to glucose with high yields. An extensive study of model compounds of varying crystallinity, lignin, and hemicellulose acetylation showed that hemicellulose acetylation plays a relatively minor role and that crystallinity is less important than removal of lignin and has a greater impact on rates than yields. Enzymatic consumption of free chain ends generated during pretreatment has been suggested to explain why cellulose digestion slows over time, but measurements of a decline in rate of hydrolysis with increasing fractional surface coverage by adsorbed enzyme have led others to conclude that steric hindrance by nearby enzyme molecules, multilayer adsorption, binding of enzyme to lignin, and cellulase getting stuck in dead-end alleys could play roles. Digestibility has also been explained in terms of the accessibility of cellulose to enzymes, and hydrolysis rates have been related to pore volume and surface area accessibility. Concerns have been raised about how accurately these features can be measured and whether the complex structure of cellulases could penetrate such pores effectively [223].

#### B. Desirable Pretreatment Attributes

The following are desirable, although not necessarily completely achievable, attributes for pretreatment processes [91,92]:

- The cost of chemicals for pretreatment and subsequent neutralization and prefermentation conditioning should be kept low.
- Generation of wastes in pretreatment should be minimized.
- Limited size reduction should be needed prior to pretreatment because biomass milling is energy-intensive and costly.
- Pretreatment should preserve hemicellulose sugars and make them more accessible for fermentation.
- Fast reactions and/or noncorrosive chemicals are desirable to minimize pretreatment reactor costs.
- The concentration of hemicellulose sugars from pretreatment should be above about 10% to keep fermentation vessel sizes reasonable and facilitate downstream recovery of the fermentation product.
- Pretreatment must promote high product yields in subsequent enzymatic hydrolysis or fermentation operations with minimal conditioning costs and little loss of sugars during conditioning.
- Hydrolysate conditioning in preparation for subsequent biological steps should not form products that present processing or disposal challenges (e.g., gypsum).
- Low enzyme loadings should be adequate to realize greater than 90% digestibility of pretreated cellulose in less than 5 and preferably 3 days.
- Pretreatment should facilitate recovery of lignin and other constituents for conversion to valuable co-products and to simplify downstream processing.



### C. Pretreatment Types

Biological, chemical, and physical methods have been evaluated to prepare cellulosic biomass for conversion of cellulose and hemicellulose to valuable products [91,92]. Biological pretreatment targets using fungi or other microorganisms to selectively remove lignin or other constituents, thereby improving accessibility to cellulase, and although the low chemical and energy use anticipated for such approaches is conceptually attractive, no controllable and sufficiently rapid system has yet been found. Known physical methods such as comminution (e.g., milling) and radiation often perform poorly and are costly. Just high-temperature steam breaks down hemicellulose into sugars and oligomers, but the total dissolved sugar yields from hemicellulose during pretreatment and subsequently from cellulose by enzymes are too limited. Passing high-temperature water without adding any chemicals or with about 0.07% sulfuric acid through biomass can reduce or eliminate acid use, achieve nearly theoretical hemicellulose sugar yields, produce much more digestible cellulose, result in liquid hydrolysate that is more compatible with fermentative organisms, lower the cost for materials of construction, use less neutralization and conditioning chemicals, and cut back on size reduction requirements. Unfortunately, dilute sugar streams and, consequently, low ethanol concentrations that increase energy requirements for distillation and pretreatment result, making current flow-through systems appear to be commercially unattractive. At this point, the most viable pretreatment options are all based on adding acid or base to lower or raise the pH, respectively, and the reader is referred to the literature for more information on biological, physical, and related pretreatment technologies [91,92].

### D. Chemical Pretreatment at Low to Neutral pH

A leading chemical approach is based on adding about 0.5–1.5% sulfuric acid to cellulosic biomass and heating the mixture to temperatures between approximately 120°C and 200°C, with about 80–90% of the hemicellulose recovered as mostly monomeric sugars. Furthermore, the cellulose left in the solid residue is highly digestible by enzymes, and hemicellulose removal also supports subsequent acid-catalyzed cellulose hydrolysis without sacrificing hemicellulose sugars that would rapidly degrade otherwise. Because high yields of sugars from both hemicellulose and cellulose are vital to economic success, many favor this approach to pretreatment [71], and dilute acid pretreatment has been the subject of over a decade of research and development [91,92]. However, dilute acid hemicellulose hydrolysis still has important limitations such as the need to use exotic and expensive materials of construction to accommodate the very corrosive environment, costs for neutralizing acid following pretreatment and disposing of the salts formed, the need to remove toxics released and formed during pretreatment in order to realize high biological processing yields, and formation of gypsum

with problematic reverse solubility characteristics when neutralized with inexpensive calcium hydroxide [84,88]. About a 7-day period with somewhat high enzyme dosages is also required to enzymatically hydrolyze cellulose pretreated by hemicellulose removal by dilute acid, and use of less enzyme and reaction for shorter times would significantly reduce costs. Most of the lignin is left with the cellulose, and adsorption of cellulase by lignin increases cellulase loading requirements.

Other acids have been evaluated to use in place of sulfuric. For example, hydrochloric acid is somewhat more effective than sulfuric, but its toxicity and highly corrosive nature make it more expensive to use [91,92]. Nitric acid performs similarly to sulfuric and is compatible with less expensive materials of construction, but the acid cost is higher. Sulfur dioxide has been used with good results as well, but this chemical presents some safety concerns with no cost advantage relative to sulfuric acid. Addition of carbon dioxide to generate carbonic acid requires such high pressures as to raise concerns about containment costs and does not appear to be as effective as sulfuric acid. Thus the low cost and good performance of sulfuric acid generally favor its use in most current designs.

An alternative pretreatment under development is based on controlling the pH between 4 and 7 with the goal of reacting hemicellulose to soluble oligomers while minimizing formation of sugar monomers that can degrade to furfural and other compounds that reduce sugar yields. This approach reduces the concentration of hydrogen ions formed by breakdown of natural acids released during pretreatment that catalyze formation and degradation of monosaccharides [224]. Reduction in release of toxic degradation products through this strategy could also benefit subsequent cellulose hydrolysis by enzymes or ethanol fermentation. In one application now under investigation, the stillage from a corn ethanol plant has proven effective in providing buffering capacity for hydrolysis of corn fiber at controlled pH. In addition, changes in physical structure such as increased pore size and decreased crystallinity are targeted to enhance enzyme penetration and effectiveness.

### E. Chemical Pretreatment at High pH

While hydrogen ions catalyze hemicellulose hydrolysis and removal at low pH, operation at a high pH of over about 10 can solubilize and remove lignin and result in improved cellulose digestibility. Over the years, various alkaline materials such as sodium and potassium hydroxide have been used for this purpose, but their costs are too high for production of low value fuels and commodity chemicals [91,92]. Use of solvents in an organosolv technique has also been considered, but the costs are too high unless cellulose is to be recovered for high value uses. On the other hand, ammonia is volatile at moderate temperatures and can be recovered and reused [225]. In particular, an ammonia fiber explosion (AFEX) process has been developed to treat cellulosic biomass with liquid ammonia at moderate temperatures of about 100°C or less followed by rapid pressure release to recover the ammonia and open up the structure



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of the solids. The physical appearance of AFEX-treated material appears similar to that prior to pretreatment, and little change in lignin or hemicellulose content is found. However, the AFEX process decrystallizes cellulose and greatly disrupts the cellulose structure, enhancing its susceptibility to cellulase. Although hemicellulose hydrolysis to monomers is very limited during AFEX, an enzyme mixture rich in hemicellulase as well as cellulase activity can achieve greater than 90% conversion of cellulose and hemicellulose to fermentable sugars for a wide variety of lignocellulosic materials including alfalfa, barley straw, corn residue, wheat straw, rice straw, corn fiber, sugar cane bagasse, switchgrass, coastal Bermuda grass, and rye grass straw. Furthermore, high yields of glucose can be obtained at low cellulase loadings of 1–10 IU of cellulase/g of dry cellulosic biomass for most of these materials, but enhanced hemicellulase activity is needed to release residual hemicellulose. The hydrolysate is also compatible with fermentative organisms and enzymes without need for conditioning, and the small amount of ammonia left in the pretreated stream is an effective nitrogen source for subsequent fermentations. The moderate temperatures and low corrosiveness of ammonia also minimize materials of construction costs. Although AFEX was originally developed as a batch process, a new continuous fiber extrusion configuration called FIBEX reduces treatment time and required ammonia levels significantly while giving similar hydrolysis results to batch AFEX [226].

Removal of lignin from biomass before biological processing improves cellulose digestibility, reduces downstream agitation power requirements, provides less sites for nonproductive cellulase adsorption, reduces dissolved lignin compounds that are toxic to fermentations, facilitates cell and enzyme recovery and recycle, and simplifies the distillation step. An ammonia recycle pretreatment (ARP) approach selectively breaks down lignin in an aqueous ammonia solution at high temperatures by an ammonolysis reaction with virtually no effect on hemicellulose or cellulose content, and the dissolved lignin is removed by circulating liquid through biomass [227]. Furthermore, ARP partially fractionates biomass into three major fractions: hemicellulose, solid cellulose, and extracted lignin. The treated solids are a low-lignin cellulosic material with high glucan content that is readily hydrolyzed by enzymes when ARP is applied to herbaceous crops, corn stover, and switchgrass. ARP pretreatment has also been studied in conjunction with additional treatment with hydrogen peroxide and dilute sulfuric acid. Although flow of liquid through biomass could prove to be too expensive, promising performance was recently observed when liquid ammonia was mixed with biomass for several days at virtually room temperature, and further development of this technology could prove very effective.

As the least expensive alkali, lime could be cost-effective for lignin removal, and recent studies have shown that lime is feasible for pretreatment at a temperature of about 100°C, a reaction time of 1–2 hr, lime loadings of about 0.1 g Ca(OH)<sub>2</sub>/g biomass, and water loadings of 5–15 g H<sub>2</sub>O/g biomass [228]. Lime enhanced enzymatic

digestibility of herbaceous biomass by as much as 10 times but was less effective on woody biomass because of its high lignin content. In addition to being inexpensive, lime is safe to handle and is widely available. Furthermore, lime can be recovered by washing biomass with water followed by reaction with carbon dioxide to precipitate calcium carbonate for feed to a lime kiln for recycle. More recently, use of lime has been studied at temperatures between 25°C and 55°C and found to remove lignin and some acetyl from biomass. Various solvents such as ethanol and methanol can also be applied to remove lignin effectively in organosolv processes, but the costs are too high to be practical for other than recovery of high-value products [91,92].

## IX. ENZYMATIC HYDROLYSIS OF HEMICELLULOSE

### A. Sources of Hemicellulases

Hemicellulases are produced by many species of bacteria and fungi, as well as by several plants. Many microorganisms produce a multiple pattern of hemicellulases to degrade the plant materials efficiently. Today, most commercial hemicellulase preparations are produced by genetically modified *Trichoderma* or *Aspergillus* strains. Most microbial hemicellulases studied are active between pH 4 and 6 and at temperatures below 70°C. More thermophilic enzymes are of great interest both in hydrolysis and other applications. Xylanases, which are stable and function efficiently at high temperatures, have been isolated especially from thermophilic bacteria. Several xylanase genes, encoding proteins active at temperatures from 75°C up to 95°C (pH 6–8), have been isolated. The most thermophilic xylanases hitherto described are produced by species of the extremely thermophilic bacterium *Thermotoga* [229]. Only few thermophilic mannanases have so far been characterized [230,231].

### B. General Hemicellulose Hydrolysis

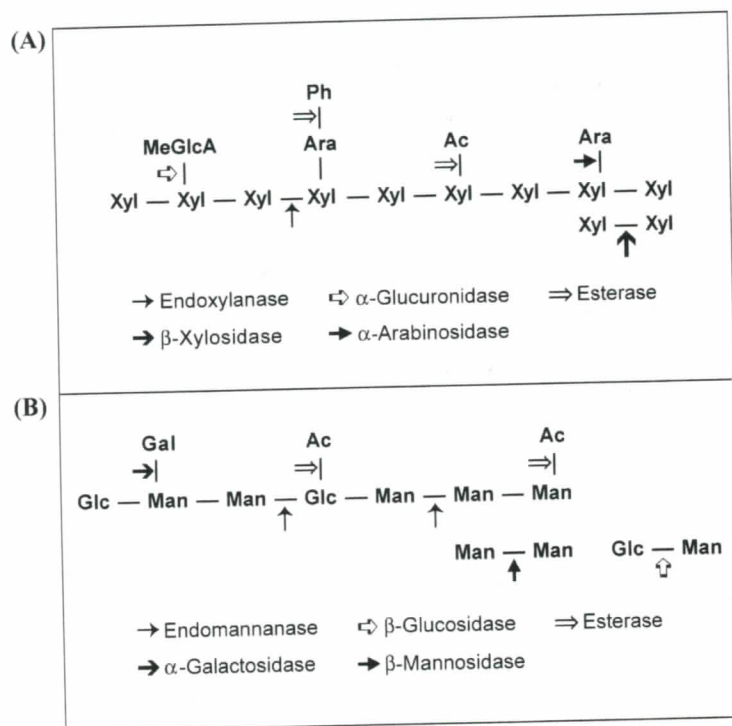
The complexity of hemicellulose structure requires a high degree of coordination between the enzymes involved in hemicellulose degradation. Most enzymes have very specific requirements for tight substrate binding and precise transition state formation, which usually leads to high catalytic turnover rates. However, even ideal catalytic sites must be “carried to the substrate” by the macromolecule within which it is housed, and enzymes are large compared to the polysaccharide oligomers under attack, especially as the particular site of action may be buried in a heterocrystalline structure of mixed polysaccharides. Of further complication is that the actions of glycosyl hydrolases often change the chemical environment of the partially degraded substrate, which in turn affects the actions of other glycosyl hydrolases. For example, most native hemicelluloses are quite water-soluble because, in part, of the substituents attached to the main chain. These side chains disrupt the water structure and help to solubilize the hemicellulose. Debranching enzymes, which

remove these substituents, may generally decrease substrate solubility when acting alone and, in turn, lower the polysaccharide's susceptibility to endo-acting hydrolases [231,232].

A major part of the published work on hemicellulases deals with the properties and mode of action, as well as applications of xylanases (reviewed by, for example, Coughlan and Hazlewood [233], Sunna and Antranikian [234], and Viikari et al. [235]). Due to their complex nature, enzymatic hydrolysis of xylans is intrinsically more complicated than most of the other plant polysaccharides. Typically, the polymer is debranched, either prior to or in conjunction with depolymerization of the backbone. Different debranching enzymes are required depending on the specific type of xylan being hydrolyzed. These include arabinofuranosidases, ferulic and coumaric acid esterases, acetyl and acetyl xylan esterases, glucuronidases, and xylosidases (Fig. 5). As the substituents are removed, xylan can become less soluble and form aggregates that sterically hinder and retard further degradation [235]. Simultaneous removal of these side chains and cleavage of the polymeric backbone synergistically enhances the rate of degradation by endoxylanase enzymes [31,232,236–241]. Thus, a xylan that has been subjected to acetyl xylan esterase is less susceptible to enzymatic degradation than a xylan subjected to a mixture of depolymerizing and debranching enzymes [242,243].

### 1. Xylan Backbone Hydrolysis

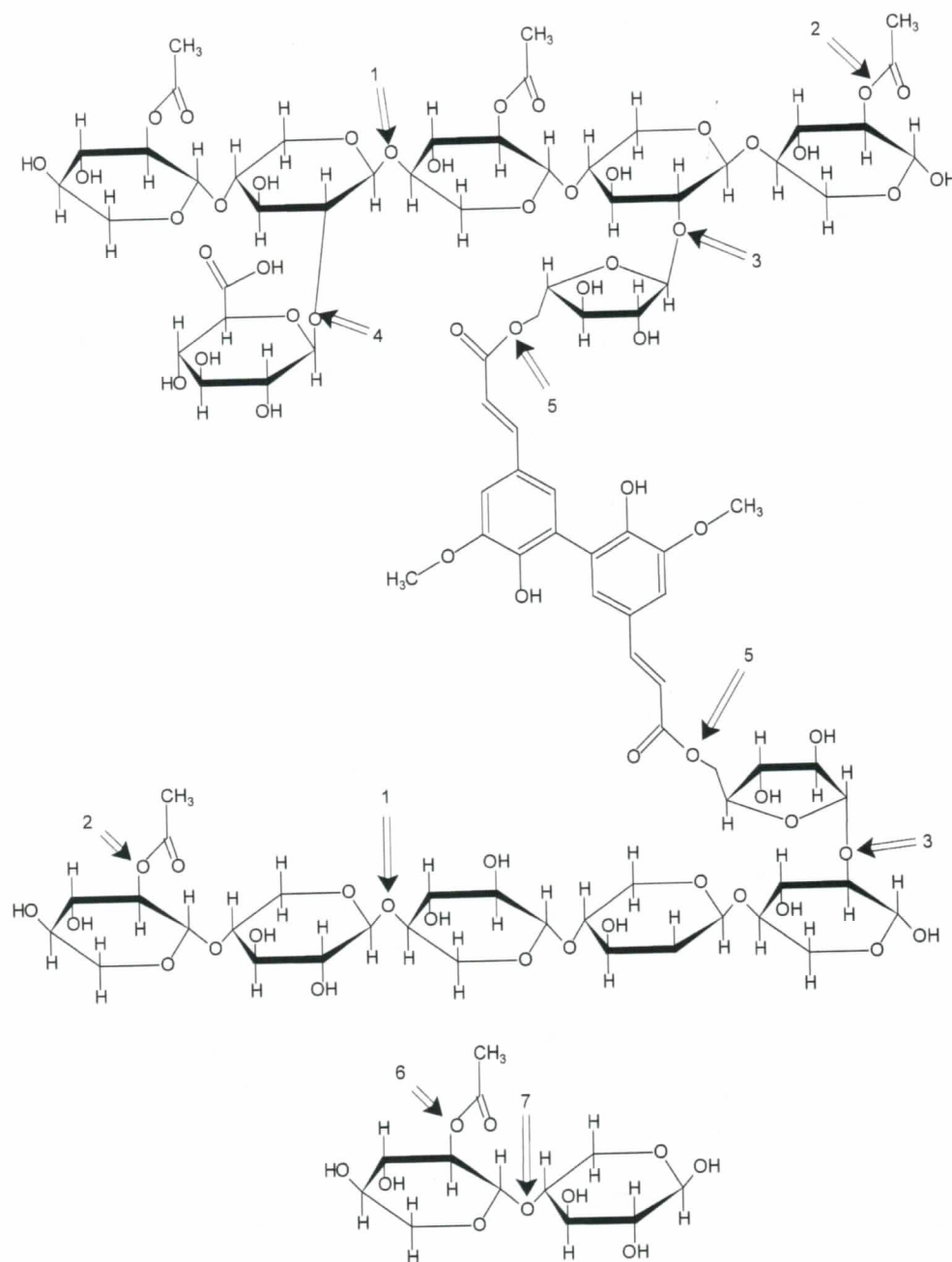
The endoxylanases (1,4- $\beta$ -D-xylan xylanohydrolases, EC 3.2.1.8) randomly cleave the main chain 1,4- $\beta$ -D-xylosidic linkages and are often quite particular about the type of linkage, type of sugar, and presence or absence of nearby substituents [243] (see Figs. 5 and 6). An endoxylanase that cleaves  $\beta$ -(1,4) linkages will usually have no effect on  $\beta$ -(1,3) linkages. In addition, an endoxylanase that cleaves main-chain linkages near an *O*-2-linked arabinose will have no effect on an open-chain xylan [244]. Although there are such specific examples of endos requiring side chains for maximal activity [245], the majority of the endo-acting hemicellulose hydrolases tend to be more active on debranched or partially debranched hemicellulose, especially in the case of xylanases. The limitation on this increased activity is probably due to solubility or the polysaccharides, which tend to become more insoluble as the debranching process continues. Decreasing chain length due to the activity of endo-hemicellulases mollifies this, allowing the shorter, less substituted fragments to remain soluble. Exo-acting enzymes, which probably fall into reducing and nonreducing end specific groups, and oligomer-hydrolyzing enzymes, also require debranching as a precursor to maximal activity. Overall, a balance must be met between removing the branching side chains from the polysaccharide backbone, decreasing the average chain



**Figure 5** Enzymes participating in the hydrolysis of xylans (A) and glucomannans (B)—Ac: acetyl; Ph: phenolic groups; Ara: arabinose; MeGlcA: methyl glucuronic acid; Xyl: xylose; Gal: galactose; Glc: glucose; Man: mannose.



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**Figure 6** Enzyme activities in depolymerization of arabinoxylan. The structure is a generalized diagram of arabinoglucuronoxylan. Enzyme activities are (1) endoxylanase, (2) acetyl xylan esterase, (3)  $\alpha$ -L-arabinofuranosidase, (4)  $\alpha$ -D-glucuronidase, (5) ferulic acid esterase, (6) acetyl esterase, and (7)  $\beta$ -xylosidase.

length, and hydrolyzing the oligomers into free monomers, all while maintaining enough solubility of the fragments to allow enzyme access. The concerted action of the various hemicellulase enzyme classes probably accounts for the high synergy observed when the enzymes are used in concert with each other [246].

Most xylanases are rather small proteins (molecular mass around 20 kDa) with a basic isoelectric point ( $pI$  8–10). Xylanases belong to the two principle xylanase groups (glycosyl hydrolase families 10 and 11) and differ from each other with respect to their catalytic properties [247]. These enzyme families are similar in that they both depolymerize

xylan via the Koshland-type, two-step catalysis that leaves products with retained stereochemistry of anomeric configuration. Family 10 enzymes typically yield lower molecular weight products (tetramers) than family 11 (pentamers) [247]. This is likely due to the difference in binding sites, with family 10 enzymes having a binding site that recognizes shorter oligosaccharides than family 11 [248,249]. Although these enzymes are active on native branched xylan, debranching may increase their activity [247,250]. Xylanases with high Mr/low pI (family 10) seem to exhibit higher catalytic versatility than the low Mr/high pI xylanases (family 11), and thus they are able to more efficiently hydrolyze highly substituted xyans.

Several three-dimensional structures of different low molecular mass xylanases belonging to family 11 have been determined, the first ones from *Bacillus pumilus* and *T. reesei* [251–253]. These enzymes have very similar structures: ellipsoidal, well-packed molecules having diameters between 30 and 45 Å. Most xylanases do not contain a separate substrate-binding domain. Some bacterial xylanases, however, have been found to contain either a cellulose-binding domain [254] or a xylan binding domain [255]. The first solved crystal structure of family 10 xylanases was the catalytic domain from *Streptomyces lividans*, which is about 1.5 times longer than the *B. pumilus* and *T. reesei* xylanases [256].

## 2. Mannan Hydrolysis

Endomannanases (1,4- $\beta$ -D-mannan mannanohydrolase, EC 3.2.1.78) catalyze the random hydrolysis of  $\beta$ -D-1,4 mannopyranosyl linkages within the main chain of mannans and various polysaccharides consisting mainly of mannose, such as glucomannans, galactomannans, and galactoglucomannans. Mannanases are generally larger proteins than xylanases (Mr 30–90 kDa) and have acidic isoelectric points. They seem to comprise a more heterogeneous group of enzymes than xylanases; that is, no clear groups based on biochemical properties have been identified. The mannanase of *T. reesei* has been found to have a similar multidomain structure as several cellulolytic enzymes; that is, the protein contains a catalytic core domain, which is separated by a linker from a cellulose-binding domain [257]. In glucomannan and galactomannan, as with xylan, degradation requires both debranching and depolymerizing enzymes, which work in synergy [241].

## 3. Enzymatic Glucan Hydrolysis

Enzymatic degradation of glucans is not as well understood as some of the other hemicelluloses. As xyloglucan is believed to be critical in governing the growth and expansion of plant cell walls, most recent research efforts appear to be focused on determination of plant enzymes responsible for control and modification of the expanding cell wall [56,258–275]. Although not as heavily branched as xyans, the xylose and other substituents on xyloglucan can make enzymatic digestion more complicated than that of cellulose and  $\beta$ -glucan. Some xyloglucanases require a specific xylose substitution pattern, while others are more general

[56]. This determination seems to be dependent on the binding subsites in specific endoglucanases, at least in *T. reesei* endo-acting  $\beta$ -glucanases [276]. A xyloglucanase from *Aspergillus niger* has been shown to be active against several  $\beta$ -glucans, but having the highest activity against tamarind xyloglucan [277]. This, combined with its lack of synergy with cellulases, indicates enzyme specificity different from traditional endoglucanases active on cellulose. A similar enzyme has been isolated from *Aspergillus aculeatus* [278]. *T. reesei* has also been shown to have endoglucanases with activity on xyloglucan [276]. A plant-specific enzyme believed to be responsible for modification of xyloglucan in the cell wall through endo-hydrolysis and glycosyl transferase activities has also been demonstrated [260,271]. A recent study on glycanases from *T. fusca* indicated that some glucan hydrolases are very specific; others have much broader substrate specificities [279]. Cel5A was shown to be active on a wide range of substrates, including  $\beta$ -glucan, carboxymethylcellulose, xyloglucan, lichenin, galactomannan, and glucomannan.

Debranching of xyloglucan has been shown to occur via  $\alpha$ -fucosidases, which specifically cleave  $\alpha$ -(1 $\rightarrow$ 2)-L-fucosides from the galactose side chains (EC 3.2.1.63) bound to the glucose backbone or are nonspecific  $\alpha$ -fucosidases (EC 3.2.1.51). As the galactose substituted onto the glucose is  $\alpha$ -(1 $\rightarrow$ 2)-linked, this is essentially a fucosylated lactose and enzymes specific for this side-chain conformation are assayed by activity against 2'-fucosyl-lactitol but show no activity against *p*-nitrophenyl- $\alpha$ -L-fucopyranoside [280]. There is an exo-acting enzyme that acts on the nonreducing end of xyloglucan oligomers. Oligoxyloglucan beta-glycosidase (EC 3.2.1.120) is produced by *Aspergillus oryzae* and removes an  $\alpha$ -xylo- $\beta$ -(1 $\rightarrow$ 6)-D-glucosyl dimer (isoprimerose) from the nonreducing end.

Because of the differences in the linkages, different enzymes are required to cleave the two forms of  $\beta$ -glucan [281–286]. Enzymatic degradation of  $\beta$ -glucan is accomplished through glycosyl hydrolase family 12 enzymes (EC 3.2.1.4). Although these endo-acting enzymes are active on  $\beta$ -(1 $\rightarrow$ 4) glycosidic linkages, they are differentiated from other  $\beta$ -(1 $\rightarrow$ 4)-acting enzymes by the distinction of being able to hydrolyze the  $\beta$ -(1 $\rightarrow$ 4) linkages in mixed  $\beta$ -(1 $\rightarrow$ 3,1 $\rightarrow$ 4)-linked polysaccharides. Glucan endo-1,3-beta-D-glucosidase [ $\beta$ -(1 $\rightarrow$ 3) glucanase] (EC 3.2.1.39) is an endo-acting glycosyl hydrolase that acts on  $\beta$ -(1 $\rightarrow$ 3) glucan, but has very limited activity on the mixed linkage  $\beta$ -glucan. Endo-1,3(4)- $\beta$ -glucanase [ $\beta$ -(1 $\rightarrow$ 3, 1 $\rightarrow$ 4) glucanase] (EC 3.2.1.6) is also an endo-acting glycosyl hydrolase. There is an exo-acting glycosyl hydrolase that is active on  $\beta$ -(1 $\rightarrow$ 3) glucan. Glucan 1,3-beta-glucosidase (EC 3.2.1.58) acts by processively releasing glucose from  $\beta$ -(1 $\rightarrow$ 3) glucan from the nonreducing end.

## 4. Enzymatic Hydrolysis of Hemicellulose Oligomers

Enzymes needed for further hydrolysis of the short oligomeric compounds produced by endo-enzymes from hemicelluloses are  $\beta$ -xylosidase (1,4- $\beta$ -D-xyloside xylohydrolase,



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EC 3.2.1.37),  $\beta$ -mannosidase (1,4- $\beta$ -D-mannoside mannohydrolase, EC 3.1.1.25), and  $\beta$ -glucosidase (EC 3.2.1.21).  $\beta$ -Xylosidases and  $\beta$ -mannosidase catalyze the hydrolysis of xylo- and manno-oligosaccharides, respectively, by removing successive xylose or mannose residues from the nonreducing termini. Exoglycanases are generally larger proteins than endoglycanases, with molecular weights above 100 kDa and they are often built up by two or more subunits.

### 5. Enzymatic Debranching of Hemicellulose

The side groups connected to xylan and glucomannan main chains are removed by  $\alpha$ -glucuronidase (EC 3.2.1.139),  $\alpha$ -arabinosidase ( $\alpha$ -L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55), and  $\alpha$ -D-galactosidase ( $\alpha$ -D-galactoside galactohydrolase, EC 3.2.1.22). Acetyl and hydroxycinnamic acid substituents bound to hemicellulose are removed by acetyl xylan esterases (3.1.1.72) and other esterases (see Fig. 5). Some accessory enzymes, such as an  $\alpha$ -arabinosidase and an esterase of *Pseudomonas fluorescens* ssp. *cellulosa* and an acetyl xylan esterase of *T. reesei*, have been found to contain a multidomain structure with a separate cellulose-binding domain [287,288]. Recently, the three-dimensional structures of acetyl xylan esterases [289,290] and feruloyl esterases [291,292] have been published.

### C. Action of Hemicellulases on Model Substrates

The enzyme activity is usually determined using isolated substrates, which may or may not be similar to the actual natural substrate. Especially with hemicelluloses, the isolation of the substrate from complex raw materials leads often to chemical and structural modification of the substrate. Some of the side groups may be removed during chemical extraction or the average degree of polymerization of the hemicellulose may change. The physical and morphological properties of the isolated substrates in the activity assay are often different from those in the practical application. Analogously with cellulose, the structure of the hemicellulose substrate changes during the hydrolysis. Therefore the kinetic modeling of the hydrolysis is difficult.

Most of the xylanases characterized act randomly and are able to hydrolyze different types of xylans, showing differences only in the spectrum of end products. The main products formed from the hydrolysis of xylans are xylobiose, xylotriose, and substituted oligomers of 2–4 xylosyl residues. The chain length and the structure of the substituted products depend on the mode of action of the individual xylanase [247]. Some xylanases, however, have rather strict substrate specificity. A unique xylanase, which requires a glucuronic acid substituent in the xylan backbone, is produced by *Bacillus subtilis* [293]. Few exoxylanases have also been isolated and characterized. These liberate xylobiose and/or xylotetraose from the nonreducing end of the xylan chain [294,295].

The main hydrolysis products of galactomannans and glucomannans by mannanases are mannobiose, manno-

triose, and various mixed oligosaccharides. The hydrolysis yield is dependent on the degree of substitution as well as on the distribution of the substituents [296]. The hydrolysis of glucomannans is also affected by the glucose-to-mannose ratio. Some mannanases are able to hydrolyze not only the  $\beta$ -1,4-bond between two mannose units, but also the bond between the adjacent glucose and mannose units [297,298]. Several endoglucanases, which hydrolyze mainly cellulose, are also able to cut the linkage between glucose and mannose in glucomannans and galactoglucomannans.

Side groups, which are still attached to oligosaccharides after the hydrolysis of xylans and mannans by xylanase or mannanase, respectively, restrict the action of  $\beta$ -xylosidase and  $\beta$ -mannosidase. The hydrolysis may stop at the substituted sugar unit. The  $\beta$ -xylosidase of *T. reesei* and  $\beta$ -mannosidases of *A. niger* are also able to attack polymeric xylan and mannan, respectively, liberating xylose and mannose by successive exo-action [299]. There are clearly different types of side-group cleaving enzymes. Some of these accessory enzymes are able to hydrolyze only substituted short-chain oligomers, which must first be produced by the backbone depolymerizing endo-enzymes (xylanases and mannanases). Others are capable of also attacking intact polymeric substrates. Most accessory enzymes of the latter type, however, prefer oligomeric substrates.

The synergy between different hemicellulolytic enzymes is usually observed by the improved action of both endoglycanases and accessory enzymes. Clear synergy between xylanases,  $\beta$ -xylosidase, and  $\alpha$ -glucuronidase of *T. reesei* was observed in the hydrolysis of deacetylated glucuronoxylan (Table 1). Pure xylanase,  $\alpha$ -glucuronidase, or their combination did not liberate notable amounts of glucuronic acid, although extensive hydrolysis of the main chain by xylanolytic activity occurred. However, addition of  $\alpha$ -glucuronidase significantly increased the hydrolysis of xylo-oligomers with the pure xylanase. When the xylanase was supplemented with  $\beta$ -xylosidase, the improving effect

**Table 1** Synergy of Different Xylanolytic Enzymes Purified from *T. reesei* in the Hydrolysis of Deacetylated Glucuronoxylan

| Enzymes                          | Hydrolysis products, (% of dry weight) |                 |                                  |
|----------------------------------|--|-----------------|----------------------------------|
|                                  | Methyl glucuronic acid                 | Reducing sugars | Xylo-oligomers ( $X_1$ – $X_5$ ) |
| XYL                              | 0.25                                   | 14.5            | 13.0                             |
| $\alpha$ -GLU                    | 0.12                                   | 0.0             | 0.1                              |
| XYL + $\alpha$ -GLU              | 0.50                                   | 18.8            | 21.1                             |
| $\beta$ -X                       | 0.12                                   | 1.4             | 1.6                              |
| XYL + $\beta$ -X                 | 0.62                                   | 20.2            | 14.9                             |
| $\beta$ -X + $\alpha$ -GLU       | 1.12                                   | 5.8             | 5.3                              |
| XYL + $\beta$ -X + $\alpha$ -GLU | 4.88                                   | 33.5            | 28.8                             |

Abbreviations— $\alpha$ -GLU:  $\alpha$ -glucuronidase 740 nkat/g;  $\beta$ -X:  $\beta$ -xylosidase 1000 nkat/g; XYL: xylanase pI 5.5, 10000 nkat/g; hydrolysis 40°C for 24 hr [300].

of additional  $\alpha$ -glucuronidase on the yield was even more significant [300]. Thus, if the substrate contains substituted xylans, the enzyme preparations should contain adequate amounts of xylanolytic activities needed.

In the case of acetylated substrates, practically no formation of sugars could be observed without addition of an acetyl esterase or an acetyl xylan esterase [301]. When purified wheat straw arabinoxylan was used as substrate, the liberation of xylose and arabinose was increased after the addition of feruloyl esterase and  $\alpha$ -arabinosidase [302]. Analogous results have been obtained with acetylated galactoglucomannans [303].

#### D. Hydrolysis of Residual Hemicellulose in the Solid Residue

The pretreatments usually aim at a complete removal of hemicellulose in order to improve the hydrolysis of cellulose. Thus the impact of the enzymatic hydrolysis of fiber-bound hemicellulose on cellulose hydrolysis has not been considered important as evaluated by the number of publications. The hemicellulolytic activity in the cellulase preparations can also be expected to be high enough to provide the necessary hydrolysis of the residual hemicellulose in the solid matrix. The enzyme activity pattern of a typical commercial cellulase (Celluclast from Novozyme), supplemented with  $\beta$ -xylosidase (Novozym 188), is presented in Table 2. As can be seen, at a frequently used cellulase dosage level of 20 FPU/g substrate, the corresponding xylanase activity is about 8200 nkat/g substrate. If estimated per gram of xylan in the substrate, the xylanase dosage becomes even higher, as the residual xylan content in the substrate is typically far below 10% of the dry weight. The activities of accessory enzymes are fairly low, but their role in the solubilization of matrix-bound xylan is expectedly less significant. The acetyl esterase activity, however, may play a crucial role if the raw material is still acetylated.

The hydrolysis of xylans in fiber-bound substrates has mostly been studied on cellulose pulps, however, with the aim of not hydrolyzing cellulose. In these experiments, it has been found that most accessory enzymes had only

**Table 2** Cellulase and Hemicellulase Activities in Celluclast Supplemented with  $\beta$ -Glucosidase (Novozym 188; \* $\beta$ -Glucosidase Activity Excluded), Calculated at a FPU Dosage of 20 FPU/g of Substrate

| Cellulase and hemicellulase activities | Activity     |
|--|--------------|
| FPU*                                   | 20 FPU/g     |
| HEC*                                   | 6,100 nkat/g |
| Mannanase                              | 1,200 nkat/g |
| Xylanase                               | 8,200 nkat/g |
| $\beta$ -Glucosidase                   | 950 nkat/g   |
| $\beta$ -Mannosidase                   | 15 nkat/g    |
| $\beta$ -Xylosidase                    | 100 nkat/g   |
| $\alpha$ -Arabinosidase                | 160 nkat/g   |
| $\alpha$ -Galactosidase                | 140 nkat/g   |

**Table 3** Hydrolysis of Steamed Birchwood Hemicellulose Fraction by Different Xylanolytic Enzymes from *T. reesei*

| Enzymes                                  | Hydrolysis products (% of dry weight) |        |           |
|--|---------------------------------------|--------|-----------|
|  | Acetic acid                           | Xylose | Xylobiose |
| XYL                                      | 4.5                                   | 6      | 6.0       |
| $\beta$ -X                               | 0.5                                   | 6      | 0         |
| AE                                       | 1.2                                   | 0      | 0         |
| XYL + $\beta$ -X                         | 5.9                                   | 25     | 1.0       |
| XYL + AE                                 | 8.5                                   | 8      | 16.0      |
| $\beta$ -XYL + AE                        | 3.2                                   | 16.0   | 0         |
| XYL + $\beta$ -X + AE                    | 9.4                                   | 42.0   | 1.0       |
| XYL + $\beta$ -X<br>+ AE + $\alpha$ -GLU | 2.0                                   | 50.0   | 3.0       |

Abbreviations—XYL: xylanase 12000 nkat/g;  $\beta$ -X:  $\beta$ -xylosidase 500 nkat/g; AE: acetyl esterase 500 nkat/g;  $\alpha$ -GLU:  $\alpha$ -glucuronidase 20 nkat/g; hydrolysis 45°C for 24 hr [305]. This substrate from the steaming process was a technical preparation containing about 70–80% xylans, the remainder consisting of extractives, tannins, and eventually pectic substances and solubilized lignin. In addition to the synergism needed for the complete breakdown of the xylan, other enzymes may also be needed, depending on the raw material.

minor effects in the hydrolysis of xylans [304]. Although the pulp substrates differ significantly from pretreated lignocellulosic raw materials, e.g., with respect to the degree of acetylation, these results are probably valid also on other substrates. It can thus be concluded that if the solid substrate contains deacetylated xylan, xylanases alone should be able to solubilize the xylan to oligomers. However, if the fiber bound contains acetyl groups, these may restrict the hydrolysis.

#### 1. Hydrolysis of Solubilized Hemicellulose

The pretreatment conditions affect the solubilization, recovery, and composition of the solubilized hemicelluloses. Depending on the raw material and pretreatment conditions, high-molecular weight fractions, oligosaccharides, monomers, or sugar degradation products are formed. Longer pretreatment times (at around 190°C) and additives, such as SO<sub>2</sub>, lead generally to better recovery of monomers. Less severe pretreatment conditions lead to solubilization of xylan and/or formation of oligosaccharides which can be hydrolyzed into monomers by enzymes [195]. The total enzymatic hydrolysis of substituted oligomers needs the synergistic action of endoenzymes and accessory enzymes. The raw material, pretreatment conditions, and thus the structure of the solubilized hemicellulose oligomers should be known for the identification of enzymes needed. The solubilized fraction from steam-pretreated birch wood contained about 10% acetyl groups, which were liberated with a culture filtrate from *T. reesei* [305]. Synergy between xylanases,  $\beta$ -xylosidase, and acetyl esterase of *T. reesei* was shown to be essential for the production of xylose from steamed birch xylan. Hydrolysis of the high-molecular weight fraction of steamed birch wood hemicellulose by xylanase alone produced only



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about 10% of the amount of xylose produced by the whole set of enzymes (see Table 3).

### X. ECONOMICS OF SUGAR PRODUCTION FROM CELLULOSIC BIOMASS

#### A. Cellulosic Biomass as a Feedstock

Cellulosic biomass is a low-cost and abundant resource that has the potential to support large-scale production of fuels and commodity chemicals. To provide a perspective on its cost, cellulosic biomass at \$44/dry metric ton is equivalent to petroleum at about \$6/bbl on an equal mass basis or about \$12–13/bbl for an equivalent energy content [73]. Some types of cellulosic biomass such as pulp and paper sludge and sugarcane bagasse can be obtained at virtually no cost in sufficient quantities to support introduction of conversion technologies, and much more substantial amounts of materials such as herbaceous and woody crops are projected to be available at less than about \$44/dry ton [1,187]. Thus it is apparent that the cost of the resource is not a dominant concern but that the primary barrier is the cost of converting cellulosic materials into valuable products. In the case of biological conversion operations, the cost of hydrolyzing hemicellulose and cellulose in biomass into fermentable sugars represents the primary cost barrier to widespread commercial use [187].

Another view of the economics of converting cellulosic biomass into fuels and chemicals can be gained by considering the basics of the conversion process. If we focus on release of sugars from the cellulose and hemicellulose portions for fermentation to ethanol as an example, about 100 gal of ethanol can be derived from a dry ton of cellulosic biomass for mature technology. Thus, a feedstock costing about \$40/dry ton would contribute about \$0.40 to the cost of 1 gal of ethanol. Then if we assume that the feedstock represents about two-thirds of the total cash and annualized capital cost of product as is typical for many commodity products, the overall cost of making ethanol would be about \$0.60/gal. These values change with the carbohydrate content, yield of sugars and ethanol, and assumed capital costs [306], but the overall concept suggests that ethanol could be produced from cellulose at low costs for mature technology. The challenge is to improve the technology sufficiently to realize these costs.

#### B. Economics of Hydrolysis-Based Technologies

Capital and operating costs have been estimated for biological conversion of cellulosic biomass to ethanol and some chemicals via hydrolysis in a number of studies over the last several years. To support such analyses, serious efforts were devoted to estimating performance through experimental research and analysis with support from various engineering firms and vendors with experience in the field, and these studies provide useful insight into cost trends for producing sugar intermediates as well as ethanol and coproducts derived from these sugars. However, it is important to note that no such processes are yet in oper-

ation, and important cost and performance assumptions for cost projections have not been verified at a commercial scale. Furthermore, many firms experienced in design, construction, and operation of such processes are reluctant to disclose information that may be released into the public domain, and the results are specific to particular process configurations, technologies, and data. In addition, biomass processing costs tend to be site-specific and will change with location. The financial structure for the project in terms of such factors as use of debt, return on investment requirements, and tax deductions will have a major impact on capital recovery costs as well. In addition, published studies often assume low interest rates with extended time scales for payment as may apply to mature technology, but higher rates of return will be demanded to finance implementation of first-of-a-kind processes. Ultimately, costs only become real when the process is fully operational. Consequently, published technoeconomic evaluations should not be viewed as providing absolute measures of process economics, but as giving useful insight that can help select promising paths for processing biomass, measure technical progress, and define research opportunities with potential impact.

Initially, a wide range of acid and enzymatically catalyzed options were considered for hydrolysis of cellulose and hemicellulose to sugars for production of ethanol and other products. Many of the concentrated acid-catalyzed approaches were projected to require costly acid recovery operations, and several present significant safety and environmental hazards. Dilute acid processes were projected to be more cost-effective but suffered from low yields. Although cellulase enzymes were expensive and did not achieve optimal performance initially, nearly theoretical yields are conceptually feasible, and it was believed that the technology could be improved through emerging tools in biotechnology [66]. In light of this opportunity and reduced federal research budgets, research was focused on improving enzymatically catalyzed conversion technologies [74].

At first, enzymatic processes were built around a sequential enzymatic hydrolysis and fermentation approach in which cellulose was first enzymatically hydrolyzed to glucose followed by fermentation to ethanol in a separate operation, and the selling price was projected to be about \$3.60/gal for this configuration in 1979 with use of a fungal strain known as QM9414 for cellulase production [74]. Changing to cellulase from an improved strain known as Rut C30 with better balance in enzyme activity components and lower end-product inhibition 3 years later dropped the projected cost to about \$2.66/gal. Next, cellulase developed by Genencor Corporation and designated as 150L improved hydrolysis further, and the projected cost dropped to about \$2.25/gal in 1985. By taking advantage of reduced end-product inhibition for the simultaneous saccharification and fermentation (SSF) configuration with this same 150L enzyme, the estimated cost of ethanol dropped to about \$1.78/gal for the year 1986. At this point, the decision was made to keep the feed rate to the processing facility constant rather than maintaining a



constant ethanol capacity, and the cost became only about \$1.65/gal through retention of economies of scale [74]. An important limitation of the technology to this point was that the five-carbon sugars arabinose and xylose could not be fermented to ethanol with high yields by known organisms, and incorporation of a newly discovered genetically engineered organism that could use all sugars dropped the total cash and capital recovery costs to only \$1.22/gal, although the capital recovery factor was increased to somewhat better reflect private capital requirements for mature technology [74]. These values have been updated in recent studies that show how sensitive the cost projections are to key technical and economic parameters [71]. Several companies now are striving to commercialize the technology but face major obstacles because of the high capital costs coupled with the risk of implementing first-of-a-kind technology.

Overall, historical improvements in projected costs for converting cellulosic biomass to ethanol can be grouped into two broad categories: overcoming the recalcitrance of cellulosic biomass and overcoming the diversity of its sugars. The first category includes advances in pretreatment that improved the recovery of sugars from hemicellulose while also opening up the structure of cellulose to promote access by cellulase enzymes, and pretreatment with dilute sulfuric acid realized both of these goals. Improvements in cellulase enzymes from the original QM9414 to Genencor 150L also enhanced yields of glucose from cellulose while reducing cellulase loadings. Although end-product inhibition limited glucose concentrations and yields, application of the integrated SSF approach allowed improvements in yields while reducing enzyme demands. Other changes such as use of cellobiose fermenting organisms in SSF and supplementation with beta-glucosidase also enhanced performance. Addition of the broth from cellulase production into the SSF fermentation improved results by taking advantage of enzyme activity otherwise lost with the fungal bodies and converting substrate left after enzyme production to ethanol [307]. Although these advances are all vital to realizing competitive product costs, overcoming the diversity of biomass sugars was essential if yields are to be sufficient to achieve low costs. In this case, insertion of two genes from the bacteria *Zymomonas mobilis* into *Escherichia coli* and other bacteria that naturally take up all hemicellulose sugars resulted in the nearly theoretical yields of ethanol needed [2].

Over the years, relatively little research has been funded on improving cellulase production for hydrolysis of pretreated cellulose, and some uncertainty and even controversy surround its cost. Early studies projected that cellulase costs were important but not a major single factor [68,187]. However, more recent investigations have projected greater impact for cellulase. For example, the cost of making cellulase from pretreated biomass with technology viewed as being currently viable was projected for a process with a feed of 2000 dry metric tons per day of cellulosic biomass [308]. For a feedstock costing \$25/dry ton, the overall cost of enzyme was projected to be about \$0.32/gal of ethanol produced, with the capital costs estimated at \$0.126/gal, electricity at \$0.107/gal, and fixed costs, feed-

stock, nutrients, and other raw materials costing about \$0.085/gal. A number of key assumptions had to be made about the performance of the enzyme production and cellulose hydrolysis process that have important implications for the cost projections. More recently, companies experienced in commercial cellulase development and production have been contracted to reduce the cost of making cellulase for large-scale application to hydrolyzing cellulose to glucose for fermentation to ethanol and other products.

### C. Application to Other Products

Although the majority of technoeconomic analyses of hydrolysis-based conversion processes have focused on manufacture of ethanol from cellulosic biomass, most of the operations used for making ethanol are applicable for producing sugars that could be fermented to several chemicals, and a few studies identified possible products to make from such sugars [309]. Furthermore, criteria were developed and applied to find economically promising products, and more detailed process designs were used to estimate costs for some of the better possibilities [310]. In general terms, most of these require fermentation yield improvements to be economically competitive with fossil-based products, although making succinic acid and a few other products appear promising now. In addition, lactic acid, 1-3 propanediol, and other new products being introduced for corn glucose fermentation could benefit from cellulosic biomass sugars.

Another study examined making both ethanol and chemicals from hemicellulose and cellulose hydrolysis sugars in a single cellulosic refinery, and important economic synergies were projected when fuels and chemicals were produced in the same facility [311]. In particular, economies of scale associated with using large equipment were projected to be significant and appeared to outweigh increases in feedstock transportation costs with facility size, similar to findings by others. Markets for many chemicals that could be made from fermentation of hemicellulose and cellulose sugars were too small to support such a large facility, while converting these sugars to ethanol for use as a fuel would require many such large operations. Thus, the low-cost sugars that would result from operating a large-scale ethanol facility make production of higher margin chemicals more profitable than they would be otherwise, and making both fuel and chemicals resulted in lower costs for either one than making just one in a dedicated facility for the same rate of return on capital. Furthermore, burning lignin and other components not fermented to products could produce all the heat and electricity needed to run the conversion process, and electricity in excess of that needed for the process could be sold at a low price for generation of base load power, further enhancing the synergies of the cellulosic biomass refinery.

### D. Opportunities for Cost Reductions

Because cellulase is costly, sensitivity studies were used to estimate how technology improvements could impact cel-



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lulase costs in one investigation [308]. Raising cellulase productivity on mixtures of hemicellulose sugars and pretreated cellulose from currently assumed values of about 75–200 FPU/L hr lowered enzyme costs rapidly to about \$0.20/gal of ethanol by reducing both equipment and power demands, two of the largest contributors to costs. However, batch cellulase production that entailed filling, operating, draining, and cleaning cycles was assumed, and only the operating portion of the total cycle is reduced as productivity was enhanced. Thus, the benefits of increases in productivity diminished rapidly if the productivity was raised above about 200 FPU/L hr, implying that a continuous enzyme production system would be needed to realize additional benefits. Increasing cellulase specific activity from 600 FPU/g of protein also dramatically reduced costs initially, but the improvements dropped off rapidly beyond about 1600 FPU/g because of the power law dependence of equipment costs on scale of operation. Cellulase costs drop nearly linearly with reductions in enzyme loading during hydrolysis, suggesting that pretreatments that make cellulose more easily hydrolyzed to glucose by enzymes would significantly reduce processing costs as well. Because of the dominance of capital, aeration, and other costs, changing feedstock costs had little effect on enzyme economics.

Another study was undertaken with the goal of determining if an advanced technology scenario could be defined that could reduce ethanol production costs enough to compete with conventional fuels in an open market [187]. In particular, advanced pretreatment technology based on hemicellulose hydrolysis and use of a single organism for cellulase production, cellulose hydrolysis, cellulose sugar fermentation, and hemicellulose sugar fermentation were substituted for previously assumed sequential process operations. Complete material and energy balances were applied for one scenario based on advanced technology that was believed to be achievable through analogy with similar commercial systems and for another scenario that assumed the best performance conceivable. Next, operating and capital costs were estimated. From this, the overall costs of making cellulosic ethanol including both cash costs and capital recovery charges were calculated to be about \$0.50/gal and \$0.34/gal for the advanced and best technology cases, respectively. Such low costs would be competitive with gasoline for use of ethanol as a pure fuel without subsidies.

The advanced pretreatment technology assumed that biomass could be pretreated with high hemicellulose yields without acids or milling to fine particles and that the hydrolysate would be fermentable without preconditioning based on observations for liquid hot-water pretreatment. Such a pretreatment is also expected to require lower-cost materials of construction. It was further assumed that use of the consolidated biological processing (CBP) for cellulose hydrolysis and sugar fermentation could realize cellulose hydrolysis yields of 92%, fermentation yields to ethanol of 90%, and an ethanol concentration of 5 wt.% in a fermentation time of 36 hr. In addition, CBP was conducted in continuous fermenters to eliminate costly seed vessels. The processing facility was also assumed to process about 2.74 million dry tons per year of feedstock

costing \$38.60/delivered dry ton to achieve some economies of scale. Overall, the key drivers in reducing costs were simplification of both the pretreatment and biological conversion steps with the potential of achieving such low costs limited if the process configuration was not changed from process designs currently projected for near-term commercial use.

It is also important to note that although concerns are sometimes raised about high-energy use and costs for ethanol purification, modern distillation and dehydration technology is able to recover ethanol in high purity at reasonable costs [71,187]. As a result, even dramatic improvements in this area would have little impact on costs, and research funds are better spent on advancing technology for hydrolyzing cellulose and hemicellulose to sugars, i.e., on overcoming the recalcitrance of cellulosic biomass.

## XI. BENEFITS AND IMPACTS OF CELLULOSIC BIOMASS CONVERSION

### A. Greenhouse Gas Reductions

Perhaps the most unique and powerful attribute of converting cellulosic biomass to fuels and chemicals is very low net greenhouse gas emissions, particularly when compared to other options for making organic transportation fuels and chemicals [2,312]. For ethanol production in particular, nonfermentable and unconverted solids and wastes left after making targeted products can be burned to provide all the heat and electricity to run the process, and no fossil fuel is required to operate the conversion plant for mature technology [68,71]. Similar results are expected for making many chemicals from biomass, provided process heat and power requirements are not excessive. Additionally, low levels of fertilizer and cultivation are needed for many lignocellulosic crops, thereby keeping energy inputs for growing biomass low [1]. The result is that most of the carbon dioxide released during ethanol manufacture and utilization in a cradle-to-grave or lifecycle analysis is recaptured when new biomass is grown to replace that harvested, and the net release of carbon dioxide is less than 10% of that for gasoline [312,313]. Use of renewable resources in other portions of the ethanol production cycle (e.g., fueling farm machinery with ethanol instead of diesel fuel) would further reduce carbon dioxide release. Furthermore, if electricity exported from the facility is assumed to displace generation by coal or other fossil fuels, essentially no net carbon dioxide is released into the atmosphere [313].

Because the transportation sector emits about one-third of all carbon dioxide, the dominant greenhouse gas, in the United States and the United States is responsible for about 25% of world greenhouse gas emissions, making ethanol from cellulosic biomass can have a large benefit if applied on a large scale. In addition, ethanol is a versatile liquid fuel, currently produced from corn and other starch crops, and is widely accepted by vehicle manufacturers and users for blending with over 10% of the gasoline consumed in the United States. Furthermore, light duty vehicles are also manufactured that use blends containing up to 85%



ethanol with gasoline. Thus, cellulosic ethanol could be rapidly integrated into the vehicle fleet and would not require extended time frames to realize its benefits.

Although fossil fuel use and greenhouse gas emissions for production of chemicals from cellulosic biomass have not been examined extensively, many such processes would be expected to also reduce greenhouse gas emissions. First, the fossil energy requirements to plant, grow, harvest, and transport cellulosic biomass are the same whether ethanol and chemicals are produced. For anaerobic fermentations to chemicals, the amount of energy to convert biomass into sugars and ferment those sugars should also be similar with some minor differences expected in the amount of energy to stir fermenters due to possible differences in residence times and fluid viscosity, a relatively minor impact. However, unlike ethanol, many chemicals are lower in volatility than water, and more energy may be required to recover some chemicals from the fermentation broth. Nonetheless, for many processes, the requirements should still be largely covered by heat and power from lignin combustion, minimizing if not eliminating the need for fossil inputs. Finally, many chemical products, such as succinic acid, have a virtually one-to-one weight yield from biomass sugars, without release of CO<sub>2</sub>, while almost half the weight of the sugars is released as CO<sub>2</sub> during sugar fermentation to ethanol. If the chemical is used quickly and ultimately burned or decomposed in landfills, the CO<sub>2</sub> balance is very similar to that for cellulosic ethanol except for whatever generally small differences result from product recovery. On the other hand, if the chemical is made into plastics or other products with long lifetimes, CO<sub>2</sub> is actually sequestered, effectively drawing CO<sub>2</sub> out of the atmosphere when we grow plants and converting that CO<sub>2</sub> into forms that have extended lifetimes. Nonetheless, low energy input processes such as anaerobic fermentations must be employed because some aerobic fermentations result in greater fossil fuel use than needed to make their petrochemical counterparts.

## B. Strategic Benefits

Despite much political clamor to reduce petroleum use during several energy crises in which petroleum supplies were tightened and prices ballooned, petroleum imports have grown to two-thirds of U.S. petroleum use, the largest fraction ever, making the country vulnerable to supply disruptions and price hikes. In addition, petroleum remains the largest and still growing source of energy in the United States, providing almost 40% of about 100 quads of total energy (quadrillion BTUs or 10<sup>15</sup> BTU) [314]. While other energy sectors are well diversified, over 96% of transportation energy is from petroleum, and the transportation sector uses about two-thirds of all petroleum consumed. Many chemicals are also derived from petroleum. Today, an interruption in oil supplies or increase in petroleum costs would badly damage or even cripple transportation and organic chemical production.

Although there is controversy in estimating biomass availability, use of petroleum for making chemicals is

far less than for fuels, and there is almost certainly more than enough cellulosic biomass to support the manufacturing of all the chemicals that could be derived from this resource [70]. Many studies estimate that enough cellulosic biomass could be available from wastes and dedicated energy crops to make a significant dent in the huge amount of gasoline consumed in the United States [1]. Furthermore, improved efficiency in fuel utilization could dramatically reduce biomass demands, making it even more likely that all light-duty vehicles could be powered by cellulosic ethanol. Many types of cellulosic biomass such as alfalfa and switchgrass contain significant levels of protein, and over half the agricultural crop land in the United States is used to grow animal feed [315]. Development of technology for recovery of animal feed quality protein from cellulosic biomass would allow land to be used to support both fuel and feed production, greatly enhancing fuel production potential. Overall, more efficient transportation coupled with more complete capitalization on the ingredients in biomass crops should make it possible to meet both fuel and feed needs from biomass. Use of cellulosic biomass has been called the "New Petroleum" because of its tremendous, although still untapped, potential [316].

## C. Solid Waste Disposal

Disposal of wastes is a mounting problem as the population continues to grow and we seek to maintain and improve the environment. For example, farmers must reduce the burning of rice straw after a harvest in northern California and less waste wood should be burned in British Columbia to improve air quality. Suppression of natural forest fires has resulted in dense forests that cause more damage to the soil and mature trees because hotter fires result when they finally rage beyond control, and many are seeking to thin the forests to restore them to their natural plant density. Converting such waste biomass into fuels and chemicals through hydrolysis of cellulose and hemicellulose would provide a valuable solution to these growing problems while also generating valuable products. However, solid waste disposal via hydrolysis to sugars for fermentation and reaction to valuable products is underappreciated and deserves far more attention. In addition, many chemicals that can be made from biomass are biodegradable, reducing the accumulation of solid wastes and facilitating waste treatment for liquids and gases. Thus, production of such materials could provide an economically attractive path to solid waste reduction.

## D. Economic Benefits

Conversion of cellulosic biomass to fuels and chemicals requires ready access to large amounts of raw material, and such facilities must be located in rural areas to keep raw material transportation costs as low as possible. Thus, relatively high-paying, skilled, manufacturing jobs for well-trained chemists, biologists, operators, and maintenance and supervisory personnel are created in rural America. In addition, new jobs must be developed on



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farms to plant, grow, and harvest cellulosic biomass, and opportunities emerge for trucking and railroad employment to ship biomass, chemicals, nutrients, and other supplies to the plant and to transport product to markets. Additional jobs are also generated in local communities through increased demand for housing, schools, stores, etc.

### E. International Fuels Market

Energy demand is growing rapidly in developing countries, and increased mobility will become more important as well, driving up the demand for transportation fuels. Given that transportation is so dependent on petroleum now, meeting this growing demand will be challenging unless new fuel sources are developed. Producing fuels from domestic sources of biomass would also reduce trade deficits for both developed and developing countries with limited petroleum resources and help grow their economies.

### F. Sustainable Production of Organic Fuels and Chemicals

When one lines up all sustainable resources and compares them to human needs, biomass provides the only known route to produce organic fuels and chemicals sustainably. Furthermore, liquid fuels provide many important attributes for transportation including ease of fueling and storage and relatively high energy density. Thus, fermenting of sugars released by hydrolysis of cellulose and hemicellulose to ethanol or other liquid fuels can provide a unique and powerful path to making fuels for transportation on an ongoing basis. It can also help diversify a transportation sector that is almost totally dependent (> 96%) on one source, petroleum, for energy. In addition, most organic chemicals also come from petroleum and other fossil resources [314], and making these products from biomass would help stabilize the price and availability of organic chemicals. Although sugar and starch now supply fermentation sugars for manufacture of several products, hydrolysis of cellulosic biomass can provide abundant, low-cost sugars that could support even larger-scale uses, provided processing costs are reduced.

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