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Review

An evaluation of dilute acid and ammonia fiber explosion pretreatment for cellulosic ethanol production

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HIGHLIGHTS

- AFEX treated corn stover resulted in larger pore size compared to DA.
- Nonspecific adsorption of cellulases was lower in AFEX treated biomass.
- Higher adsorption of cellulase onto AFEX treated cellulose than DA.
- AFEX treated hydrolysate was superior to DA for ethanol production.

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ABSTRACT

The challenge associated with cellulosic ethanol production is maximizing sugar yield at low cost. Current research is being focused to develop a pretreatment method to overcome biomass recalcitrance in an efficient way. This review is focused on two major pretreatments: dilute acid (DA) and ammonia fiber explosion (AFEX) pretreatment of corn stover and how these pretreatment cause morphological and chemical changes to corn stover in order to overcome the biomass recalcitrance. This review highlights the key differences of these two pretreatments based on compositional analysis, cellulose and its crystallinity, morphological changes, structural changes to lignin, enzymatic reactivity and enzyme adsorption onto pretreated solids and finally cellulosic ethanol production from the hydrolysate of DA and AFEX treated corn stover. Each stage of the process, AFEX pretreated corn stover was superior to DA treated corn stover.

1. Introduction

The biochemical platform which includes pre-treatment, enzymatic hydrolysis and fermentation is one of the promising pathways for lignocellulosic biofuel production. Although it is, there are many challenges yet to be addressed especially in pre-treatment and enzymatic hydrolysis process (Balat et al., 2008). For example, an efficient pre-treatment method that can overcome low yields of sugar recovery and relatively slow kinetics of enzymatic hydrolysis (Himmel, 2007) is yet to identify. Different pre-treatments such as dilute acid (DA), steam explosion (SE), ammonia fiber explosion (AFEX) and ionic liquid (IL) have been demonstrated on a pilot scale to overcome biomass recalcitrance for cellulosic ethanol production (Brodeur et al., 2011). Each pretreatment has its own approach and mode of action to interact with plant cell wall and its components (da Costa Sousa et al., 2009). A large number of research has been carried out to understand how different pre-treatments affect the plant cell wall composition and its impact on enzymatic hydrolysis (Zhang et al., 2009). However, a deeper knowledge in relation to the interaction between pre-treatment (catalyst and severity) and plant cell wall would be much valued as the yield of enzymatic hydrolysis depend upon the composition and structure of pre-treated biomass (Singh et al., 2015). A major challenge in linking the interaction between plant cell wall and pre-treatment is the diversity of plant cell wall due to the difference in structure and organization (Ong et al., 2014). Hence, the choice of pre-treatment and optimum conditions may vary from biomass to biomass depending upon mode of pre-treatment mechanism and cell wall structure. Despite a large number of publications in lignocellulosic ethanol production, literature is still lacking information about comparative evaluation of pre-treatments based on a single substrate and the effect of different pretreatments on the structure and chemistry of biomass. Moreover, there are many pretreatment methods for biomass conversion, however, in the near future (based on 5–8 year time frame for implementation), the dilute acid (DA) or ammonia fiber

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explosion (AFEX) seems to be the most probable scenario towards commercialization of bioethanol production from lignocellulose (Kazi et al., 2010). Hence, this review is focused on assessing two pretreatments namely, DA and AFEX and its effect on biomass composition, structure and enzymatic hydrolysis of corn stover. At this stage, the feedstock variability is out of scope as the same batch of corn stover was used for both pre-treatments by Gao et al. (2014).

2. Lignocellulosic biomass and its recalcitrance

Plant cell walls are made up off complex cross-linked polysaccharide networks, glycosylated proteins, pectins and lignin (Ritter, 2008). The cell wall structure is intimately interconnected by lignin–carbohydrate linkages. For example in grasses, lignin–carbohydrate linkage is mediated by ferulates attached to arabinoxylans (Hatfield et al., 1999; Yang et al., 2011). Thus, the term recalcitrance is being derived or caused by the non-cellulosic components and their interactions present in the biomass. These non-cellulosic components can be structural compounds such as lignin and hemicellulose, pectin, acetyl group contents, glycosylated proteins and uronic acids (Zhao et al., 2012) and might play significant role in determining the structure of cell wall on a molecular level. The cell walls are highly resistant towards chemical or biological degradation as the primary goal of cell wall is to protect the whole plant cell polysaccharide from microbial attack (Malinovsky et al., 2014). This property of natural resistance against biological or chemical catalysts by the cell wall is known as recalcitrance. The term biomass recalcitrance cannot be generalized for any biomass as the cross linked polysaccharide network and cellulosic to non-cellulosic components ratio might vary from biomass to biomass and even within the different phenotypes of the same biomass. Moreover, a possible delay in cellulose biosynthesis or a slight modification in integrating the polymers into the cell wall might cause possible variation among cell type (Harris et al., 2010). Examples include changes in cellulose crystallinity and degree of polymerization (DP), the types of hemicellulose and its associated side chains, lignin monomer composition and lignin distribution within the cell wall and lignin–carbohydrate cross linking. In addition, physical characteristics such as cell wall thickness, amount and distribution of vascular tissue and pore volume and its distribution may contribute to the diversity (Zhao et al., 2012). Furthermore, challenges faced by enzymes to act on an insoluble substrate and inhibitors generated during the conversion process may contribute to recalcitrance of lignocellulosic biomass to enzymes (Himmel, 2007).

Overcoming cell wall recalcitrance is the primary step towards a cost effective lignocellulosic biofuel production (Balat et al., 2008; Lee et al., 2014; Pattathil et al., 2015). In order to overcome the biomass recalcitrance in an efficient manner, a detailed understanding about the cell wall structure or architecture linking to pretreatment is essential as the cell wall composition varies in different plant species and even between cell types (Zeng et al., 2014; Pu et al., 2013). For example, glucuronoarabinoxylans are abundant in the primary cell walls of grass (monocot) with minor portions of xyloglucans, pectic polysaccharides and structural proteins (Pattathil et al., 2015). In contrast, dicot plants are abundant in xyloglucans and gymnosperm are abundant in mannans and glucomannans as the major hemicelluloses followed by pectic polysaccharides and structural proteins (Vogel, 2008; Pattathil et al., 2015). Thus, the recalcitrance is caused by these higher order organizations of plant cell wall. For example, access to crystalline cellulose is limited by the coating of amorphous cellulose, hemicellulose and lignin which might create mass transport limitations for the delivery of catalysts such as enzymes (Himmel, 2007).

3. AFEX and DA pretreatment of corn stover

AEFX is a physico-chemical pretreatment where the biomass is exposed to ammonia at higher temperature and pressure for limited period of time (Balat et al., 2008; Behara et al., 2014). In AFEX pre-treatment, ammonia penetrates the cell wall and in the presence of water, the ester linkages are cleaved by various ammonolyic and hydrolytic reactions that ends up in the production of amides or acids (Chundawat et al., 2010). The cleavage of diferulate linkages which cross link polysaccharides, lignin ferulate and lignin diferulate linkages facilities the solubilization of hemicellulose oligomers and extractives to outer cell wall surfaces (Chundawat et al., 2011). At the end of pretreatment, rapid pressure release cause the decompression of ammonia at the cell wall periphery causing large pores in the middle lamella and outer cell wall. AFEX ensures loss of almost no hemicellulose or lignin. The pore size of AFEX treated biomass was larger than 10 nm which allows the accessibility of cellulose by cellulase (Chundawat et al., 2011). For example Cel7A from *Trichodermaressei* has a radius of approximately 5 nm and length of 12 nm (Donohoe et al., 2009). Hence, the enzyme cellulase have better access to AFEX treated corn stover which explains increased enzyme activity on AFEX treated biomass. Though AFEX treated corn stover have better enzymatic reactivity, economics of ammonia pretreatment and its recovery is an issue for commercial scale application. Though recycling ammonia could overcome the cost factor, but ammonia recycling unit may add the total installed equipment cost higher than DA (Kazi et al., 2010). AFEX seems to be effective for herbaceous and low lignin content biomass but less effective as lignin content increases (Brodeur et al., 2011).

Dilute acid pretreatment is very well known and is effective at higher temperature and pressure for lignocellulosic ethanol production (Balat et al., 2008; Alvira et al., 2009). The acid pretreatment is mainly removes hemicellulosic fraction of biomass, especially xylan. Though acid pre-treatment is effective for lignocellulosic biomass with higher sugar yield, the formation of inhibitory compounds have negative impacts in downstream processing which increases the cost of the process. Xylose and glucose released during pretreatment can further dehydrated into inhibitors such as furfural and hydroxymethylfurfural (HMF) (Mosier et al., 2005). In addition, due to the partial breakdown of lignin, phenolic compounds are also released during pretreatment. These compounds can be inhibitory to the microorganisms or enzymes during the next stages of process. More number of unit operations such as detoxification and washing are required to remove these toxic compounds which increases the production cost (Behara et al., 2014). In addition, DA may be less attractive due to corrosion, toxicity and maintenance cost.

3.1. Compositional difference in AFEX and DA pre-treated corn stover

The AFEX and DA pretreatment have different mechanisms as evident from the preliminary compositional analysis of pretreated biomass (Uppugundla et al., 2014). AFEX is a dry process, a little change or no major change occurs to the carbohydrate content of corn stover (Table 1). Mode of action of AFEX pre-treatment is the swelling of biomass, which causes an increase the accessible surface area, disruption of biomass fibers, decrystalization of cellulose and break down of lignin carbohydrate linkages (Agbor et al., 2011; Behara et al., 2014). Lau and Dale (2009) observed no major changes in the carbohydrate content of AFEX treated corn stover. A same trend was observed by Falls et al. (2011) when switchgrass was pretreated using AFEX though a minor change in acid insoluble lignin was observed. Reduced acid insoluble lignin content in AFEX treated biomass may correlate to unknown modifications

Table 1
Compositional analysis of untreated AFEX and DA treated corn stover (Uppugundla et al., 2014).

Composition	Untreated	AFEX	DA
Glucan	33.4	33.5	59.1
Xylan	24.9	24.8	6.5
Arabinan	3.7	3.3	3.6
Acetyl	2.1	0.0	0.6
Acid Insoluble Lignin	17.2	12.2	22.2
Ash	3.6	4.4	2.5
Extractives	10.4	24.8	15.4

to lignin (Uppugundla et al., 2014). In AFEX treatment, a partial solubilization of lignin occurs (increases the biomass porosity) and a portion of acid insoluble lignin may later relocated to the biomass surface. This portion of acid insoluble lignin might get extracted during hot water and ethanol extraction as a part of sample preparation for the compositional analysis (Uppugundla et al., 2014). A higher amount of extractives were obtained after pre-treatment may be due to the release of partially soluble compounds precipitated on the outer surface of biomass during pretreatment (Behara et al., 2014). In addition, a complete removal in acetyl group was observed from AFEX treated biomass, as the alkaline pretreatment cleaves ester linkages present in biomass (Uppugundla et al., 2014). AFEX pretreatment changes the structure of the biomass which increases the enzymatic digestibility and water holding capacity by reducing the hydrophobic interactions (Agbor et al., 2011; Behara et al., 2014) present in the biomass. Minor amount of solid material may solubilize during AFEX treatment with no major loss in hemicellulose or lignin.

In DA pretreated corn stover, compositional analysis indicated a significant drop in xylan followed by acetyl group was observed. A significant increase in percentage of glucan and acid insoluble lignin was observed corresponding to decrease in xylan percentage (Singh et al., 2015). An increase in acid insoluble residue might be correlated to repolymerization of polysaccharide degradation products or polymerization with lignin to form lignin like materials called pseudo lignin (Sun et al., 2014; Pu et al., 2013). A dramatic increase in pseudo-lignin content was observed as the severity of pretreatment increases (Kumar et al., 2013). The structural comparison indicate that the pseudo lignin was not derived from native lignin and the pseudo lignin has more C=O groups and possess more aliphatic structures (Hu et al., 2013) than native lignin. Hu et al. (2012) compared the enzymatic hydrolysis of holocellulose at varying ratios with pseudo lignin and EMAL (enzymatic mild acidolysis lignin) and concluded that pseudo lignin reduced significantly the overall enzymatic conversion of cellulose to glucose. Thus, during acid pre-treatment, care is required to avoid the formation of pseudo lignin. AFEX was able to hydrolyse the ester linkages completely; however, DA was unable to achieve 100% removal of ester linkages may be due to incomplete hydrolysis. In addition to ester linkages, DA can cleave ether linkages present in lignin. The efficiency of DA pre-treatment might vary depending up on type of reactor used for pre-treatment. Ciesielski et al. (2014) compared the effect of mechanical disruption on the effectiveness of DA pretreated corn stover using 3 types of different reactors Zipperclave (ZC), steamgun (SG) and horizontal screw (HS) reactor and concluded that SG and HS reactor had higher conversion than corn stover treated with ZC reactor system. Though the difference in composition was negligible between the pretreated biomass, a higher productivity in SG and HS system could be explained by micro and nano scale change such as reduced particle size, cellular dislocation, increased surface roughness, delamination and nanofibrillation generated within the biomass particles during pre-treatment (Ciesielski et al., 2014).

3.2. The effect of AFEX and DA on cellulose and its crystallinity

Cellulose is the predominant polysaccharide that contributes up to 45% of lignocellulosic biomass in the form of linear fibrils of approximately 30–40 hydrogen bonded chains of β 1–4 glycopyranoids with degree of polymerization of approximately 10,000–15,000 (Yang et al., 2011). Cellulose accounts up to 15–30% of dry mass in primary cell wall and up to 40% in secondary cell wall where it is found in the form of microfibrils (Sticklen, 2008). The cellulose fibril networks are embedded in non-cellulosic polysaccharide matrixes composed with lignin and structural proteins. Cellulose is synthesized by cellulose synthase complexes (CelS) known as rosettes. The CelS complex synthesizes a basic cellulose unit, known as the elementary fibril, which contains 36 β-D-glucan chains, are 5–10 nm in diameter, many micrometers in length, and spaced 20–40 nm apart (Ding and Himmel, 2006). Three different CesA proteins, encoded by members of the CesA gene family, are required for formation of functional CelS (Wightman and Turner, 2010). According to Appenzeller et al. (2004) at least 3 of the 12 CesA genes were involved in secondary wall synthesis of maize tissues. These microfibrils are then cross-linked by hemicelluloses/pectin/lignin matrixes during cell growth and maturation (Ding and Himmel, 2006). Due to strong inter chain hydrogen bonding between the adjacent chains in a cellulose sheet and weaker hydrophobic interactions between cellulose sheets, the crystalline cellulose is highly resistant to chemical and biological hydrolysis. These hydrophobic interactions makes the crystalline cellulose more resistant due the formation of a dense layer of water near hydrated cellulose surface (Matthews et al., 2006; Himmel, 2007; Behara et al., 2014). Two different types of intramolecular hydrogen bonding and one intermolecular hydrogen bonding occurs in cellulose I. The first type intramolecular hydrogen bonding is between the endocyclic oxygen (oxygen atom in the ring) and the hydrogen atom in the hydroxyl group of the C3 carbon (Cheng, 2009). The second type is between the oxygen atom in the hydroxyl group of the C6 carbon and the hydrogen atom in the hydroxyl group of the C2 carbon of a neighboring glucose unit (Mann and Marrinan, 1958; Marchessault and Liang, 1960). There is single intermolecular hydrogen bonding between the hydrogen atom in the hydroxyl group of C6 carbon and the oxygen atom in the hydroxyl group of the C3 carbon atom (Cheng, 2009). The native cellulose occurs in two distinct allomorphs cellulose I_α (one chain triclinic) and cellulose I_β (two chain monoclinic). Cellulose I_β is the dominant form in plant cells (Nishiyama et al., 2010).

Cellulose crystallinity and its effect on enzymatic hydrolysis are of controversial concern. It is widely accepted that cellulose crystallinity has negative impact on enzymatic hydrolysis especially during initial period of hydrolysis and the rate of hydrolysis is expected to decrease with increasing the hydrolysis time (Hall et al., 2010). Though the correlation exists between enzyme adsorption and hydrolysis, the initial rate of enzymatic hydrolysis increased with decreasing the crystallinity of biomass at the same amount of bound enzymes (Hall et al., 2010). The exact role of crystallinity on enzymatic hydrolysis is not clearly understood. Some authors proposed hydrolysis rate is depend on crystallinity and others found opposite effect. Though there are different conclusions about cellulose crystallinity, it is quite clear that crystallinity can change during pre-treatment and can affect biomass recalcitrance (Sun et al., 2014). In addition to crystallinity, other factors including both substrate (accessible surface area and porosity) and enzyme related factors (nonspecific adsorption, jamming, clogging deactivation, etc.) were responsible for this slowdown of enzymatic hydrolysis (Mansfield et al., 1999; Xu and Ding, 2007). The pre-treatment is aimed to reduce the biomass recalcitrance which can enhance its depolymerization rate during enzymatic hydrolysis. It is widely accepted that strong hydrogen-bonding

and stacking forces together with accessible surface area and microfibril shape, giving rise to the extraordinary stability of crystalline cellulose nanofibers that has strong resistance against chemical or biological degradation (Chundawat et al., 2011).

A reduction or an increase in crystallinity index (Crl) is mainly depending upon the mode of the pre-treatment used. In case of dilute acid pre-treatment, the Crl of biomass increases due to the removal of amorphous portions hemicellulose and a minor portion of lignin (Singh et al., 2015). Mapping out the structural changes to native and dilute acid pretreated corn stover indicate that crystallinity of cellulose was increased from 20% to 38% after dilute acid pretreatment (Zhang et al., 2013), though it can vary based on pretreatment severity. In case of AFEX, not much change in Crl was observed. In AFEX, the cleavage of lignin–carbohydrate complex (LCC) causes ultra-structure modifications that improve the enzymatic digestibility. Thus AFEX and DA can be classified into two based on their mode of action. The DA mainly removes the hemicellulose, a minor portion of lignin and thus increases the enzyme accessibility to the crystalline cellulose fibrils. AFEX disrupts the cellulose crystallinity and thus increases the glycosidic bond accessibility. In AFEX, the native form of cellulose, Cellulose I_β may be converted to other polymorphs of cellulose such as cellulose III by treatment with liquid ammonia or amines. The hydrogen bonding pattern are different in cellulose I_β and cellulose III. Cellulose I_β is dominated by intrasheet hydrogen bonding (2&6 and 3&5) followed by intersheet hydrogen bonding (3&6). Cellulose III is mainly stabilized by intersheet O2—O6 hydrogen bonds that are entirely missing in cellulose I_β (Chundawat et al., 2011). This transformation of cellulose I_β to cellulose III might result in the reduction of intrasheet hydrogen bonds and an increase in intersheet H-bond. Cellulose III allomorph is expected to be more hydrophilic which may progress its binding to cellulose via carbohydrate binding module (CBM) (Gao et al., 2013). The enzymatic hydrolysis rate of cellulose allomorphs can be arranged in the following order amorphous cellulose $\geqslant$ cellulose III $\geqslant$ cellulose II $\geqslant$ cellulose I. The different forms of cellulose and its properties are given in Table.2. The cellulose II allomorph can be generated from native cellulose by caustic mercerization or regeneration with ionic liquids (Weimer et al., 1991; Zugenmaier, 2001). Igarashi et al. (2007) observed 5 times higher cellobiose production from cellulose III as compared to cellulose I_β. This reorganization of H-bond network increased the hydrolysis rate of crystalline cellulose as close to amorphous cellulose. Thus the AFEX pretreatment can alter the structure of lignocellulosic biomass in two ways either by changing the crystalline structure of cellulose I_β to another cellulose allomorph form i.e. cellulose III, which is considered to be more amorphous than cellulose I_β or by cleaving the ester and biferulate linkages which cross link polysaccharides with lignin.

3.3. Surface roughness and morphological changes to dilute acid and AFEX treated corn stover

Singh et al. (2015) studied the surface morphologies of AFEX and dilute acid treated corn stover using confocal fluorescence and atomic force microscopy. Confocal florescence microscopy analysis indicated that the dilute acid treated corn stover had morphological changes occurred to the cell wall. However, in case of AFEX, there was no visible morphological changes occurred to cell wall. Though, cellulose fibers remain unaltered in both size and shape the partially dissolved lignin was displaced to the surface of corn stover during AFEX pre-treatment. It was further confirmed by AFM (Atomic force microscopy). The DA pretreated cellulose fiber were smaller as compared to AFEX treated corn stover and the average width of the cellulose fibers for dilute acid and AFEX treated biomass were 209 ± 34 nm and 685 ± 119 nm, respectively. AFM analysis indicates the cellulose fibers vary in size and are separated and piled together. This provides an indication of disintegration of fibrils might have occurred during DA treatment (Inouye et al., 2014). According to Inouye et al. (2014), the diameter of the fibrils generated from DA was about 10% lesser and no major changes was observed in the diameter of the crystalline portion of DA treated corn stover whereas Singh et al. (2015) observed much lower cellulose fibers after DA pre-treatment. A difference in digestibility was observed between the fragments generated and the fibrils that are unbroken even after pre-treatment. The sugar recovery in DA is mainly from individual cellulose chains and fragments generated during pretreatment (Inouye et al., 2014). Singh et al. (2015) concluded that the cellulose fibers in DA treated corn stover was composed of many cellulose nanocrystals. These nano crystals may be the unbroken cellulose fragments after DA pretreatment. A possible explanation for this might be the removal of amorphous regions such as hemicellulose and minor amount of lignin present in corn stover during dilute acid pre-treatment. The small angle neutron scattering (SANS) measurement, the surface roughness analysis of corn stover before and after pre-treatment indicated that an increase in surface roughness in pretreated samples may be due to the removal or redistribution of cell wall components during pre-treatment (Singh et al., 2015). This is a clear indication of surface changes occurred to corn stover after pretreatment and a possible explanation may be the lignin precipitation/condensation onto the biomass surface.

Thermo gravimetric analysis (TGA) of untreated corn stover, treated with dilute acid and AFEX were studied by Singh et al. (2015) and the TGA profile was found to be different for DA and AFEX treated corn stover. For untreated corn stover, the first weight loss peak occurred at 278 °C (hemicellulose zone: 245–290 °C) followed by the weight loss at 336 °C (cellulose zone: 290–350 °C). The dilute acid treated corn stover shows a peak in cellulose region and it indicates the absence or minor of hemicellulose region in dilute acid treated material. This is in agreement with compositional analysis, where the residual hemicellulose present in the pre-treated corn stover represents only about 6%. In case of AFEX, opposite trend in TGA curves were observed. In hemicellulose region, an increase in decomposition temperature in comparison with untreated followed by a decrease in decomposition temperature in cellulose region were observed. A possible explanation for this increase in the decomposition temperature might be the partial conversion of cellulose into low molecular weight cellulose or the conversion of cellulose I_β into cellulose allomorphs such as cellulose III. This low molecular weight cellulose or cellulose III have similar properties as that of amorphous cellulose. Thus, the decomposition temperature of depolymerized cellulose falls in the range of hemicellulose. This suggests that in AFEX

Table 2
Different types of cellulose polymorph and its properties.

Polymorph	H bonding alignment	Chain orientation	H-bonding pattern
Cellulose Iα	Inter-sheet	Parallel	2–6 and 3–5 (intra) 3–6 (inter) 5–3–6 (bifurcated)
Cellulose Iβ	Inter-sheet	Parallel	2–6 and 3–5 (intra) 3–6 (inter) 5–3–6 (bifurcated)
Cellulose II	Through-sheet	Antiparallel	3–5 (intra) 5–3–6 (bifurcated) 2–6 and 6–2 (inter)
Cellulose III	Through-sheet	Antiparallel	3–5 (intra) 5–3–6 (bifurcated) 2–6 and 6–2 (inter)

treatment, cellulose is getting converted into cellulose III which is much more like amorphous properties (Singh et al., 2015).

3.4. Structural changes to lignin during AFEX and dilute acid pre-treatment

Lignin in biomass is made up off three individual units namely guaiacyl (G), sinapyl (S) and p-hydroxyphenyl (H) units linked by β aryl ether (β-O-4), biphenyl ether linkages (5-O-4) and condensed (C—C) biphenyl linkages or a combination of above (Li et al., 2012). Lignification starts once the growth ceases, it originates from cell corner and extends into primary cell wall, followed by secondary cell wall layers (S_1, S_2 and S_3). The cell wall is arranged in a fashion that lignin–hemicellulose and cellulose–hemicellulose linkages are alternatively arranged in a sandwich form (Zeng et al., 2014). An efficient lignin removal may be achieved by the cleavage of aromatic rings of lignin monomers, linkages between lignin units and by cleaving of ester and ether linkages between lignin and hemicellulose (Zeng et al., 2014).

Singh et al. (2015) studied the HSQC NMR spectra of untreated, DA and AFEX treated corn stover. Untreated corn stover showed β-aryl ether units, resinol units, dibenzodioxin units, cinnamyl alcohol end groups and methoxy group in the aliphatic region. The anomeric region of untreated corn stover accounts for polysaccharide linkages including β (1-4)-D-glucopyranosyl units, β (1-4)-D-xylopyranosyl units and α (1-3)-L-arabinofuranosyl units and the aromatic region of untreated corn stover contains syringyl (S), guaiacyl (G) and p-hydroxyphenyl (H) units. S/G ratio of native corn stover with low levels of H units was 1.42 (Singh et al., 2015) and found to be in agreement with Li et al. (2012). In addition, ferulate and *p*-coumarates were also found in untreated corn stover. The HSQC NMR spectra of AFEX treated corn stover showed significant reduction in dibenzodioxocin units and complete removal of acetylated xylopyranosides and the depletion of β-correlation of ferulate and *p*-coumarates. The decrease in ferulate and *p*-coumarates was also observed by Li et al. (2012) during alkaline pretreatment. Approximately 20% reduction in β-aryl ether indicates that AFEX does not have strong effect in cleavage of β-aryl ether units. However, in DA nearly 60% reduction in β-aryl ether units correlate to lignin de-polymerization and a significant decrease in xylan correlation were observed in the HSQC NMR (Heteronuclear Single Quantum Coherence NMR) spectra of DA treated corn stover (Singh et al., 2015). However, the presence of acetyl group indicates the occurrence of residual hemicellulose in the pretreated corn stover. Cross peaks observed in the spectra may be the indication of overlapping of polysaccharides and lignin side chains. This might have occurred due to lignin condensation and depolymerization. In case of dilute acid pretreatment, Moxley et al. (2012) observed an increase in phenolic groups with increasing pretreatment severity and the rate of increase of S was higher than that of G. With increasing pretreatment severity, S/G ratio increased may correlate to the degradation of β-O-4 linkages.

3.5. Enzymatic reactivity of dilute acid and AFEX treated corn stover

The primary step towards enzymatic hydrolysis is the cellulase adsorption onto pretreated solids and the rate of hydrolysis is directly related to the amount of adsorbed enzymes (Lynd et al., 2002; Kumar and Wyman, 2009). A good correlation was found between adsorbed enzymes and glucose release for the first 24 h. After 24 h, the glucose release could not correlate may be due to substrate, enzyme features and other parameters such as enzyme inhibition by sugars and their oligomers (Kumar and Wyman, 2009; Zhang et al., 1999) The enzymatic reactivity of corn stover treated with dilute acid or AFEX was compared by Gao et al. (2014) at optimum enzyme mixtures (cellulase, xylanase and pectinase) over a period of 120 h. The glucose release from the DA and AFEX treated biomass had same trend during enzymatic hydrolysis. During the first 8 h, a faster glucose release was observed at lower (3 mg protein/g glucan) and higher enzyme loading (30 mg protein/g glucan) and glucose yield was increased with increasing enzyme loading. The deposition of lignin droplets might negatively affect the early stages of enzymatic hydrolysis (Li et al., 2014). Hence, a higher enzyme loading might accelerate the rate of enzymatic hydrolysis which leads to a higher glucose yield. Hydrolysis inhibition by deposited lignin droplets decreased with increasing hydrolysis time (Li et al., 2014). Singh et al. (2015) studied about the glucose release from 1 h to 72 h at different enzyme loading and approximately six times higher glucose was released from 1 h hydrolysis of DA treated corn stover with varying the enzyme loading from 3 to 30 mg protein/g glucan. In case of AFEX treated corn stover, the glucose release was about 10% lower than DA treated corn stover at higher enzyme loading. By comparing AFEX and DA treated corn stover, one can conclude that AFEX treated corn stover did not benefit from higher enzyme loading. Therefore, enzymes may not be the limiting factor for AFEX treated corn stover, however in case of DA pre-treatment, the enzymes may be limiting. The nonspecific adsorption of enzymes onto lignin of acid treated corn stover might be the cause as the glucose yield was increased with increasing enzyme loading. Hence a higher enzyme loading might require for acid pretreated biomass.

The effect of xylan and lignin removal on enzymatic digestion indicate the xylan removal did not show a clear trend in 1 h glucose release. However, the glucose release had a correlation with lignin release at all enzyme loading. This is in agreement with other studies where lignin is inhibitory to the enzymatic hydrolysis either by reducing the cellulase activity or due to unproductive enzyme binding (Li et al., 2014; Kumar and Wyman, 2009). It was reported that xylooligomers can act as strong inhibitory agent to enzymatic hydrolysis. Gao et al. (2014) studied the release of xylooligomers and concluded that the release of xylooligomers was lower in DA as compared with AFEX. A lower glucooligomer and xylooligomer release from DA treated corn stover was observed and the reason is yet to be clear. During 72 h hydrolysis of DA and AFEX treated biomass, the glucose yield increased significantly even though the xylooligomer concentration remained the same. Hence, we can conclude that the performance of enzymatic hydrolysis depends upon the lignin removal, unproductive binding of enzyme and any structural changes occurs to the biomass during pretreatment. This was contradictory to the study conducted by Qing et al. (2010), where the xylooligomers was found to be the stronger inhibitors for enzymatic hydrolysis than glucose and cellobiose.

Considering the standard compositional analysis AFEX had shown minimal variation in composition compared to untreated corn stover and it has been difficult to explain the mechanism and the causes for improved digestibility of AFEX pretreated materials in the past. The study conducted by Singh et al. (2015) indicates that disruption of lignin–carbohydrate linkages of polymeric lignin contribute to the efficiency of AFEX pretreatment. DA pretreatment appears to start with significant lignin de-polymerization, with 50% of the lignin re-condensed and precipitated back to the pretreated corn stover. DA pretreated corn stover was found to be thermally more stable, however, fiber width was measured to be significantly smaller than AFEX pretreated corn stover. The small fragments resulted from DA pretreatment may hydrolyze during the initial phase of enzymatic hydrolysis and the presence of re-condensed lignin onto biomass surface may explain the slow hydrolysis kinetics of DA treated corn stover at low enzyme loading. These comparative results might be useful for further development and optimization of pretreatment and

Table 3
Comparitive evaluation of DA and AFEX pretreatment of corn stover.

Biomass	Pretreatment and conditions	Hydrolysis	Sugar yield (%)	Reference
Corn stover	AFEX: Parr reactor at 62.5% solid loading at 1:1 Biomass to ammonia loading	At 2% Biomass loading, Spezyme CP, Novozyme 188, Multifect Xylanase and Multifect Pectinase.	88	Lau et al. (2009)
	Dilute acid: Parr reactor, 5 and 7.5% solid loading, 1% dilute H_2SO_4		82	
Corn Stover	AFEX: Liquid ammonia added to moist biomass before heating reactor, 5 min reaction time.	Spezyme CP	96	da Costa Sousa et al. (2009)
	Dilute acid: Soaked overnight in 3% acid solution before pretreatment		92	
Corn stover	AFEX: 90 °C, 220 psi, 1:1 NH_3 to Biomass, 5 min	Spezyme CP or GC 220 cellulase, Multifect Xylanase, β-glucosidase	79	Kumar and Wyman (2009)
	Dilute acid:			
	Sunds System 180 °C, 0.03H_2SO_4:Dry wt, 90 s, 25% solids		140	
	Parr Reactor 140 °C, 0.01H2SO4:Dry wt, 40 min, 5% solids		55	
Corn stover	AFEX: 1:1 ammonia to biomass, 140 °C for 15 min.	Cellic® CTec2	80	Gao et al. (2014)
	Dilute acid: 0.5%, 160 °C for 20 min	Cellic® HTec2 Multifect® Pectinase	92	
Corn Stover	AFEX: Parr reactor at 140 °C, 15 min, 1:1 ammonia to biomass	Cellic CTec 2	79	Uppugundla et al. (2014)
	Dilute acid: Parr reactor 160 °C, 20 min, 10% solid loading, 0.5% acid loading.	Cellic HTec2 Multifect Pectinase	88	

enzymatic hydrolysis process for cellulosic ethanol production (see Table 3).

3.6. Enzyme adsorption on to AFEX or DA treated lignin

The enzyme-substrate interactions were different for AFEX and DA pretreated corn stover. Kumar and Wyman (2009) observed that AFEX treated lignin had the lowest cellulase adsorption capacity, whereas lignin from DA pre-treatment had the highest. DA lignin had a maximum cellulase adsorption capacity of 53 mg/g lignin and that of AFEX was 38.7 mg/g lignin. The cellulase adsorption onto cellulose was found to be much higher for AFEX treated (270 mg/g cellulose) corn stover than DA treated (131 mg/g cellulose) corn stover (Kumar and Wyman, 2009). A low cellulase adsorption onto lignin from AFEX treated corn stover may indicate that ammonia might have reduced the hydrophobicity of lignin which ends up in much lower unproductive binding to lignin. Although, enzyme adsorption on to solids vary with mode of pretreatment employed, an equilibrium was achieved within 1.5 h after the incubation. A higher enzyme adsorption onto AFEX treated corn stover indicates the importance of disrupting lignin–carbohydrate linkages (may cause increase in pore size) than the xylan removal for increasing accessibility to cellulose. The addition of enzymes such as laccases improves the enzymatic synergetic mechanism of cellulose hydrolysis by releasing the cellulases from the nonproductive binding sites of lignin thereby increasing the concentration of free cellulases in solution.

Kumar and Wyman (2009) suggest that nonspecific binding of cellulase enzyme onto lignin droplets may not be the only reason for retarding the enzymatic hydrolysis. Cellulases initially act on cellulose microfibril surface and then moves down layer by layer (Igarashi et al., 2011). Hence, lignin droplets deposited on the surface of pretreated biomass might hamper the enzymatic hydrolysis in two ways: (1) either by blocking the access to cellulose (2) or by obstructing the enzyme movement along the surface. The slowdown of enzymatic hydrolysis due to lignin droplet reduces with increasing the hydrolysis time. A possible explanation may be the fact that during slowdown of enzymatic hydrolysis, Igarashi et al. (2011) observed a traffic jam in cellulase movements when there was disturbance on the surface of cellulose (may be due to lignin droplet), resulting a slowdown in the enzymatic hydrolysis. The hydrolysis was continued when subsequent enzyme molecules was found to lead a push that eliminated the obstacle. Based on this, a new hypothesis was proposed by Li et al. (2014) i.e. the enzymatic hydrolysis inhibition starts with the formation of lignin droplets which block or reduce the speed of the enzyme action by causing a traffic jam. With the accumulation of more enzymes and changes in the surface chemistry of adjacent cellulose chains, lignin droplets are either peeled off from the cellulose surface, allowing the hydrolysis to continue. With the peeling of more droplets as the hydrolysis proceeds, the inhibition is getting reduced. The inhibition is getting stopped, when the surface cellulose have been hydrolyzed at the stage where the inhibition stops.

Current research has been focused on removing hemicelluloses and lignin from biomass and thus improving the access to cellulose by cellulolytic enzymes. However, a few works has been carried out to understand how the soluble components (e.g. sugar oligomers, sugar degradation products such as furfural, HMF, formic acid, levulinic acid and lignin-derived compounds) released during pretreatment and enzymatic hydrolysis of cellulose (Yang et al., 2011). Currently, the pretreated solids are thoroughly washed to remove any soluble lignin derivatives including vanillin, syringaldehyde, trans-cinnamic acid and hydroxybenzoic acid-which are potential inhibitors to the cellulose hydrolysis (Ximens et al., 2011; Yang et al., 2011). The sugar yield was reduced when pretreated solids used for hydrolysis without washing process (Brownell and Saddler, 1987). Though washing improves the sugar yield, water recycling would be required which again increase the overall cost. Hence, the enzymatic hydrolysis of whole slurry or solids without washing is required to reduce the operating and capital cost of cellulosic ethanol production.

3.7. Cellulosic ethanol production from AFEX/dilute acid treated corn stover

Despite a large number of publications on cellulosic ethanol production, industrially relevant approaches towards commercialization are still lacking due to number of unit operations involved in cellulosic ethanol production process. The cost of cellulosic ethanol is estimated to be two to three times more than the petroleum fuels on energy equivalent basis (Carriquiry et al., 2011; Balan, 2014). For example, the quality of enzymatic hydrolysate depends up on the mode of pre-treatment applied to open up the structure of the lignocellulosic biomass. Before fermentation, enzymatic hydrolysate may have to undergo various downstream operations such as washing, nutrient supplementation, detoxification which are costlier unit operations in cellulosic ethanol process (Lau and Dale, 2009). The direct lignocellulose to ethanol production without washing, detoxification and nutrient supplementation may contribute significantly to the commercialization of the cellulosic ethanol process.

Considering the two pretreatments i.e. AFEX and DA, AFEX seems to be generating a high quality hydrolysate with much reduced levels of inhibitors than DA and also preserves nutrients in biomass for fermentation (Lau and Dale, 2009). AFEX has the advantage of no sugar loss or degradation of sugars into inhibitors. The hydrolysate obtained from AFEX was rich in nutrients may be due to the ammonia binding onto biomass (Lau and Dale, 2009). Lau and Dale (2009) compared the fermentability of AFEX and dilute acid treated hydrolysate and concluded that AFEX treated corn stover was more fermentable with respect to cell growth and sugar consumption. No loss of carbohydrates occured during AFEX treatment, whereas in case of DA, approximately 15% of xylose degraded to byproducts that can be inhibitory to the enzymes or microorganisms.

Kazi et al. (2010) studied the techno economic analysis of different pretreatment technologies for biochemical conversion of corn stover into ethanol. The aim was to estimate the total capital investment (TCI) and ethanol production cost (PV) including 10% return on investment. The total installed equipment cost was lower for DA ($164 million) followed by AFEX ($167 million). An additional capital expense of $10.8 million is incurred for DA pretreatment scenario for conditioning the pretreated slurry prior to fermentation. Though, the AFEX pretreatment reactor cost ($9.15 million) is lower than compared to dilute acid ($22.5 million), the addition of ammonia recycle unit results in total installed equipment cost slightly above dilute acid pretreatment scenario. The study was further looked onto ethanol production cost (PV) for the pretreatment scenario. The lowest ethanol production cost of $1.32/LGE (Liter of Gasoline Equivalent) was from AFEX pretreated biomass at 20% solid loading. In the case of dilute acid, the lowest ethanol production cost was $1.36/LGE. The ethanol product value (PV) for DA and AFEX was varying from $1.36/LGE to $1.44/LGE and $1.32/LGE to $1.66/LGE, respectively. The study suggested that the PV is more sensitive to pretreatment retention time, xylan conversions, solids loading and cellulose conversions.

According to Lau and Dale (2009), 17% more energy is present in the insoluble residue left over after enzymatic hydrolysis (after subtracting for glucan and xylan in the unhydrolyzed solids; assuming 90% lignin) of AFEX treated corn stover in comparison to dilute acid pretreated corn stover. However, the life cycle analysis (LCA) of these experimental data should be conducted to estimate the greenhouse gas savings of the pretreatment technologies. Pourbafrani et al. (2014) studied the impact of pretreatment technologies and co-products on GHG emissions and concluded that DA results in higher ethanol yield and lower net energy use than AFEX. In contrast to this, Spatari et al. (2010) concluded that AFEX showed more promise than DA for reducing life cycle GHG emissions. Based on the well to wheel analysis of six pathways, pathways with lower ethanol yield have lower greenhouse gas emissions (McKechnie et al., 2011; Pourbafrani et al., 2014). The ethanol yield is inversely related to lignin pellet production. A lower ethanol yield might result in more residual biomass for co-product (for example: electricity generation or lignin pellet production). According to Pourbafrani et al. (2014), adding co-products such as pellet production displaces GHG-intensive coal use in biomass co-fired power plants and results in much lower GHG emissions. Considering all these factors, the choice of pretreatment may be one of the crucial step that might have an enormous role in determining the sustainability of bioethanol production from lignocellulosic biomass.

4. Conclusion

The choice of pretreatment will have an enormous role the overall economics of the cellulosic ethanol process. By comparing AEFX and DA, the performance of AFEX was superior to DA in terms of maximum sugar recovery at lower enzyme loading, minimal sugar loss, inhibitor formation and reduction in number of unit operations such as washing or detoxification of hydrolysate. Adding to this, AFEX based cellulosic technology is expected to have 17% more available energy from insoluble lignin than DA which could be used for steam or electricity generation and thus reduction in greenhouse gas emissions.

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