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Overview to Ammonia Pretreatments for Lignocellulosic Biorefineries

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ABSTRACT

Development of environmentally sustainable and economically viable technologies for plant cell wall deconstruction to fermentable sugars has been impeded due to native plant cell wall recalcitrance to thermochemical and biological based processing. Lower severity alkaline-based pretreatment processes like Ammonia Fiber Expansion (AFEX^{TM}) can overcome several limitations of traditional pretreatment approaches (e.g., acidic pretreatments) to producing cellulosic biofuels and biochemicals. Here, we give an overview of chemical reactions taking place during alkaline pretreatments including reactions between ammonia and polysaccharides/lignin (e.g., ammonolysis, hydrolysis and Maillard-type reactions). AFEX^{TM} based pretreatments enhance enzymatic digestibility and fermentability of lignocellulosic biomass through various chemical and ultra-structural modifications within the cell wall. An improved mechanistic understanding of the AFEX^{TM} process has led to the development of novel alkaline pretreatments that are briefly discussed in this review.

Keywords: AFEX, biomass, conversion, decomposition products, pretreatment
Abbreviations: AFEX^{TM}, Ammonia Fiber Expansion; **G**, guaiacyl unit; **H**, p-hydroxyphenyl unit; **LCC**, lignin-carbohydrate complex; **S**, syringyl unit

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BACKGROUND

We have recently experienced dramatic increases in the real price of crude oil (Administration EI 2012), with dramatic social and economic repercussions throughout the world. Lignocellulosic biomass is an attractive alternative to petroleum due to its renewability, high energy content, high availability and high accessibility. Cellulosic biofuels can also improve the environment; generate local jobs and provide energy security (Huber and Dale 2009). One of the processes used for the production of biomass-derived fuels and chemicals is based on the pretreatment of lignocellulosic biomass, followed by enzymatic hydrolysis and fermentation of soluble sugars to desired products. These sugars can either be used as a carbon source for fermentative microorganisms to produce chemicals or utilized via thermocatalytic conversion. One of the leading pretreatment technologies for lignocellulosic biomass is Ammonia Fiber Expansion (AFEX^{TM}) (Balan *et al.* 2009). This technology exhibits interesting features which include operation at relatively low temperatures and low residence times, showing good effectiveness for agricultural residues (e.g. corn stover, switch grass) during enzymatic hydrolysis and fermentation without need for any external nutrient supplementation or detoxification. Recently, important chemical mechanisms that occur during AFEX^{TM} pretreatment

Received: 4 December, 2011. Accepted: 31 March, 2012.

Invited Review

Table 1 Composition of lignocellulosic materials in cellulose, hemicellulose and lignin (Pettersen 1984; Sun and Chen 2002; Monti *et al*. 2008).

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Hardwood	40-55	24-40	18-25	< 1
Softwood	45-50	25-35	25-35	< 1
Grasses	25-40	35-50	10-30	1-12

and a relationship between structural changes in the biomass and its digestibility have been reported (Chundawat 2009). These fundamental studies have helped make further improvements to the process.

In this review we cover important structural features of lignocellulosic biomass, and structural and chemical changes that occur in the biomass during ammonia based pretreatment. This analysis identifies reactions that favor the overall digestibility of the biomass and determine what conditions drive those changes to take place.

CHARACTERIZATION OF LIGNOCELLULOSIC BIOMASS

The main components of the plant cell wall are cellulose, hemicelluloses, lignin and protein and ash. The ratio of these components varies among families of plants, like grasses, softwoods and hardwoods (**Table 1**).

Plant cell wall components form a complex structure of cellulose, hemicelluloses and lignin that can provide both support and provide a defense mechanism against invading microbes (Pettersen 1984; Monti *et al.* 2008; Huber and Dale 2009). This is one of the reasons why plant cell walls are highly recalcitrant towards fungal and bacterial enzymes and why a pretreatment is necessary to "open-up" the complex cell wall prior to enzymatic hydrolysis (Wyman *et al.* 2005; da Costa Sousa *et al.* 2009).

Cellulose

Cellulose is the main polysaccharide in plant cell walls. Its structure is identical for all plants; however woody biomasses have a higher cellulose content compared to grasses. The cellulose component of lignocellulosic biomass has two prominent supra-molecular forms: crystalline and amorphous. Crystalline cellulose is present in significant quantities in cell walls and is composed of rigid micro-fibrils (**Fig. 1**). These micro-fibrils are composed of organized assemblies of β-(1,4)-D-glucan chains that have intra- and inter-molecular hydrogen bonds to each other (Cosgrove 2005).

Hemicelluloses

These are the second major polysaccharides found in plant cell walls and are mainly composed of aldopentoses (i.e., arabinose, xylose and galactose) and play an important role in cross-linking cellulose micro-fibrils. In grasses, the most abundant hemicelluloses are xyloglucan and arabinoxylans.

Xyloglucan has a backbone similar to cellulose; however it has xylose branches on every 3 of 4 glucose residues (**Fig. 2**). This polymer is thought to either bind spontaneously to adjacent cellulose micro-fibrils, maintaining their link, or bind covalently to pectin polysaccharides (Cosgrove 2005). Arabinoxylans have a β-(1,4)-D-xylan backbone, branched with arabinose residues. Glucuronic and ferulic acid branches are also present in the arabinoxylan structure. Ferulic acid residues are responsible for cross-linking arabinoxylans to lignin. There are between nine and ten ferulic acid ester-ether bridges for every 100 C6-C3 lignin monomers (Jeffries 1994; Chundawat 2009).

In softwoods the major hemicelluloses are galactoglucomannan (the most important), glucomannan and arabinoxylan. They are composed of a β-(1,4)-linked D-mannopyranose and D-glucopyranose backbone decorated with D-galactopyranose residues, which are linked as single-unit side chains by α-(1-6)-bonds (Jeffries 1994) (**Fig. 3**). The hydroxyl groups at position C2 and C3 in the backbone units are partially substituted by *O*-acetyl groups, on average one group per 3-4 hexose units (Jefferies 1994). The arabinoxylan structure in softwoods is the same as in grasses, but grasses contain more arabinoxylans than hardwoods and softwoods (**Fig. 2**). Other hemicelluloses, like arabinogalactan, xyloglucan and other glucans, are present in small amounts in the plant cell wall.

In hardwoods the main hemicellulose component is *O*-acetyl-4-*O*-methylglucurono-β-D-xylan (**Fig. 4**). As in softwoods and grasses, the backbone consists of β-(1,4)-D-

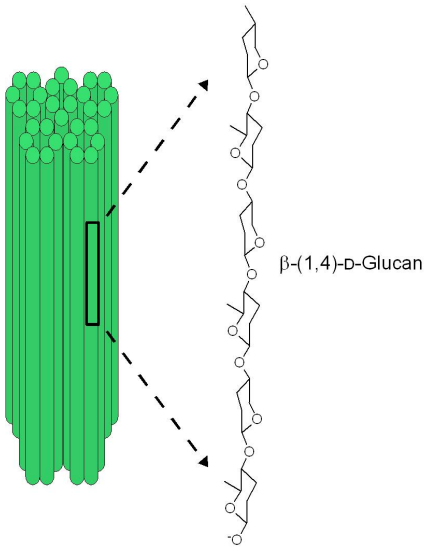

Fig. 1 Graphical representation of the structure of cellulose microfibrils. Cellulose microfibrils (green) are composed of chains of β-(1,4)-D-glucose molecules.

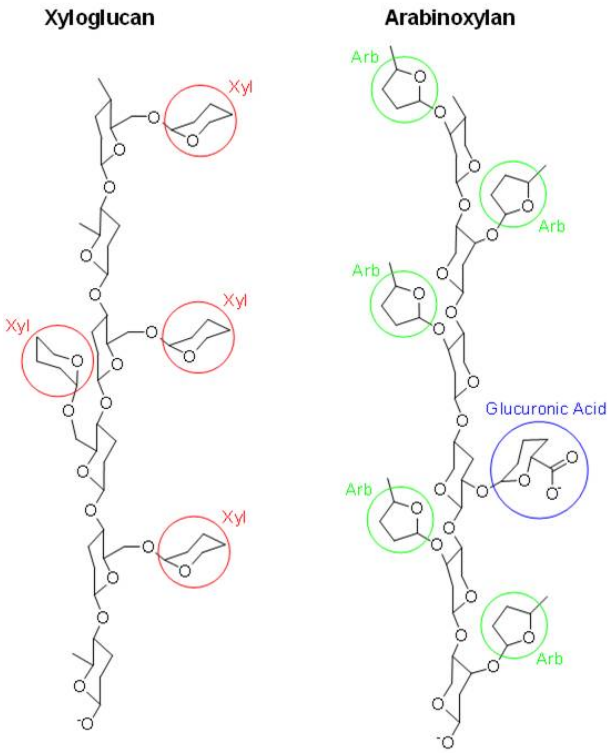

Fig. 2 Graphical representations of the major hemicellulose structures in grasses. (**A**) xyloglucan; (**B**) arabinoxylan. Xyloglucan is composed of a structural backbone similar to cellulose but with additional xylose branches (red). Arabinoxylan has a β-(1,4)-D-xylan backbone with attached arabinose residues (green) and an occasional glucoronic acid residue (blue).

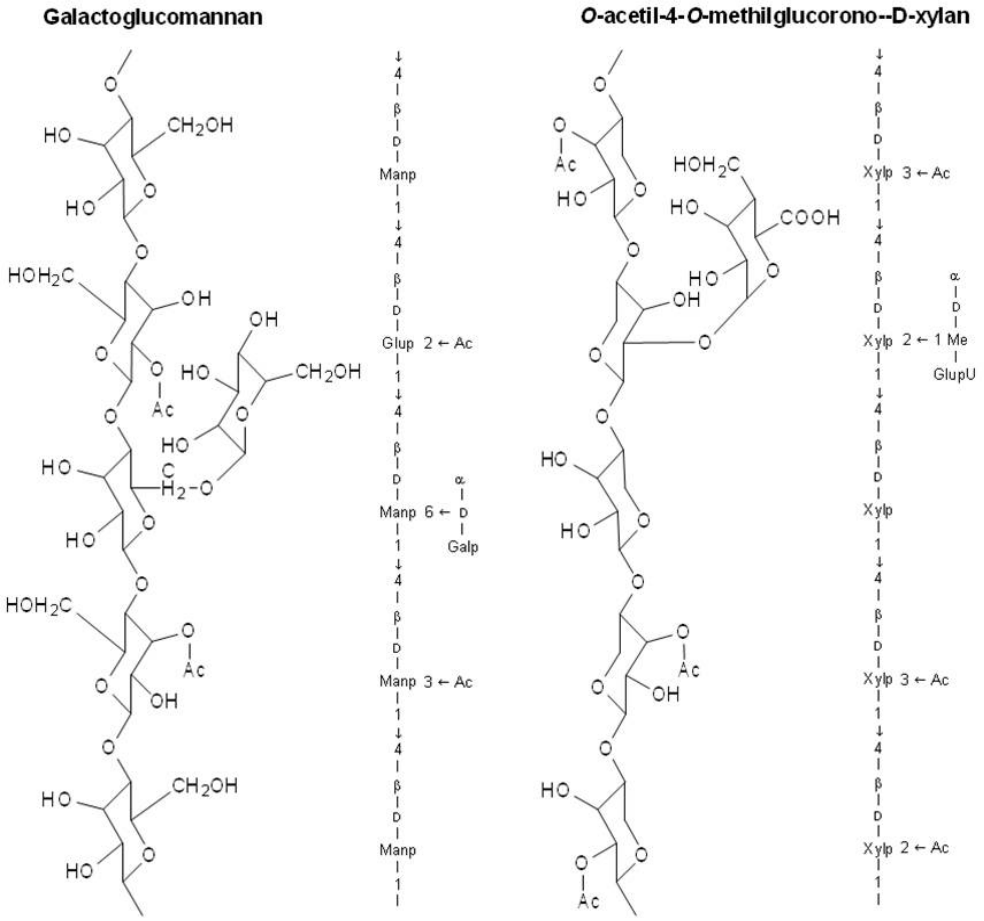

Fig. 3 Graphical representations of the primary hemicellulose structures in (A) softwoods and (B) hardwoods. Softwoods are primarily composed of galactoglucomannan, while hardwoods primarily contain *O*-acetil-4-*O*-methilglucorono-β-D-xylan.

xylan units; however in hardwoods most of the hydroxyl groups at the carbons C2 and/or C3 are substituted by acetyl groups. In addition, xylose units are substituted with α-(1-2)-linked 4-*O*-methylglucoronic acid residues, normally at every 10th xylose unit (Jefferies 1994). Their xylan backbone does not contain arabinose in their side chain.

Pectins

Pectins are a complex and heterogeneous group of polysaccharides that are covalently linked to each other and affect primary cell wall porosity. Some important pectins include rhamnogalacturan I and II (**Fig. 4**), galactans and arabinans (**Fig. 5**). Rhamnogalacturan I is a polymer constituted of alternating residues of galacturonic acid and rhamnose. Rhamnogalacturan II is constituted of a complex structure of 11 different sugar residues linked to a backbone chain of galacturonic acid molecules, called homogalacturonan when no side chains are attached. When side chain residues of xylose are covalently bound to this chain, it is called xylogalacturonan. Arabinans are branched polymers composed of arabinose which can be linked to a backbone of β-(1,4)-D-galactan to give rise to arabinogalactans. The neutral arabinans and arabinogalactans can be linked to the acidic pectins, modulating the accessibility to the cell wall (Cosgrove 2005; Laine 2005).

Lignins

In angiosperms, the lignin polymer is produced by dehydrogenative polymerization of three different cinnamyl alcohols (p-coumaryl, coniferyl, and sinapyl alcohol) that differ in the degree of methoxylation at the C3 and C5 positions of the aromatic ring (**Fig. 6**). When these alcohols are transformed in lignin polymers, they are called p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the polymer, respectively. In addition to these three main components, lignin polymers incorporate compounds that derive from incomplete biosynthesis of components H, G and S, as well as other phenylpropanoid units such as acetates, hydroxycinnamyl aldehydes and others (Sederoff *et al.* 1999).

A variety of chemical linkages, including ether and carbon-carbon bonds, connects the units of a complex network of different compounds. This polymer complexity and heterogeneity depends on the relative proportions of the three main monolignol units (H, G and S). Lignin from grasses incorporates G and S units at comparable levels and more H units than hard woods. In softwoods, lignin is essentially made of G units while in hardwoods it is made of G and S units. Lignin rich in G units has relatively more carbon-carbon bonds than lignin rich in S units. Thus, grasses and softwoods are less susceptible to Kraft delignification than hardwoods. Kraft delignification is used by the paper industry, breaking down the non-condensed ether β-*O*-4-linkages in lignin using alkali agents (e.g. NaOH). The carbon-carbon bonds are more resistant to chemical degradation, so they are not affected by this process.

AMMONIA FIBER EXPANSION (AFEX™) PRETREATMENT PROCESS

Close inspection of various ammonia based pretreatments reveals that ammonia was either used in its liquid state (30-99% concentration), supercritical state, or as dilute ammonium hydroxide (0.1-28%) (e.g., ammonia recycled percolation). AFEX™ is a novel alkaline pretreatment process that has been successful in improving lignocellulosic biomass degradability by hydrolytic enzymes (Balan *et al.* 2009). Concentrated anhydrous ammonia is added to the system which associates with water to form ammonium and hydroxide ions, as shown below:

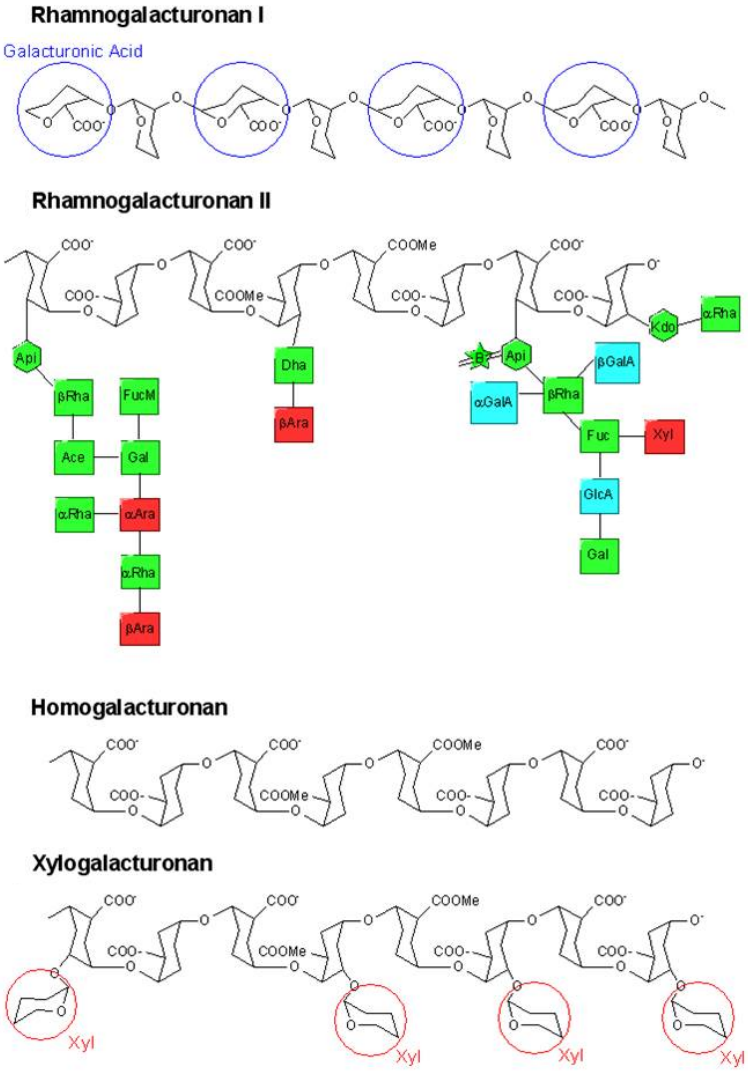

Fig. 4 The structural composition of two of the major pectin domains in plants. (**A**) rhamnogalacturan I; (**B**) rhamnogalacturan II. Rhamnogalacturan I consists of alternating residues of galacturonic acid (blue) and rhamnose. Rhamnogalacturan II has a complex structure of eleven different sugar residues linked to a backbone chain of galacturonic acid molecules. This backbone is called (**C**) homogalacturonan when no side chains are attached. When side branches of xylose (red) are covalently bound to the homogalacturonan backbone, it is called (**D**) xylogalacturonan.

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \quad (1)$$

This is an exothermic reaction (ΔH of formation of NH_4OH is -87.59 Kcal/mol at 25°C) which rapidly increases the temperature of the biomass in the reactor.

When the desired reaction temperature is reached, the system is maintained at constant temperature and pressure for the necessary residence time, after which the reactor pressure is released rapidly, decreasing the temperature of the system to approximately 20-30°C. Several kinds of biomasses have been pretreated using AFEX™ (Belkacemi *et al.* 1998; Alizadeh *et al.* 2005; Bals and Balan 2006; Murnen *et al.* 2007) and the optimal reaction parameters vary depending on the cell wall type. The pretreatment reaction variables include reaction temperature, ammonia to biomass loading, moisture content and residence time. Typical conversion for untreated and AFEX™ treated feedstocks are shown in **Fig. 7**. In these studies, temperatures ranged from 70 to 140°C, the ammonia to biomass loading varied from 0.6: 1 to 2: 1, moisture contents ranged between 60 and 230%, and the residence time varied from 15 to 45 min. AFEX™ has tremendous potential to pretreat grasses such as corn stover and switch grass, improving their degradability by up to 90% of theoretical yield (Teymouri *et al.* 2005a, 2005b). These improvements result from physicochemical alterations of the lignocellulosic biomass ultrastructure during AFEX™.

One advantage of using ammonia during pretreatment is easy recovery of ammonia (about 97-98% can be recovered, while the remaining is either reacted or left behind along with the biomass) owing to its volatility. Among various ammonia pretreatments AFEX™ is unique in that no separate liquid stream is formed, AFEX™ is thus a dry to dry process and AFEX™-pretreated biomass has a long shelf life. Hence, AFEX™ treated biomass can be easily pelleted, transported, stored, and used as animal feed in addition to being used as a biorefinery feedstock, perhaps in regionally dispersed biomass processing depots (Carolan *et al.* 2007).

CHEMICAL CHANGES OCCURRING IN LIGNOCELLULOSIC BIOMASS DURING ALKALINE PRETREATMENTS

Since AFEX™ is an alkaline pretreatment, it is important to understand the biomass structural changes when it is exposed to these conditions. Due to the great interest of the Kraft pulping industry, some work has already been done analyzing the degradation products of alkali treatments. This section describes chemical changes that occur in polysaccharides and lignin under alkaline conditions. Moreover, the major degradation products of AFEX™ pretreated corn stover will be discussed relative to their inhibitory effect to enzymes.

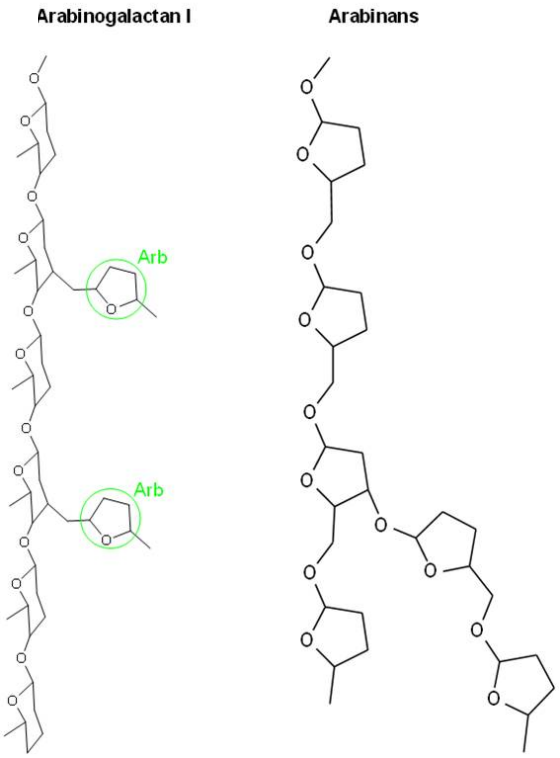

Fig. 5 Graphical representations of the remaining two major pectin domains. (**A**) galactans; (**B**) arabinans. A β-(1,4)-D-galactan backbone supporting a number of arabinose branches (green) is called arabinogalactan. Arabinans are composed of chains of arabinose molecules.

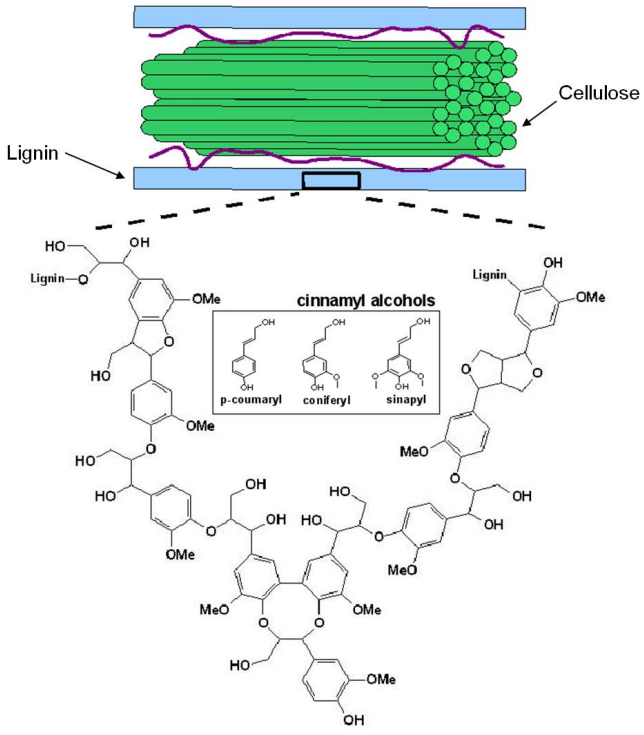

Fig. 6 Lignin (blue) is a component of the plant cell wall which provides structural support and protection to cellulose (green). Lignin polymers are composed of chains of cinnamyl alcohol derivatives (p-coumaryl, conifryl, and sinapyl alcohols), compounds that derive from the incomplete biosynthesis of components H, G and S, and phenylpropanoid units.

Reactions with polysaccharides

Initial reactions under alkaline conditions include solvation of hydroxyl groups by hydroxyl ions, causing the biomass to swell. At high temperatures, many chemical reactions can occur (Fengel and Wengener 1989), including: (i) Dissolution of undegraded polysaccharides; (ii) Peeling of end-groups and the formation of alkali stable end-groups; (iii) Alkaline hydrolysis of glycosidic bonds and acetyl groups, and (iv) Degradation and decomposition of dissolved polysaccharides and peeled monosaccharides.

Loss of polysaccharides and a decrease in the degree of polymerization during alkali treatments are in large part due to peeling and hydrolytic reactions. At temperatures around 100°C, the degradation of polysaccharide chains starts from the existing reducing end groups, commonly called the primary peeling reaction. At temperatures around 150°C the chains start to be cleaved by alkaline hydrolysis and the new reducing ends formed in this process will also be subject to peeling reactions (secondary peeling) also called endwise peeling reactions (Sjöström 1977).

The peeling reaction of polysaccharides involves the elimination of the reducing end groups of polysaccharides, forming any number of different carboxylic acid compounds. Sjöström (1977) has proposed a mechanism for endwise peeling (**Fig. 8**). In the initial step, the reducing end group (A) is isomerized to a ketose (B) which is in equilibrium with the corresponding 2,3–enediol. The C4-substituent is alkali labile in this conformation (B), which promotes its cleavage, leading to the formation of a new reducing end in the polysaccharide chain. The resulting monomeric sugar can be tautomerized to a dicarbonyl compound that can be rearranged to yield isossaccharinic acids (C). Other degradation products that can result from these reactions may be lactic acid (D), 2-hydroxybutanoic acid and 2,5-dihydroxypentanoic acid (E).

Hemicelluloses are genetically much more vulnerable to these types of chemical reactions in an alkaline media than cellulose. However, one can notice differences among the different hemicellulose components. Xylans are more stable than glucomannans and arabinans. Actually, the easier cleavage of arabinose side-groups in softwood xylans has a stabilizing effect against alkaline peeling, since an alkali-stable metasaccharinic acid end group is formed after the loss of the arabinose side group (Fengel and Wengener 1989).

The reaction that occurs during endwise peeling terminates when competing reactions start to take place. These reactions are called stopping reactions and are very important in preventing the disruption of the polysaccharide fibers and their degradation (Fengel and Wengener 1989). These reactions are initiated by a β-hydroxy elimination at the C2 position, producing a tautomeric intermediate that is converted to an alkali stable metasaccharinic acid group (VII) or C2-methylglyceric acid (IX) (Sjöström 1977).

Other low molecular weight acids are also formed during alkaline conditions, including acetic acid and formic acid. Formic acid is a product of peeling reactions of polysaccharides while acetic acid is formed by cleavage of the acetyl side chain groups present in grasses and hardwoods xylans. Glucuronic acid side groups of xylan are also hydrolyzed during intensive alkaline pretreatment (Sjöström 1977; Fengel and Wengener 1989).

Reactions with lignin

The most labile lignin bonds in alkaline conditions are the aryl-ether bonds, which are mostly cleaved during Kraft pulping treatment. Also, aryl-alkyl or alkyl-alkyl bonds are typically destroyed in alkaline medium, but to a lesser extent than the aryl-ether bonds. Diaryl ether and C-C bonds are normally stable in these conditions, and usually remain unaltered during alkaline treatment.

In hardwoods and softwoods the most common linkages are the α- and β-aryl ether types and are therefore the most

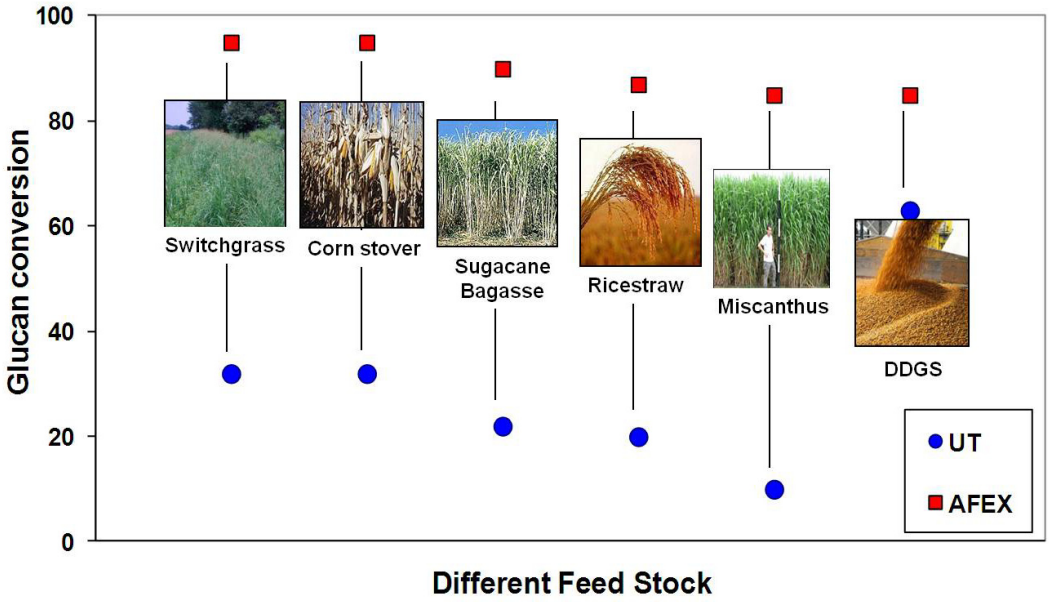

Fig. 7 Typical biomass conversion observed for both untreated and AFEX^{TM}-treated samples. Hydrolysis experiments were done at 1% glucan loading of 25 mg of Cellulase and 2.5 mg of xylanase/g of glucan and the reaction conditions were, 50°C, 200 rpm, 168 h. About 70% xylan conversion notices for most of the feed stock.

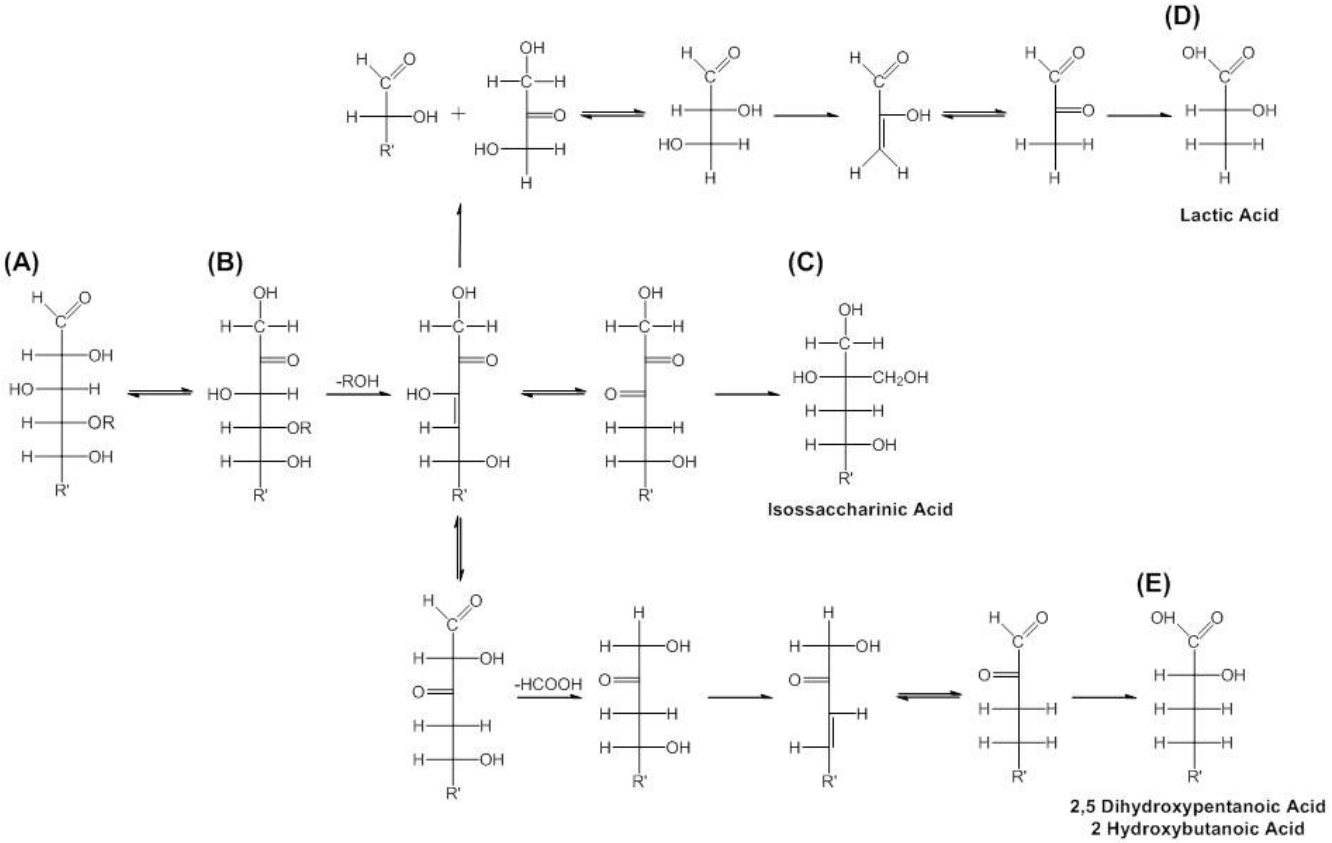

Fig. 8 Endwise peeling reactions of polysaccharides. In the initial step, the reducing end group (**A**) isomerizes to a ketose (**B**). The resulting product can be tautomerized to a dicarbonyl compound that can be rearranged to yield isossaccharinic acids (**C**). Other degradation products that can result from these reactions are lactic acid (**D**), 2-hydroxy-butanoic acid and 2,5-dihydroxypentanoic acid (**E**).

important linkages to cleave to promote lignin degradation. As α- and β-aryl ether linkages are relatively easy to cleave, their cleavage is independent of the hydroxyl ion concentration and is normally the main reaction that occurs in the initial phase of the alkaline pulping processes (Sjöström 1977). During alkaline treatment of biomass, many compounds can be formed from lignin. Most are aromatic acids, aromatic aldehydes and phenolic compounds, which can have an inhibitory effect on enzymes and microorganisms.

Reactions between ammonia and lignocellulosic biomass

1. Ammonolysis reactions

In the special case of AFEX^{TM} pretreatment of lignocellulosic biomass, ammonia is not only a source of hydroxyl groups but is also a reactant itself. Amination of organic compounds via ammonolysis occurs during AFEX^{TM} pretreatment, as evidenced by the considerable amounts of acetamide and phenolic amides found in water extracts of

Table 2 Extent of competitive reactions (ammonolysis and hydrolysis) when reacting with esters of different molecular weights (French and Wrightsman 1938).

Acetate	Course of the reaction at 25°C			
	Molecular weight	Hours	% Reaction	Ratio of ammonolysis: Hydrolysis
Methyl	74.1	20	90	10.3:1
Ethyl	88.1	100	97	5.1:1
n-Propyl	102.1	100	89	5.4:1
n-Butyl	116.2	100	87	4.8:1
n-Amyl	130.2	100	78	6.1:1
Isobutyl	116.2	100	76	4.1:1
Benzyl	150.2	15	73	5.6:1
Isoamyl	130.2	100	72	6.2:1
Isopropyl	102.1	200	44	1.6:1
s-Butyl	116.2	200	44	1.4:1
t-Butyl	116.2	200	5	0.7:1

AFEX™ treated corn stover (Chundawat 2009; Chundawat *et al.* 2010).

The dissociation of ammonia in aqueous solutions produces ammonium and hydroxyl ions. Both ions target several linkages, including ester linkages, which are considered among the most reactive linkages. French and Wrightsman (1938) studied the effect of aqueous ammonia solutions on different esters and found that the ratio of ammonolysis to hydrolysis decreases with increasing molecular weight of the alcohols associated with the different esters (**Table 2**). Moreover, the reactivity decreases by increasing the molecular weight of the ester, especially for the case in which the alcohol associated with the ester had side chains close to the ester bond (e.g., isopropyl acetate) likely due to the stereochemistry of the chemical reaction and the size of ammonium ion compared to the hydroxyl ion.

Depending on the type of reaction, the resulting products will be carboxylic acids or amides. For example, the acetyl groups present in xylans and mannans will produce acetic acid or acetamide depending on whether they are engaged in hydrolysis or ammonolysis reactions, respectively.

2. Maillard-type reactions

Reactions of ammonia (or amino compounds) with polysaccharides, also called Maillard reactions, comprise an extremely complex network of reactions. More than fifty reaction products have been identified after reacting ammonia with D-glucose, including pyrazines, imidazoles, ketones, aldehydes and amides (Shibamoto *et al.* 1979), and reaction pathways are still debated. Hodge (1955) presented a simplified mechanism to understand Maillard reactions, dividing them in the following stages: I, Initial Stage (a, sugar-amine condensation; b, Amadori rearrangement); II, Intermediate Stage (a, sugar dehydration; b, sugar fragmentation; c, amino acid degradation) and III, Final stage (a, aldol condensation; b, aldehyde-amine condensation and formation of heterocyclic nitrogen compounds). Some of the pyrazine derivatives which were identified in AFEX™ treated corn stover are listed in **Table 3**.

AFEX™-CATALYZED DECOMPOSITION PRODUCTS

Decomposition products formed/released from monocot cell walls during AFEX™ (Chundawat *et al.* 2010) have been identified and quantified. In this work, corn stover was pretreated using AFEX™ at 130°C, with a biomass to ammonia ratio of 1:1, 60% moisture and 15 min residence time. The decomposition products were extracted from AFEX™ treated corn stover and analyzed by various analytical techniques (e.g., LC-UV, LC-RI, GC-MS and LC-MS/MS). **Table 3** depicts the average concentration of various products found in AFEX™-pretreated corn stover water extracts.

The origin and mechanism of formation of some of the

Table 3 Maillard reaction products produced in AFEX™-treated corn stover (Chundawat *et al.* 2010).

(µg analyte/g AFCS)	
Maillard reaction products	**AFEX™**
Pyrazine	4
2-Methylpyrazine	45
2,5-Dimethylpyrazine	5
2,6-Dimethylpyrazine	16
2-Ethylpyrazine	4
2,3-Dimethylpyrazine	5
2-Ethyl-6-methylpyrazine	3
2-Ethyl-5-methylpyrazine	3
2,3,5-Trimethylpyrazine	5
2-Ethenylpyrazine	4
3-Ethyl-2,5-dimethylpyrazine	1
2,6-Diethylpyrazine	3
Tetramethylpyrazine	3
6-Methyl-2-ethenylpyrazine	3
Acetylpyrazine	3
2-Acetyl-5-methylpyrazine	2
3-Methyl-2-pyrazinyl methanol	15
6-Methyl-2-pyrazinyl methanol	265
2-Methyl-1-H-imidazole	31
2,4-Dimethyl-1-H-imidazole	108
4-Methyl-1-H-imidazole	418

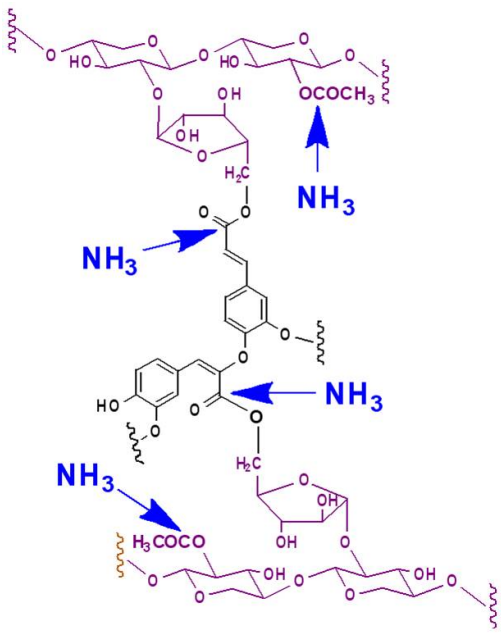

Fig. 9 The ester linkages present in biomass which are susceptible to cleavage during the AFEX™ process.

degradation products presented in **Table 4** discussed above. This paper reported for the first time the major cell wall decomposition products formed during AFEX™ and compared it against the products formed during dilute acid pretreatment (Chundawat *et al.* 2010). Certain compound classes which were identified include aliphatic organic acids, furans, aromatic aldehydes, phenolic acids, amides and gluco- and xylo-oligosaccharides. Ammonolysis of ester linkages during AFEX™ resulted in the formation of acetamide (25 mg/g AFEX™ treated corn stover) and various other phenolic amides (15 mg/g AFEX™ corn stover) (**Fig. 9**). Maillard reactions with carbonyl-containing intermediates (e.g., pyrazines, imidazoles) represent the second largest sink for ammonia during AFEX™. Currently, 50% of the nitrogen reacted during AFEX™ (for corn stover as substrate) is still unaccounted for. Several biologically inhibitory carboxylic acids and furans formed during dilute acid pretreatment were also present in trace quantities for AFEX™-treated corn stover. The presence of water soluble extractives from AFEX™ treated biomass resulted in slight

Table 4 Cell wall decomposition products identified from AFEX™-treated corn stover. BM is biomass (Chundawat *et al.* 2010).

Average (μg analyte/g BM)		
Cell wall decomposition products	**Untreated**	**AFEX™**
malonic acid	62	88
lactic acid	126	318
maleic acid	3	4
cis-aconitic acid	346	895
methylmalonic acid	76	1
succinic acid	282	596
fumaric acid	249	356
trans-aconitic acid	898	2,898
levulinic acid	171	24
glutaric acid	5	8
itaconic acid	5	22
2-hydroxy-2-methylbutyric acid	3	3
gallic acid	ND	ND
adipic acid	3	3
acetic acid	1,610	4,610
formic acid	324	912
2-furoic acid	4	6
5-hydroxymethyl furfural	71	642
furfural	1	3
total fructose	16,027	3,403
total glucose (monomers + oligomers)	18,400	18,500
total xylose (monomers + oligomers)	1,200	50,000
total arabinose (monomers + oligomers)	300	10,000
acetamide	ND	25,040
total phenolic amides (monomers + dimers)	ND	14,761
total pyrazine and imidazole derivatives	ND	945
other likely nitrogenous species	ND	2,630
3,4-dihydroxybenzoic acid	7	7
3,5-dihydroxybenzoic acid	ND	ND
2,5-dihydroxybenzoic acid	ND	1
3,4-dihydroxybenzaldehyde	3	6
salicylic acid	24	32
4-hydroxybenzeldehyde	12	93
vanillic acid	25	46
homovanillic acid	ND	4
4-hydroxyacetophenone	1	20
caffeic acid	7	5
syringic acid	12	50
vanillin	13	195
4-hydroxybenzoic acid	ND	1
benzoic acid	11	9
syringaldehyde	3	11
p-coumaric acid	161	1,080
ferulic acid	35	103
sinapic acid	2	2
3-hydroxy-4-methoxycinnamic acid	3	42
4-hydroxycoumarin	ND	ND
ortho-toluic acid	ND	1
para-toluic acid	18	19

inhibition of both enzymes and microbes (Lau and Dale 2009; Chundawat *et al.* 2010).

There was a 300% increase in the total nitrogen content of AFEX™ treated corn stover (36 ± 3.1 mg NH_3 equivalents/g biomass) compared to untreated corn stover (9.3 ± 1.6 mg NH_3 equivalents/g biomass). A significant proportion of this increase is attributable to residual ammonia (5.5 ± 1.1 mg NH_3/g biomass). Soluble nitrogenous compounds accounted for 88% of total reacted nitrogen for AFEX™ treated corn stover, while the remaining 12% are likely amidated forms of insoluble lignin-phenolics and/or hemicellulose. Insoluble amidated lignin is likely produced due to ammoniation of phenolics that are ether-linked to lignin and ester-linked to hemicelluloses. Previous work on ammonia treated hardwoods has attributed insoluble amides to amidated uronic acid side-chain residues bound to xylan (Wang *et al.* 1964). Ammonolysis by-products (i.e. acetamide and phenolic amides) account for nearly 36% of the total nitrogenous products incorporated within corn stover during AFEX™ (8.6 mg total reacted NH_3 equivalents/g biomass). Hence, loss of ammonia due to ammonolysis alone cannot account for all the ammonia reacted during AFEX™ (i.e. 21.1 mg NH_3 equivalents/g pretreated corn stover).

In addition to these decomposition products, AFEX™ corn stover water extractives contain considerable amounts of gluco- and xylo-oligosaccharides. Most of the oligomers released during AFEX™ range between degree of polymerization (DP) 2 and 6. However, there is a significant proportion (10–20% of total) of higher DP oligomers (DP > 10) present as well. Water extractable arabinoxylan content for AFEX™ treated corn stover was 22% and 24% of theoretical xylan and arabinan content, respectively. Oligosaccharides in the liquid extract were acid hydrolyzed and the resultant monomeric sugars were quantified by high performance liquid chromatography (HPLC) (Chundawat *et al.* 2010). This gave a 0, 41, and 33-fold increase in glucose, xylose, and arabinose concentrations, respectively, when compared to untreated corn stover (**Table 4**). Compared to untreated corn stover, AFEX™ pretreated samples have decreased monomeric glucose concentration while there is not much change in monomeric xylose and arabinose concentrations. This could be due to alkali and/or thermal induced degradation of these monomeric sugars during pretreatment. These findings strengthen our hypothesis that AFEX™ cleaves lignin-hemicellulose ester linkages, thereby releasing water extractable arabinoxylans oligomers along with other phenolics from within the cell wall. However, the exact structure of oligosaccharides released during AFEX™ is unclear and is currently being investigated.

MECHANISTIC UNDERSTANDING OF AMMONIA-BASED PRETREATMENTS

A fundamental understanding of the chemical reactions occurring during biomass pretreatment and the resultant physiochemical changes that improve enzymatic digestibility will allow further improvements to the process. Based on several spectroscopy and microscopy studies for untreated and AFEX™ pretreated cell walls, we have identified a series of chemical and physical changes that occur during AFEX™ (Chundawat 2009; Chundawat *et al.* 2011a) and that are responsible for the improved enzyme accessibility of the pretreated substrate (**Fig. 10**). Some of the major effects of AFEX™ are: (i) Ammonia and hydroxyl ions catalyze the cleavage of ester-linkages between lignin and hemicellulose that allow their subsequent removal to outer wall surfaces; (ii) Extraction of lignin and hemicellulose after LCC bond cleavage away from cellulose creates nano-pores (10-100 nm in size) within the cell wall; (iii) AFEX™ degradation products are removed and deposited on the outer periphery of the cell walls by diffusion and migration through the nano-porous tunnel network (as visualized via 3D TEM based tomography); (iv) Hemicellulose is deacetylated via ammonolysis (to acetamide) and hydrolysis (to acetic acid). Various other degradation products are formed in AFEX™ as well, and are listed in **Table 3**; (v) cellulose crystal structure is modified by anhydrous liquid ammonia from Cellulose I to III only for certain AFEX™ conditions, and (vi) enzyme accessibility and rate of hydrolysis increases significantly due to improved porosity (and accessible internal surface area) of the cell wall ultra-structure after AFEX™ (Chundawat *et al.* 2011a).

The current mechanism of AFEX™ is based on a recent study characterizing the effect of AFEX™ on corn stover derived secondary cell walls (Chundawat 2009; Chundawat *et al.* 2011a). Ammonia is thought to penetrate the outer walls and middle lamella where it catalyzes ammonolytic and hydrolytic (in the presence of water) cleavage of various ester linkages (e.g., hemicellulose acetates, ferulates) (Chundawat *et al.* 2010). Cleavage of LCC ester linkages facilitates the removal of hemicellulose and other extractables to outer wall surfaces, exposing the cellulose microfibrils. These extractives have been found to be abundant in arabinoxylan oligomers, amides, minerals and other cell

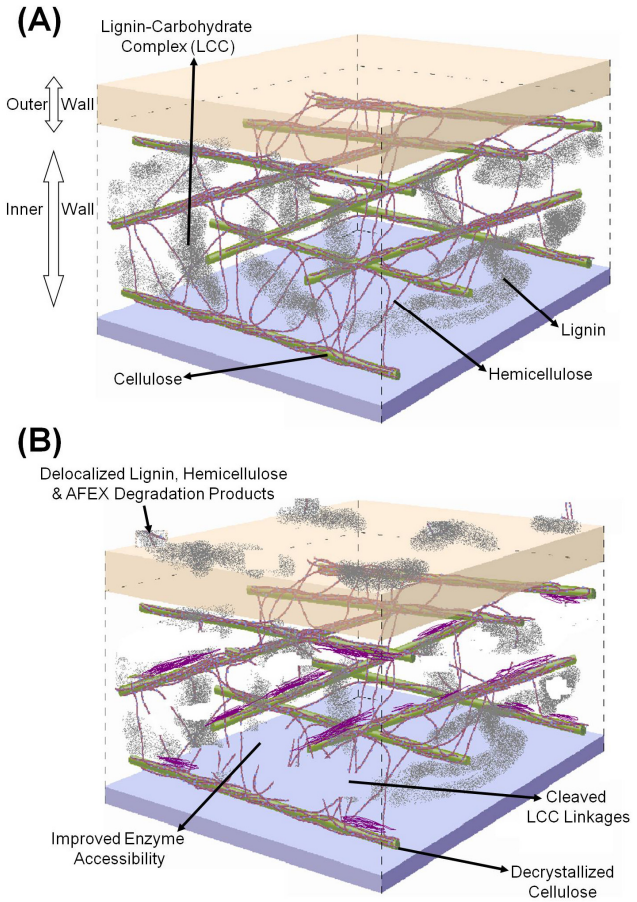

Fig. 10 (**A**) Untreated generic cell wall depicting cellulose (green fibrils), hemicellulose (purple strands) and lignin (grey matrix). (**B**) AFEX™-treated cell wall. This figure was adapted from Somerville *et al.* (2004).

wall degradation products released/formed during AFEX™ (Chundawat *et al.* 2010). A sudden pressure drop at the end of AFEX™ causes convective transport of ammonia-water and cell wall extractables towards the lumen and cell corners. The decompression of ammonia forms large pores in the middle lamella and outer secondary cell walls. Pores larger than 10 nm should facilitate improved accessibility of cellulases (3-10 nm in size) for pretreated cell walls. AFEX™ pretreatment conditions are also known to significantly influence the ultra-structure of the cell walls.

Absence of water during AFEX™ results in the coalescence of lignin as globular structures surrounded by polysaccharides associated with the outer periphery of the globules. Temperature plays an important role in the delocalization of lignin (i.e., lignin migration at temperatures > 130°C). No significant amounts of cellulose III were formed during conventional AFEX™ (1:1 ammonia to biomass loading, 60% moisture, 140°C, 15 min residence time) employing ammonia-water mixtures. However, in the absence of water cellulose III is produced. Cellulose III is known to have higher enzymatic digestibility than native cellulose (Chundawat 2009; Chundawat *et al.* 2011b), suggesting that it would be beneficial to alter AFEX™ conditions to produce cellulose III, while possibly simultaneously extracting lignin (Chundawat *et al.* 2011c). There were no major modifications seen in the structure (i.e., β-ether linkages are intact) of lignin after conventional AFEX™. However, more work is needed to examine the role of quinone-methide intermediates reacting with ammonia during AFEX™ (Sewalt *et al.* 1997). A detailed mechanistic under-standing of the kinetics of ammonolysis of diferulates and other LCC linkages within the cell wall during AFEX™ is also currently lacking.

It is generally assumed that removal of hemicellulose and lignin during pretreatment into a separate liquid phase is necessary to improve cellulase accessibility to underlying cellulose. However, pretreatments that remove lignin and hemicellulose may also remove other essential nutrients that negatively influence downstream fermentation. AFEX™ pretreatment employing much lower solvent loadings improves polysaccharide digestibility via cleaving lignin-carbohydrate ester linkages, partially solubilizing cell wall extractables and delocalizing these extractables to exterior cell wall surfaces, thereby creating a porous, interconnected tunnel-like network that is much more accessible to cellulolytic enzymes (Chundawat *et al.* 2011a). The nutrients (e.g., proteins, free sugars, ammonia, minerals) left behind in the cell wall promote greater fermentability of AFEX™ treated substrates, unlike most other pretreatments.

OTHER POSSIBLE CONFIGURATIONS FOR AMMONIA-BASED PRETREATMENTS

Gaseous ammonia pretreatment (GAP) process

This is an improvised, modified AFEX™ process which uses hot ammonia gas to pretreat the biomass. Ideal pretreatment using ammonia should meet the following conditions as much as possible: homogeneous, with negligible mass-heat transfer issues, low residence times, low ammonia/water usage, and without complex ammonia-water separation procedures. The GAP process using a biomass fluidization method achieves most of these conditions (Balan *et al.* 2011).

For GAP pretreatment hot ammonia gas is delivered to the pre-wetted biomass, where it condenses on the biomass and reacts with water. The desired temperature (100-140°C) is achieved rapidly due to the exothermic reaction between water and ammonia. In this approach, there is no expansive release of pressure at the end of the pretreatment, allowing significant energy savings during recycling of ammonia. This new process can be made continuous using recycled streams of (a) ammonia gas, (b) ammonia/steam gas mixture, (c) ammonia gas combined with an inert or other carrier gas, or (d) ammonia/steam gas mixture combined with an inert/carrier gas in any of the following reactors: fluidized bed reactor, a semi-fluidized, packed bed reactor, or a fixed bed reactor. Only a small portion of ammonia (~0.5-2 wt%, of total ammonia) will be reacted during the GAP approach and the remaining ammonia can be recycled in its gaseous state (Yoo *et al.* 2011).

Extractive Anhydrous liquid ammonia based process (E-AFEX™)

We have developed another cellulosic biomass pretreatment process using ammonia which results in the formation of the highly digestible cellulose III allomorph and can also simultaneously extract cell wall components (i.e., lignin, lignin decomposition products, xylo-oligosaccharides, amides) using essentially anhydrous liquid ammonia pretreatment (Chundawat *et al.* 2011b, 2011c). Cellulose crystallinity, lignin-carbohydrate complex (LCC) ester linkages and non-specific enzyme binding to cell wall components (such as lignin) are the major rate-limiting steps to efficient cell wall deconstruction. Non-crystalline cellulose (or amorphous cellulose) is known to have a 4-5 fold higher rate of enzymatic hydrolysis than native crystalline cellulose. However, conventional processes utilizing ionic liquids and concentrated phosphoric acid for making amorphous cellulose are expensive and require high levels of chemicals and water. Pretreating cellulose using inexpensive liquid ammonia could also modify cellulose crystal structure to make it more easily digestible without completely transforming it to amorphous cellulose. Cellulose III allomorph formed from native cellulose I during E-AFEX™ was found to have a 2-5 fold (depending on initial cellulose crystallinity) higher rate of enzymatic hydrolysis (Chundawat 2009; Chundawat *et al.* 2011b). The presence of added water during ammonia based pretreatments does not allow

Table 5 Varying ammonia-based pretreatment methods along with their respective pretreatment conditions and sugar conversion.

Ammonia-based Pretreatments	Typical Temperature Pressure	Co-solvent Addition	% Ammonia in solution	Ammonia: Biomass: Co-solvent Loading	Residence Time (min.)	Cellulase Loading	% Cellulose Conversions
Super Critical Ammonia (SCA) (Weimer *et al*. 1986, Weimer and Chou 1986)	>132.5°C, >1653 psi	None	>95% ammonia	1.5-3.5 : 1 : 0	15-60	5-10 IU/g biomass	90-100
Soaking Aqueous Ammonia (SAA) (Kim and Lee 2005b, Lin *et al.* 2010)	25-30°C, 15 psi	Yes (water)	15-30% ammonia	1.5 : 1 : 8.5	1440-8640	15 FPU/g glucan	80-90
Ammonia Recycle Perculation (ARP) (Kim *et al.* 2003, Kim and Lee 2005a)	150-180°C, 350 psi	Yes (water)	15% ammonia	0.5 : 1 : 3	10-60	10 FPU/g glucan	90
Ammonia Fiber Expansion (AFEX™) (Teymouri *et al.* 2005a, Balan *et al.* 2009)	100-140°C, 200-350 psi	Yes (water)	50-70% ammonia	1-2 : 1 : 0.6	15-30	15 FPU/g glucan	80-100
Gaseous Ammonia Pretreatment (GAP) (Balan *et al.* 2011, Yoo *et al.* 2011)	100-140°C, 200-350 psi	Yes (water)	No solution added	0.6-0.1 : 1: 0.6	15-30	15 FPU/g glucan	80-100
Extractive-AFEX™ (E-AFEX™) (Chundawat *et al.* 2011a, 2011b)	100-140°C, 400-1200 psi	Yes (non-aqueous)	50-100% ammonia	3-6 : 1: 3-6	15-60	15 FPU/g glucan	90-100

significant conversion to cellulose III. Reduced levels of water using the new pretreatment method can also significantly reduce costs for ammonia recovery.

It is well known that enzymes and microbes are inhibited due to interaction with lignin and other cell wall decomposition products produced by pretreatment (Sun and Cheng 2002). The extent of these inhibitions depends on the pretreatment conditions and how the cell wall is modified during pretreatment. However, it is virtually impossible to completely avoid enzymatic and microbial inhibition when lignin is present in the pretreated biomass. Removal of degraded lignin (and other cell wall decomposition products) should significantly reduce enzyme loading (and improve hydrolyzate fermentability) and consequently improve the economical viability of a cellulosic biorefinery. In most pretreatment technologies, where it is possible to observe the delignification phenomenon, the final enzymatic digestibility of the pretreated biomass is improved with respect to the control. With the E-AFEX™ process, it is possible to obtain two fractions, of which one would be cellulose rich and the other hemicellulose-lignin rich (after stripping with ammonia). While the cellulose-rich fraction could be used to produce biofuels (after enzymatic hydrolysis and fermentation), the hemicellulose fraction could be separated from lignin and used for other applications (e.g., soluble inducers for microbes to produce cellulases/hemicellulases, fuels and other products using thermo-chemical catalysis). The isolated lignin can also be used in several applications including combustion to produce electricity, chemical synthesis through catalysis or as resin/binders in the production of biomaterials.

Other ammonia-based processes

Different variations to the ammonia treatment process have been reported in the literature as highlighted in **Table 5**. Some examples of this include; supercritical ammonia (SCA) treatment, ammonia recycled percolation (ARP), soaking in aqueous ammonia (SAA) and ammonia-peroxide pretreatment (Weimer *et al.* 1986; Kim and Lee 1996; Kim *et al.* 2003; Kim and Lee 2005a, 2005b; Hennessey *et al.* 2007; Lin *et al.* 2010). These pretreatments vary from the conventional AFEX™ process mostly in the thermodynamic state of ammonia or ammonia-water mixtures employed during pretreatment. SCA pretreatment is the only ammonia pretreatment process that is carried out under the super critical state of ammonia, while all other pretreatments are carried out under sub-critical conditions. Most aqueous ammonia pretreatments like the ARP process are carried out in a flow-through mode by percolating dilute ammonium hydroxide solutions (5-15% concentration, w/w) through a packed bed reactor under high pressure (2-3 MPa) and at high temperatures (160-180°C) to selectively extract lignin and hemicellulose; while extractive AFEX™ (or E-AFEX™) is a novel ammonia treatment process carried out using concentrated, anhydrous ammonia solvent mixtures (60-99% concentration, w/w). In recent years MBI International (www.mbi.org) has led the development of a packed bed AFEX™ process to carry out low cost AFEX™ pretreatment with minimal energy requirements to recover ammonia, unlike the conventional processes. More details on these pretreatments can be found in a recent publication (Chundawat *et al.* 2012).

CONCLUSIONS

In this work we presented evidence that different pretreatment conditions generate different degradation products at different ratios. The important reactions determining the actual degradation of the biomass is a key question that can only be answered by such a study. Also, understanding the relevant reactions allows us to design conditions promoting reactions that favor downstream processes.

ACKNOWLEDGEMENTS

This work was funded by DOE Great Lakes Bioenergy Research Center (www.greatlakesbioenergy.org) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through Cooperative Agreement DEFC02-07ER64494 between The Board of Regents of the University of Wisconsin System and the U. S. Department of Energy. We appreciate financial support, in initial stages of the project, from Michigan State Research Foundation (SPG grant). AFEX™ is a trademark of MBI International (www.mbi.org).

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