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Insights into the effect of dilute acid, hot water or alkaline pretreatment on the cellulose accessible surface area and the overall porosity of *Populus*†

Xianzhi Meng,[a] Tyrone Wells Jr.,[b] Qining Sun,[b] Fang Huang[b] and Arthur Ragauskas*[c]

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Pretreatment is known to make biomass more reactive to cellulase by altering the chemical compositions as well as physical structures of biomass. Simons' staining technique along with mercury porosimetry was applied on the acid, neutral, and alkaline pretreated materials to measure the accessible surface area of cellulose and pore size distribution of *Populus*. The results indicated that acid pretreatment is much more effective than water and alkaline pretreatment in terms of cellulose accessibility increase. Further investigation suggests that lignin does not dictate cellulose accessibility to the extent that hemicellulose does, but it does restrict xylan accessibility which in turn controls the access of cellulase to cellulose. The most interesting finding is that severe acid pretreatment significantly decreases the average pore size, *i.e.* 90% average size decrease could be observed after 60 min dilute acid pretreatment at 160 °C; however, the nano-pore space formed between the coated microfibrils increased after pretreatment, especially with the acid pretreatment, suggesting that this particular type of biomass porosity is probably the most fundamental barrier to effective enzymatic hydrolysis.

Rapid developments in biotechnology, engineering, and plant genetics are leading to a manufacturing concept for converting lignocellulosic biomass, representing the most abundant carbon-neutral renewable resources, to biofuels and biomaterials.[1] However, this process is significantly hindered by innate biomass recalcitrance which refers to the characteristics of lignocellulose to protect its carbohydrates from degradation by cellulases.[2] In an effort to assess the effects of the substrate characteristics, such as hemicellulose and lignin content, cellulose crystallinity, and the degree of cellulose polymerization, intensive research has focused on modification and correlation of these substrate characteristics with biomass recalcitrance.[3,4] Some of the studies, however, report conflicting trends in the individual effects of these characteristics, which are mainly due to the fact that biomass recalcitrance does not come from a single structural factor and interactive effects naturally exist between these factors.[5] Unlike other factors, the accessible surface area of cellulose also known as cellulose accessibility has been consistently recognized as one of the most critical factors affecting the enzymatic hydrolysis yield and rate.[6–8] Grethlein reported a linear relationship between the initial cellulase reaction rate and the pore volume of the substrate accessible to a nominal diameter of 5.1 nm, which represents the diameter of a typical cellulase.[9,10] Several pretreatment technologies have been developed to change the structure of lignocellulosic biomass physically, chemically, biologically, or in combination. Though the fundamental mechanisms for each pretreatment, particularly how they alter the chemical compositions or physical structures of biomass, have not yet been fully understood, the final objective of pretreatment is always to render biomass more accessible to enzymes for efficient and rapid sugar generation using low protein loading.

The ideal pretreatment should fractionate cellulose, hemicellulose, and lignin cost-effectively so that cellulase can react with pure cellulose, and at the same time minimize the loss of sugars and formation of degradation products that inhibit enzymatic hydrolysis and fermentation. Dilute acid (DA), hot water (HW) and dilute alkaline pretreatment methods are the three most commonly used pretreatment technologies that have included significant research efforts over the past few years. Hemicellulose, located on the outer surface of cellulose fibers as well as inter-fibrillar space, has been shown to be most susceptible to changes under pretreatment conditions.[11]

[a]*Bioenergy Science Center, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332, USA*
[b]*Renewable Bioproducts Institute, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332, USA*
[c]*Bioenergy Science Center, Department of Chemical and Biomolecular Engineering, Department of Forestry, Wildlife, and Fisheries, University of Tennessee, Knoxville, Tennessee 37996, USA. E-mail: aragausk@utk.edu*
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Lignin could also act as a protective matrix making the target polysaccharides inaccessible to microbes, hence slowing down the deconstruction process. Obviously, the content of lignin and hemicellulose in the plant cell wall affects the degree of substrate digestibility, and understanding the relative importance of the removal of one of these two components over the other is critical for further optimization of the current pretreatment techniques.[12] Comparisons of pretreatment effectiveness in terms of increasing cellulose accessibility or reducing biomass recalcitrance based on literature data are hindered by the fact that various studies use different feedstocks, enzyme loadings and pretreatment conditions. At the same time, a majority of the studies that tried to highlight the importance of cellulose accessibility made use of highly digestible pure cellulosic substrates such as filter paper which are not really indicative of how real heterogeneous lignocellulosic biomass might behave.[13] In this study, *Populus* was pretreated with DA, HW and NaOH under three different pretreatment conditions (Table 1), producing substrates differing substantially in thecomposition and structure. Considerable amounts of work have been done to develop surface area measurement techniques that can be performed on cellulosic substrates.[14] One of the approaches that can be used as an alternative to represent the amount of the accessible surface area of the substrate is the measurement of porosity using probing molecules, such as water in NMR cryoporometry and relaxometry techniques, mercury in the mercury porosimetry technique and a set of dextran molecules in the solute exclusion technique.[15] Other techniques such as nitrogen adsorption, water retention value (WRV), Simons' staining and protein adsorption methods directly measure the adsorption of a given molecule on a lignocellulosic substrate.[15] Some of these techniques such as nitrogen adsorption require prior drying of the substrates which makes it typically less effective due to fiber hornification, while other techniques such as WRV suffer from the fact that the size of water molecules is much smaller than cellulase enzymes resulting in over-estimation of cellulose accessibility.[14] Solute exclusion and NMR techniques can measure lignocellulosic substrates in their wet state, but they are laborious and expensive.[6] A recent study by Wang *et al.* measured the total substrate accessibility to cellulase based on the maximum adsorption capacity of cellulose for a non-hydrolytic fusion protein named TGC, containing a green fluorescent protein and a cellulose binding module, and the results correlated quite well with the classic solute exclusion technique.[15] Simons' staining method and mercury porosimetry were used to measure different and complementary information on the cellulose accessibility of substrates prepared by dilute acid, hot water and alkaline pretreatments, providing insights into the effect of pretreatment on cellulose accessibility as well as the role of cellulose accessibility in the fundamentals of biomass recalcitrance.

The chemical composition of each of the substrates was determined by the Klason protocol according to TAPPI standard method T-222 (Fig. 1). The majority of the hemicellulose (98%), typically characterized by xylan, is removed within 10 min of DA pretreatment. The DA and HW pretreatment is ineffective at removal of lignin, and in fact the Klason lignin content actually increases after pretreatment due to the formation of pseudo-lignin.[16] On the other hand, 35% of lignin can be removed *via* 60 min NaOH pretreatment at 120 °C while only 28% of xylan is degraded.

The native and pretreated *Populus* was subjected to enzymatic hydrolysis for 24 h at a consistency of 1% (w/v) in 50 mM citrate buffer (pH 4.8) with cellulase and β-glucosidase loadings of 20 FPU g^{-1} and 40 CBU g^{-1}, respectively. The glucose and xylose yield (Fig. 2) was analysed by high-performance anion exchange chromatography with pulsed amperometric detection. Severe DA pretreatment resulted in the highest glucose yield as compared to other pretreatments, and approximately 500 mg of glucose per gram of dry pretreated biomass could be released after 60 min 160 °C DA pretreatment. Under the same pretreatment conditions (120 °C, 10 min), alkaline pretreated *Populus* actually has the highest glucose release, approximately 320 mg g^{-1} of dry biomass. Alkaline pretreatment also released much more xylose compared with the other two pretreatments, primarily due to the

Table 1 Conditions for dilute acid, hot water and alkaline pretreatment of *Populus*

Pretreatment	Temperature (°C)	Time (min)	Impregnation agent
Dilute alkaline	80	10	1% (w/w) NaOH at 5% solid loading
	120	10	
	120	60	
Hot water	120	10	DI water at 5% solid loading
	160	10	
	160	60	
Dilute acid	120	10	1% (w/w) H_2SO_4 at 5% solid loading
	160	10	
	160	60	

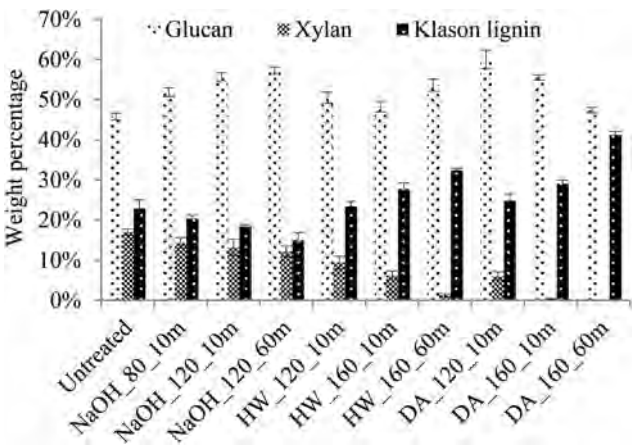

Fig. 1 Glucan, xylan, and Klason lignin contents of native, dilute alkaline, hot water and dilute acid pretreated *Populus*.

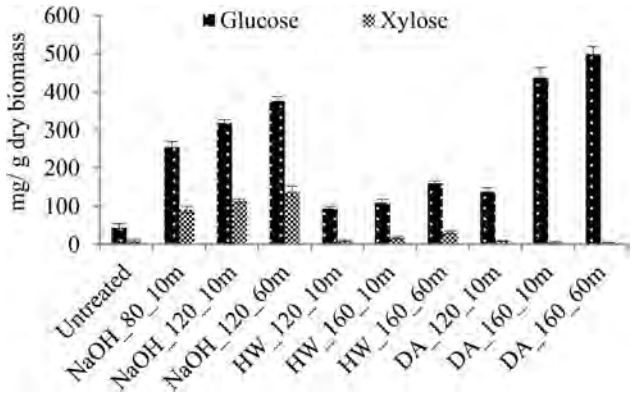

Fig. 2 Glucose and xylose yield (mg per g dry biomass) after 24 h enzymatic hydrolysis of native, dilute alkaline, hot water and dilute acid pretreated *Populus*.

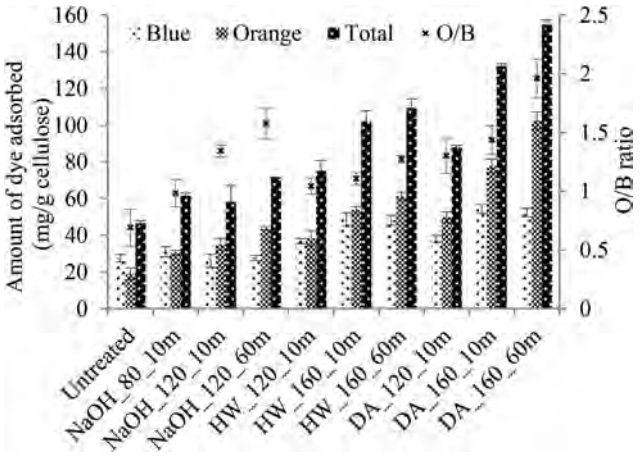

Fig. 3 Simons' staining results for the biomass accessible surface area represented by the amount of adsorbed dye (mg dye per g of cellulose) and the relative biomass porosity represented by the ratio of the adsorbed large orange dye to small dye (O/B).

significant solubilization of xylan during HW and DA pretreatment.

The different sugars released from *Populus* after different pretreatments could be related to its compositional analysis data or other cellulose structural parameters such as crystallinity or the degree of polymerization. However, the direct factor that affects sugar release is probably the accessible surface area of cellulose because the prerequisite step for enzymatic hydrolysis to occur is the intimate contact between the cellulase and the reactive cellulose surface.[15] Therefore, an accurate description of cellulose accessibility change upon biomass pretreatment, along with the composition and enzymatic hydrolysis results could thus provide a better understanding of the effect of the lignin/hemicellulose content on cellulose accessibility and the role of cellulose accessibility in biomass recalcitrance.

Simons' staining (SS), a two color differential staining technique, has been shown to be a semi-quantitative method for the estimation of the accessible surface area of lignocellulosic substrates by applying two dyes: Direct Orange (DO) 15 and Direct Blue 1 (DB).[17] DB 1 has a molecular diameter of ~1 nm, while DO 15 is a polymer with a molecular diameter in the range of ~5–36 nm for the high molecular weight fraction and it also has a much higher binding affinity for the hydroxyl group on cellulosic surface compared to DB 1. Therefore, when lignocellulosic substrates are treated with a mixture of DO and DB dyes, the DB molecules will populate the smaller pores of the fiber, whereas the DO molecules enter the larger substrate pores or the surface. The ratio of DO and DB (O/B) adsorbed by the substrates can therefore be used to indicate the relative amount of large pores to small pores. In addition, because DO dye has a very similar diameter compared to a typical enzyme, the amount of DO dye adsorbed (A_O) can be used to estimate the accessible surface area of cellulose to cellulase.[18] A recent study has proposed that the use of A_O(O/B) as a correction factor for the shape of the pore size distribution curve contributing to the enzyme-accessible surface area shows better correlation with other accessibility measurement techniques such as water retention value.[19] A modified Simons' staining assay based on previously developed procedures was applied to provide insights into the pore surface area (Fig. 3).

As indicated by the increase of O/B and orange dye adsorption, all these pretreatments significantly increase the biomass porosity and the total accessible surface area of cellulose. For each type of pretreatment, as the pretreatment severity extended, the cellulose accessible surface area is also increased. For example, O/B and orange dye adsorption increased from 0.69 and 18.9 mg g^{-1} to 1.30 and 49.7 mg g^{-1} respectively after 10 min 120 °C DA pretreatment. These numbers further increased to 1.44, 77.8 mg g^{-1} and 1.96, 102.5 mg g^{-1} as the pretreatment temperature and time increased to 160 °C and 60 minutes, respectively. A very interesting finding is that a 312% increase (18.9 to 77.8) in orange dye adsorption could be noticed after 10 min pretreatment, while only a 32% increase (77.8 to 102.5) is obtained after the remaining 50 minutes. This phenomenon also applies to the other two pretreatments, suggesting that the increase in the accessible surface area of cellulose primarily occurs in the first 10 min of pretreatment and though continues through the rest of the pretreatment time, it occurs at a significantly slower rate. In addition, DA pretreatment is found to be much more effective than the other two pretreatments in terms of accessible surface area increase, while HW and alkaline treatments under the same pretreatment conditions show very similar data, 38.4 and 35.0 mg g^{-1} respectively.

With compositional, accessibility and digestibility data available for a series of pretreated *Populus* samples, a comprehensive investigation of the effect of removal of each cell wall component by different pretreatment techniques on cellulose accessibility as well as the relationship between cellulose accessibility and substrate digestibility can be performed. Although the current understanding of the cell wall structure is quite limited, it has generally been recognized that elementary cellulose fibrils are coated with other non-cellulosic poly-

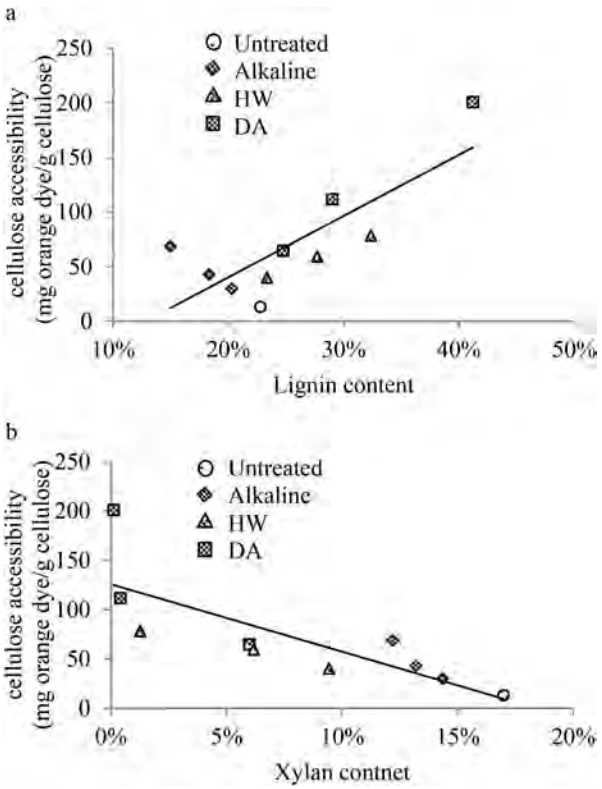

Fig. 4 Effect of lignin (a) and xylan (b) removal by different pretreatments on cellulose accessibility for a series of alkaline, HW and DA pretreated *Populus*. A correction factor A_O(O/B) was used to represent the cellulose accessibility, where A_O is the orange dye adsorption, and O/B is the ratio between orange and blue dye adsorption.

saccharides to form microfibrils, which are then cross-linked by hemicellulose/pectin matrices to form macrofibrils.[20] The relative importance of removing lignin *versus* xylan was obtained by comparing the cellulose accessibility of *Populus* substrates after DA, HW and alkaline pretreatment as indicated by Simons' staining method (Fig. 4). Obviously, the cellulose accessibility of *Populus*, pretreated with an alkaline solution, HW and DA under different pretreatment conditions, is inversely proportional to the amount of xylan retained (Fig. 4b), while the relationship between the cellulose accessibility and the Klason lignin content is not quite obvious (Fig. 4a). As a matter of fact, data shown in Fig. 4a suggest that the cellulose accessibility is inversely proportional to the lignin content for the three alkaline pretreated substrates, but has a general trend of a positive relationship with the lignin content for DA and HW pretreatments. This is mainly because the decrease of lignin content after alkaline pretreatment is accompanied by the decrease of xylan, while on the other hand the increased lignin content after DA and HW pretreatments was accompanied by a dramatic decrease of xylan content which helps increase the cellulose accessibility as shown in Fig. 4b. This is consistent with a recent review with in-depth analysis of removal of the lignin/hemicellulose content to improve the substrate digestibility from last 5 years of published literature work, which suggests that cellulose saccharification is linearly proportional to the amount of xylan removal but has a general trend of an inverse relationship with lignin removal for a series of acid-based pretreatments due to the fact that this increased lignin removal is normally achieved at the expense of hemicellulose removal.[8] Jungnikl *et al.* investigated the implication of chemical extraction treatments on the cell wall nanostructure of spruce wood using small-angle X-ray scattering, indicating that delignification had only a moderate effect on the structural organisation of the cell wall, while further extraction of hemicellulose with NaOH induced considerable nanostructural changes.[21] An inverse relationship was also observed between the lignin content after alkaline pretreatment and the extent of xylan conversion in a 24 h period (Fig. S2†). Wang *et al.* also showed that the xylan conversion efficiency was more sensitively affected by the variation of NaOH pretreatment conditions than the glucan conversion efficiency.[22] It therefore can be concluded that lignin probably doesn't directly dictate cellulose accessibility but rather restricts xylan accessibility which in turn controls the access of cellulase to cellulose. However, it is worth mentioning that near complete removal of both lignin and xylan may cause aggregation of cellulose microfibrils resulting in decreased cellulose accessibility.[23]

The relationship between cellulose accessibility and substrate digestibility was also analyzed to determine whether accessibility is a dominant factor affecting saccharification of different pretreated lignocellulosic substrates (Fig. 5). It was found that substrate digestibility is always proportional to the cellulose accessibility for each pretreatment technique under different pretreatment conditions, including DA, HW and alkaline pretreatment. Furthermore, for the same type of pretreatment that exhibits a similar degradation mechanism, *i.e.* HW and DA pretreatment, a strong positive relationship between cellulose accessibility and substrate digestibility can also be obtained. However, when alkaline pretreatment is involved,

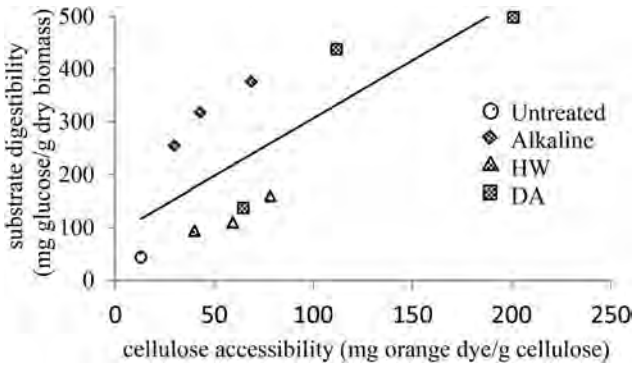

Fig. 5 Relationship between cellulose accessibility measured by Simons' staining (mg dye per g dry biomass) and substrate digestibility (mg glucose per g dry biomass) for a series of alkaline, HW and DA pretreated *Populus* samples. A correction factor A_O(O/B) was used to represent the cellulose accessibility, where A_O is the orange dye adsorption, and O/B is the ratio between orange and blue dye adsorption.

this linear relationship becomes less obvious. The likely reason is that lignin can not only physically limit the cellulose accessibility but also bind to cellulase unproductively through functional groups such as lignin phenolic hydroxyl groups thereby reducing the effectiveness of the enzymatic hydrolysis, and the relative contribution of these two negative roles of lignin has not yet been fully quantitatively understood.[24] Several studies have shown that unproductive binding of enzymes to lignin could be responsible for the requirement of high enzyme loading.[25] However, a recent study demonstrated that the effect of unproductive adsorption is minimal for most cases at typical hydrolytic reaction concentrations and the steric hindrance of lignin remained a major limiting factor.[26] In our study, removal of lignin by alkaline pretreatment probably didn't increase cellulose accessibility to the extent that HW/DA did as shown previously by Simons' staining; however, the negative binding effect of lignin has been decreased to some extent during the subsequent enzymatic hydrolysis process, resulting in the highest sugar release when compared to HW and DA pretreated substrates under the same pretreatment conditions. In other words, although alkaline pretreatment increases the cellulose accessibility and substrate digestibility, it seems reasonable to argue that the increase of cellulose accessibility by NaOH pretreatment is probably not the main reason causing the high substrate digestibility. However, as the DA pretreatment severity increased, the accessibility increased to a certain level that it became the dominating factor, causing higher sugar release despite retaining a large lignin fraction. A recent study also showed that the lignin-binding cellulase can be potentially recovered by addition of a sufficient quantity of cellulosic substrate with an increased surface area.[27] It can therefore be concluded that the cellulose accessible surface area appears to be a strong indicator of the ease of enzymatic hydrolysis only when the same or the same type of pretreatment is applied, and this direct cause–effect relationship as discussed above, cannot be easily obtained for substrates produced using different types of pretreatments. Other biomass or cellulose structural relevant factors such as cellulose crystallinity, the degree of polymerization, or here in this case the irreversible enzyme adsorption by lignin might need to be considered in order to predict the substrate digestibility better. Kumar and Wyman reported that delignification of corn stover by peracetic acid greatly enhanced enzymatic hydrolysis, but had a very limited effect on cellulose accessibility.[28] In contrast, Rollin *et al.* showed that high levels of delignification by soaking in aqueous ammonia without a significant increase in cellulose accessibility did not result in a large increase in glucan digestibility of switchgrass.[29] All these published reports support the conclusion that delignification may have a limited effect on cellulose accessibility; however, the exact role of lignin content in biomass recalcitrance is much more complicated and most of the time depends on substrates and pretreatment methods being used. Therefore, different pretreatment strategies are required to be adopted when trying to engineer different plants for efficient reduced recalcitrance.

It has been generally accepted and largely cited in the literature that pretreatment increases biomass porosity. However, there are several porosity scales in biomass from the cell lumen, intercellular space, pits to the nano-pores formed between coated microfibrils.[30] The following classifications that comply better with wood anatomy than the IUPAC definition of pore-size classes were proposed: macropores comprise the cell lumina, approximately 5 to 400 μm; micropores include pit apertures, pit membrane voids, 100 nm to 5 μm; nanovoids include the pores in the cell wall and space between cell wall components ranging in diameter of less than 100 nm.[31,32] With a majority of pore size data focused on the native biomass or pure cellulosic pulp, the description of the effect of different pretreatments on different scales of biomass porosity ranging from nanometers to macrometers is still quite limited. From this perspective, a porosimetry technique that can generate the actual pore size distribution (PSD) curves other than Simon' staining would be necessary. However, most of these techniques require a prior drying of the substrate which makes it typically less effective in determining the pore volume due to the fact that water removal from non-rigid porous materials such as biomass could produce partial irreversible collapse of pores known as fiber hornification. Zauer *et al.* showed that the pore diameter of native hardwood ranging between 4 and 400 nm decreased considerably due to thermal drying at 200 °C for 4 h.[33] Organic solvent exchange drying is a technique that has been used in surface/pore size measurement such as nitrogen adsorption and mercury porosimetry, which shows minimal pore collapse upon drying of lignocellulosic substrates.[34] To avoid this pore collapse, the untreated and pretreated *Populus* samples were solvent exchanged in Soxhlet apparatus with wet methanol, absolute dry methanol, and dry toluene using molecular sieves to absorb all water diffusing from the substrates before the final oven drying. In this manner, water is removed from biomass step by step preserving the maximally swollen pore structure of the wood samples in the absolutely dry state. Mercury porosimetry which can provide a wide range of information, *e.g.* the pore size distribution, total pore area and volume, average pore diameter, and the pore tortuosity was performed on these organic solvent exchanged untreated and pretreated substrates. Briefly, non-wetting liquid mercury was penetrated into the pore under external pressure, and the mercury volume infiltrated into the pore was measured as a function of the external pressure with an AutoPore IV 9500 porosimeter (Micromeritics, Atlanta, Georgia, USA). Intrusion pressure was then directly converted to the corresponding pore size by using the Washburn equation. The pore size distribution curves for dilute alkaline, HW and acid pretreated samples along with untreated *Populus* are shown in Fig. 6.

The PSD of alkaline and HW pretreated samples presents multi-modal hierarchical pore distributions with similar average diameters, while DA pretreatment results in a wide unimodal distribution. The pores with a diameter of ~100 000 nm are probably due to the inter-particle space of granules.[35] The major part of macropores and micropores

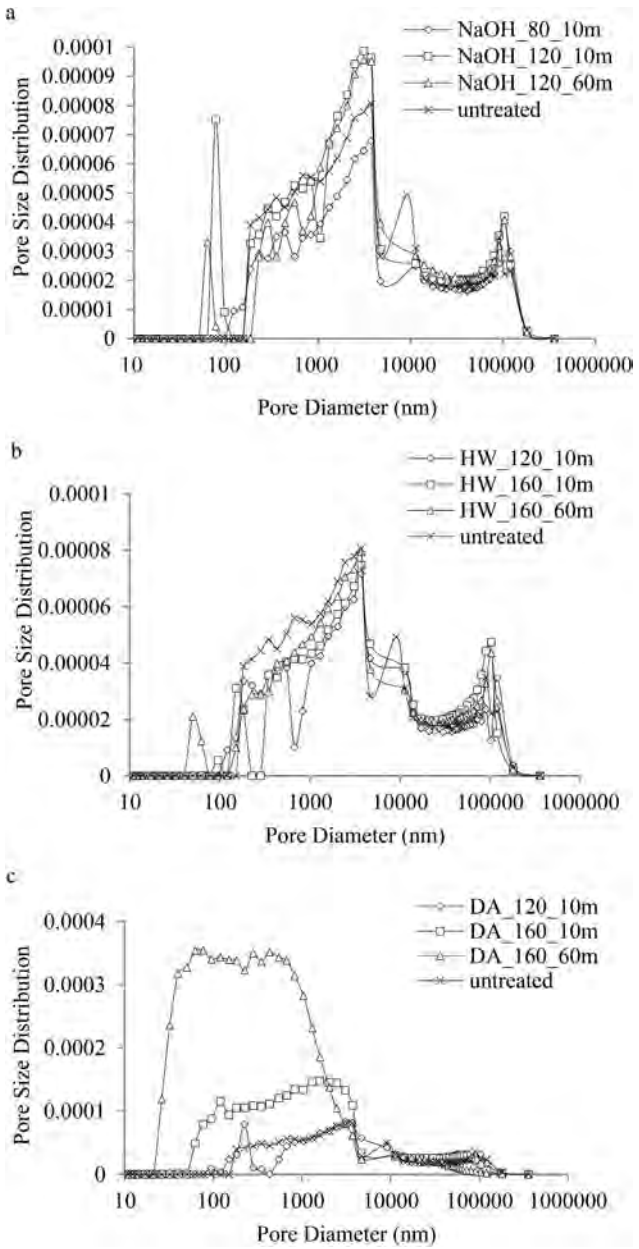

Fig. 6 Pore size distributions of *Populus* before and after pretreatment. (a) Alkaline pretreatment; (b) hot water pretreatment; (c) dilute acid pretreatment. Pore size distribution is represented using the fundamental theorem of calculus, d*v*/d*x*, where the pore volume *v* is a function of the pore diameter *x* given by the Washburn equation.

shown for alkaline and HW pretreated samples has a diameter of about 1000–10 000 nm, and their distribution of all three HW pretreated samples and one of the NaOH pretreated samples with the lowest severity is actually narrower than that of untreated biomass. The untreated biomass also has much greater volumes of pores with a diameter of around 10 000 nm compared to the pretreated sample. After pretreatment, fiber cells were separated from each other and the cell wall was destroyed into fragments, which can block the fiber cell lumen and pit in the cell wall, decreasing the corresponding pore volume.[36] Moreover, some volumes of nanopores between 50 and 100 nm were also observed on the distribution curves of all the pretreated samples, while no pores with a diameter lower than 100 nm were found for the untreated sample. This increase of pore size in the nano-space is much more obvious for DA pretreatment compared to the other two pretreatments. Indeed, 160 °C DA pretreatment significantly increases the pore volume between 10 and 1000 nm, primarily due to its near complete removal of hemicellulose and redistribution of lignin. Xu *et al.* also investigated the effect of acid treatment on fiber structures by small-angle X-ray scattering, suggesting that microvoids representing a needle-shaped space adjacent to cellulose increases from 790 nm to 1319 nm after 40 min 160 °C DA pretreatment for sorghum, likely due to the "peeling-away" of the plant cell wall components such as xylan.[37] At the most fundamental level, enzymatic hydrolysis only occurs when enzymes diffuse, bind and react on readily activated cellulose fibrils, and synergism can only occur when large amounts of enzymes with complementary activities occupy the same reaction volume.[38,39] Therefore, this significant nano-pore expansion by severe DA pretreatment could increase the synergistic activities, causing a high sugar release. Table 2 summarizes the major pore characteristics of these substrates, of which the pretreated samples always have a larger total pore area. Meanwhile, DA pretreatment has the largest pore area among these three pretreatments while HW and alkaline pretreatments result in a very similar pore area, which are in accordance with the Simons' staining results. Both HW and alkaline pretreatments slightly increase the average pore diameter, while the two DA pretreatments at 160 °C actually significantly decrease the average pore diameter, *e.g.* 90% decrease of average pore diameter was observed after 60 min 160 °C DA pretreatment. The results also indicated that DA pretreatment increased the pore tortuosity which is consistent with the literature results by Foston and Ragauskas using water self-diffusion experiments.[34] The importance of pore size distribution in enzymatic hydrolysis of biomass has also been highlighted in the literature. Luterbacher *et al.* proposed a pore-hindered diffusion and kinetic

Table 2 Pore area, diameter and tortuosity of the tested untreated and pretreated *Populus* from mercury intrusion porosimetry

Substrates	Total pore area (m^2 g^{-1})	Average pore diameter (nm)	Tortuosity
Untreated	0.86	17 427.2	1.723
NaOH_80_10m	1.12	22 983.4	1.585
NaOH_120_10m	1.23	17 827.0	1.706
NaOH_120_60m	1.36	21 111.1	1.639
HW_120_10m	0.94	22 480.5	1.507
HW_160_10m	1.01	20 768.7	1.847
HW_160_60m	1.04	21 245.3	1.671
DA_120_10m	1.99	18 068.2	1.949
DA_160_10m	2.34	6998.9	2.439
DA_160_60m	5.85	1627.4	3.649

model that can be used to predict cellulose hydrolysis with time using pore size distribution and initial composition data.[40] Chundawat *et al.* reported that nanoporous tunnel-like networks as visualized by 3D-electron tomography can be formed within the cell wall after ammonia fiber expansion, and the shape, size (10 to 1000 nm), and spatial distribution of pores depended on their location within the cell wall and the pretreatment conditions.[41] To the best of our knowledge, it is the first report showing that the unique significant nano-pore expansion caused by severe DA pretreatment despite its small average pore size should be responsible for the high sugar release, therefore suggesting that this nano-pore space formed between coated microfibrils is probably the most fundamental pore-scale barrier for efficient enzymatic hydrolysis.

Conclusions

A comprehensive investigation of the effect of DA, HW and alkaline pretreatment on cellulose accessibility was performed in this study. The results indicated that the accessible surface area of cellulose is an important factor governing the extent of hydrolysis; however, effectiveness of different types of pretreatment methods cannot be simply judged solely on this common basis. Delignification through alkaline-based pretreatment is found to be less effective than removal of hemicelluloses using an acid in terms of cellulose accessibility increase. Lignin also plays a negative role in the processes of enzymatic hydrolysis by binding to cellulases, and this negative effect of lignin could be compensated by the positive effect of cellulose accessibility, especially under severe DA pretreatment conditions. Pore size distribution analysis indicated that the most fundamental barrier in terms of the biomass porosity scale for efficient enzymatic hydrolysis is the nano-pore space formed between coated microfibrils, although some of the porous architecture such as the cell lumen and pit could be severely destroyed after pretreatment. Cellulose structural relevant factors such as crystallinity and the degree of polymerization might also play some roles, but the relative contribution of these factors compared with cellulose accessibility and whether some side effects from these factors can be avoided with the significant increase of cellulose accessibility after pretreatment need further analysis.

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