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Review

X-ray scattering studies of lignocellulosic biomass: A review

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ABSTRACT

The high processing cost of lignocellulosic ethanol is one of the most important barriers to its profitable commercialization. Pretreatments have been used to change the structure of biomass significantly and to improve sugar and ethanol yield. Great efforts have been made to understand the structural changes of biomass during these processes, including the molecular assembly of crystalline cellulose. Wide-angle and small-angle X-ray scattering are powerful techniques in studying the biomass structure at a molecular level. In this review, after we introduce the basic structure of lignocellulosic biomass, the effects of commonly used pretreatment methods on biomass structure, and the principle of X-ray scattering technique, the application of X-ray scattering, including studies of crystallinity, crystallite size, orientation distribution, and pore structure, and the related results in biomass conversion are summarized and discussed. Future study of biomass with X-ray scattering also is proposed.

Contents

1. Introduction

Cellulosic ethanol made from low-cost lignocellulosic biomass has been proposed as a next-generation transportation fuel with economic and environmental advantages. In 2007, former United States President George W. Bush declared the "Twenty in Ten" initiative in which gasoline usage in the United States is to be reduced by 20% in 10 years. According to this initiative, about 35 billion gallons of fuels will be produced from renewable and alternative substrates by 2017. Since the current ethanol production from starch or sugar sources cannot meet this goal and an overuse of these food sources risk increased food prices, lignocellulosic biomass is a promising alternative.

Scientists and engineers with different backgrounds, including chemical engineering, agricultural engineering, and biochemistry, have spent more than 30 years on biomass conversion on the sugar platform (Ladisch, Ladisch, & Tsao, 1978; Tsao, Ladisch, Voloch, & Bienkowski, 1982). Pretreatment methods have been developed

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to process lignocellulosic biomass (Mosier et al., 2005). Genetic engineering technology has been employed successfully to develop advanced microorganisms that effectively utilize hydrolyzed carbohydrates (Lin & Tanaka, 2006). Research with different ideas or designs has been conducted to study how to convert low-cost biomass to ethanol, and many of these processing technologies have been reported as effective and efficient (Alizadeh, Teymouri, Gilbert, & Dale, 2005; Kim et al., 2008; Zhang, Shao, & Lynd, 2009). Full-scale production of ethanol has been disclosed in patents (Hogen & France, 2009; Warzywoda, Ballerini, & Monot, 2009).

Despite these efforts, a successful profitable commercialization has not been reported (Moresco, 2008). Several technical barriers have hindered the large-scale, cost-effective production of cellulosic biofuels. One of the most important obstacles is that many approaches are considered uneconomic due to relatively high capital and processing costs (Hinman, Schell, Riley, Bergeron, & Walter, 1992; Wooley, Ruth, Sheehan, et al., 1999; Wyman, 2003). Bioethanol production from renewable biomass generally goes through several steps, including physico-chemical pretreatment, enzymatic hydrolysis, and fermentation. According to technoeconomic evaluation (Foust, Aden, Dutta, & Phillips, 2009; Wooley, Ruth, Glassner, & Sheehan, 1999), the cost of lignocellulosic biomass itself represents about one-third of the total cost of cellulosic ethanol production. Another major cost is from processing, especially pretreatment cost (Gregg & Saddler, 1996; Wyman, 2007). Lignocellulosic biomass has naturally evolved a complicated structure that makes biomass resistant to environmental degradation and attack. A number of processing technologies have been developed to overcome its recalcitrance (Mosier et al., 2005). The objective of pretreatment is to make polysaccharides, including cellulose and hemicellulose, easily degradable by enzymes at a low cost. According to a recent report (Dogaris, Karapati, Mamma, Kalogeris, & Kekos, 2009), most research showed considerable sugar yield with enzyme hydrolysis, but the possibility of scale-up was not discussed in most cases, and processing costs were barely reported. An important reason is that those processes were barely designed with a detailed investigation of the specific structure of biomass. Meanwhile, research about structural changes during pretreatment has been limited in composition analysis, mass crystallinity comparison, specific surface area (SSA) comparison, and qualitative microscopic imaging, etc. Detailed structural information of biomass would help develop high-efficiency and low-cost processes.

Structural characteristics and structural changes during processing have received attention because of the call for renewable energy (Wyman, 2003). A large number of papers targeting different biomass with different methods have been published in the past 30 years (Fan, Lee, & Beardmore, 1980; Liu, Yu, & Huang, 2005; Mansfield, Mooney, & Saddler, 1999; Sinitsyn, Gusakov, & Vlasenko, 1991). The basic structure of lignocellulosic biomass has been modeled (Somerville et al., 2004), although the detailed structure varies from one type of biomass to another. Cellulose is considered the only contributor to the crystalline part, whereas hemicellulose and lignin are the amorphous parts (Kelley, Rials, & Glasser, 1987). Structural changes of biomass vary depending on pretreatment methods and hydrolysis conditions on the sugar platform. For example, sulfuric acid pretreatment is effective in hemicellulose removal (Saha, Iten, Cotta, & Wu, 2005; Xu et al., 2011). Aqueous ammonia was used in corn stover pretreatment and showed considerable ability to remove lignin (Kim, Kim, Sunwoo, & Lee, 2003). Removal of the amorphous parts of biomass not only increases the SSA of cellulose for the following enzyme treatment, but also reduces possible inhibition to enzyme as reported earlier (Hidaka, Takizawa, Fujikawa, Ohneda, & Fukuzumi, 1984). One of the important structure factors is crystallinity which is generally referred as the ratio of crystal part to the whole mass. Crystallinity increase is found in many pretreatment processes as a result of removal of the amorphous part; however, limited studies have been done on the structure changes of cellulose during processing. It is important to understand how cellulose, including crystalline and amorphous cellulose, and other polymers change during different pretreatments. Other factors, including substrate factors (e.g., lignin distribution) and enzyme factors (e.g., synergism), are also critical to biomass processing (Mansfield et al., 1999).

Numerous analytical techniques, including X-ray scattering (Zugenmaier, 2007), Fourier transform infrared (FTIR) spectroscopy (Šturcová, His, Apperley, Sugiyama, & Jarvis, 2004), solid-state nuclear magnetic resonance (NMR) (Šturcová et al., 2004), Raman spectroscopy (Sivakesava, Irudayaraj, & Demirci, 2001), scanning electron microscopy (Zeng, Mosier, Huang, Sherman, & Ladisch, 2007), transmission electron microscopy (Donohoe, Decker, Tucker, Himmel, & Vinzant, 2008), confocal laser scanning microscopy (Bak et al., 2009), and atomic force microscopy (Zhang et al., 2007), have been used to study biomass. Among these techniques, X-ray scattering has been considered powerful due to its ability to provide information about molecular assembly in 3D. For example, macrostructure and microstructure of polymers could be investigated by wide-angle and small-angle scattering, respectively (Alexander, 1969). In-depth studies could be performed to provide detailed information about polymer structure, such as degree of crystallinity, preferred orientation, lattice distortion, and crystallite size. Therefore, X-ray scattering plays an irreplaceable role in the structural study of biomass. Unveiling biomass structure is possible by employing X-ray scattering with other complementary techniques.

Until now, a detailed summary of X-ray scattering study on lignocellulosic biomass has not been available. In this review, we briefly introduce the basic structure of lignocellulosic biomass, the effects of frequently used pretreatment methods on biomass structure, and the principle of X-ray scattering technique. Then, we provide a detailed summary of the current X-ray scattering applications in biomass conversion, as well as the related results, to explain debates and problems in the study of biomass structure. Suggestions and possible approaches for the study of biomass structure are also proposed.

2. Progress in study of biomass structure and pretreatment

2.1. Structural features

The natural structure of plant cell wall is excellently "designed". Functionally, the cell wall provides a rigid barrier that protects the cell from outside erosion and allows turgor that is the motive force of cell expansion (Ray, Green, & Cleland, 1972). Cell wall composition varies in different biomass and even in different parts of a particular biomass (Duguid et al., 2009). Primary and secondary walls are found to play different roles (Giddings, Brower, & Staehelin, 1980). Cell walls comprise three main components: cellulose, hemicellulose, and lignin (Table 1).

Cellulose, the main structural component in cell wall, is a long chain of hundreds of glucose molecules with β(1–4) glycosidic linkage (Kraessig, 1993). Cellulose can be found in 4 types (I, II, III and IV) (Perez & Samain, 2010; Zugenmaier, 2001), in which cellulose I, the native cellulose (Updegraff, 1969), has two crystalline modifications: cellulose Iβ is primarily found in cell wall of plants, and cellulose Iα is considered to enrich some microbes, such as bacteria (Sugiyama, Vuong, & Chanzy, 1991). The glycosidic bonds that connect glucose units could easily be broken by cellulase (endoglucanases and exoglucanases) to glucose and cellubiose, a disaccharide that could be hydrolyzed to glucose by β-glucosidase (Sternberg, Vijayakumar, & Reese, 1977). Cellulose

Table 1
Composition comparison of corn stover, switchgrass and wheat straw.[a,b].

	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Corn stover	36.1–40.8	26–35	17.2–21
Switchgrass	32–36.6	21.5–26.6	18.5–21.4
Wheat straw	37.1–48.6	23.2–31.7	8.2–19.2
Hard wood	40–55	24–40	18–25
Soft wood	45–50	25–35	25–35
Sorghum stover	37.9–40.5	26.2–29.8	15.5–20.3

[a] Numbers do not sum to 100% because other minor components, such as ash, are not included.
[b] Data from sources Alizadeh et al. (2005), Lloyd and Wyman (2005), Saha et al. (2005), Sun and Chen (2008), Sun and Cheng (2002), Suryawati et al. (2008), Theerarattananoon et al. (2010), Xu et al. (2010), Zeng et al. (2007), Zhao et al. (2009) and Zhu et al. (2006).

in biomass comprises crystallite because of highly ordered repeating units, which make it possible to use X-ray scattering to analyze the crystalline structure. Hemicellulose has different structural characteristics compared with cellulose and is noncovalently connected with cellulose microfibrils by hydrogen bonds (Tolbert, 1980). These bonds are very strong, which make obtaining purified cellulose from plant cell wall without cellulose loss challenging (Haigler, 1985). Hemicellulose is a complex of different polysaccharides (linked polymers of glucose, mannose, xylose, etc.) that show a considerable degree of chain branching without ordered sequence (Atalla, Brady, Matthews, Ding, & Himmel, 2008). Thus, hemicellulose is considered as amorphous as lignin in X-ray scattering study. Lignin comprises amorphous phenolic polymers, and both aliphatic and aromatic constituents are found in lignin. It is widely accepted that the biosynthesis of lignin stems from the polymerization of three types of phenylpropane units, also referred to as monolignols (Whetten, MacKay, & Sederoff, 1998). They are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (El Mansouri & Salvadó, 2007), which give rise to the p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of the lignin polymer, respectively. The exact native structure of lignin remains unknown. Because lignin is not only physically but also chemically connected (through covalent bonds) with hemicellulose (Sun, Sun, Fowler, & Baird, 2005), it is difficult to extract pure lignin polymer. Meanwhile, lignin is a competitive inhibitor in cellulase hydrolysis because cellulase could be irreversibly absorbed by lignin (Palmqvist, Hahn-Hägerdal, Galbe, & Zacchi, 1996).

Some other minor components, including proteins, also exist in plant cell walls, and are involved in different functions. The structural model of an *Arabidopsis* leaf cell has been reported (Somerville et al., 2004), but the structural characteristics of the plant cell wall, including the exact assembly of three major components, still need an in depth investigation. The composition of biomass varies depending on breed types and locations. Table 1 compares the major components in some lignocellulosic biomass currently used for bioethanol production. The content of the carbohydrates, which can be converted to fermentable sugars, is significant, and attention should be paid to how to change the structure cost-effectively either for making those carbohydrates ready for enzymatic hydrolysis or for obtaining end-product directly. For this purpose, it is critical to understand what happens during pretreatment.

2.2. Effects of pretreatment methods on lignocellulosic biomass structure

Lignocellulosic biomass biorefinery, including bioethanol production at the sugar platform, generally goes through feedstock harvest and storage, pretreatment (including physical and chemical), enzymatic hydrolysis, fermentation, and product recovery (Lee, 1997). Pretreatment is usually conducted before enzymatic hydrolysis to break up the tight structure of lignocellulosic biomass and expose polysaccharides for further digestion and recovery. Size reduction is an effective physical way to break up biomass chips, which increases SSA for subsequent processing. Different pretreatment methods have been developed and the effects of these methods on structural change of lignocellulosic biomass are summarized in Table 2.

Table 2
Effects of pretreatment on biomass structure.[a]

Methods	Mass loss	Change crystallinity[c]	Increases SSA	Removal of amorphous part
Diluted acid	●	+	●	●
Hot water	●	+	●	●
Alkali	●	+	●	●
AFEX	○[b]	○	●	○[b]
Steam explosion	●	+	●	●
Ionic liquids	●	–	●	●

SSA, specific surface area.
●, significant; ○, insignificant; +, increased; –, decreased.
[a] Data from sources Balan et al. (2009), Lee et al. (2010), Li et al. (2011), Mosier et al. (2005), Silverstein et al. (2007), Sun et al. (2000), Thompson et al. (1992), Wu et al. (2011), Zeng et al. (2007) and Zhao et al. (2008).
[b] Could become significant if a washing step is employed after pretreatment.
[c] Mass crystallinity summarized.

Extensive reviews on biomass pretreatment have been reported (Hendriks & Zeeman, 2009; Mosier et al., 2005). For instance, acid (diluted and concentrated) pretreatment has been used to break plant cell walls (Teixeira, 1999), in which cellulose could be hydrolyzed to glucose because its glycosidic linkage is susceptible to acid treatment (Atalla, 1979), whereas hemicellulose could be effectively removed, resulting in an increase in accessible surface area of cellulose (Xu et al., 2011; Zeng et al., 2007). An increase in biomass crystallinity also was reported in most of the related research because of the removal of the amorphous part (Thompson, Chen, & Grethlein, 1992). Liquid hot water pretreatment, with the advantage of no chemicals, has been used for pretreatment of some lignocellulosic biomass (Mosier et al., 2005). Because liquid hot water at high temperature (about 200 °C) reacts with hemicellulose to form acids, which results in a decrease of pH in the reaction system, the whole process is comparable to acid pretreatment (Weil et al., 1997). Alkali pretreatment is also used for enhancing biomass digestibility (Curreli et al., 1997; Silverstein, Chen, Sharma-Shivappa, Boyette, & Osborne, 2007; Zhao, Wang, Zhu, Ragauskas, & Deng, 2008). The glycosidic bonds in cellulose are generally considered stable toward alkali at low temperature (Johansson & Samuelson, 1975), which enables a reduction in cellulose degradation during mild conditions. Alkali (such as sodium hydroxide) generally functionalized cellulose starting from the reduced terminal, which makes the cellulose with short chains dissolve at the beginning of treatment (Johansson & Samuelson, 1975). Thus, the decrease of polymerization (DP) may not be significant with this reaction. Delignification (Kim & Holtzapple, 2006) and deacetylation (Kong, Engler, & Soltes, 1992) are other characteristics of alkali treatment, including lime treatment. Steam explosion, in which biomass is treated with high temperature and high pressure followed by a sudden reduction of pressure, is frequently reported. This method could cause hemicellulose removal and lignin transformation with an increase in pore surface area (Grous, Converse, & Grethlein, 1986), and could limited cellulose loss under controlled conditions. Similar to steam explosion, ammonia fiber explosion/expansion (AFEX) utilizes both physical (high temperature and pressure) and chemical (ammonia) processes to change biomass structure and increase enzymatic digestibility (Foster, Dale, & Doran-Peterson, 2001). Delignification is one of the most important characteristics of this treatment because of the depolymerization of lignin (Mosier et al., 2005).

Although AFEX is considered to promote cellulose decrystallization (Balan et al., 2009; Gollapalli, Dale, & Rivers, 2002), whether the crystalline structure of lignocellulosic biomass changes during this treatment is debated (Lee, Jameel, & Venditti, 2010), and the relationship between crystallinity and enzymatic digestibility remains uncertain.

Recently, ionic liquids (ILs) have emerged as a promising solvent for biomass pretreatment (Liu, El Abedin, & Endres, 2006). By replacing the inter- and intra-molecular hydrogen bonds of cellulose with its anion, ILs become ideal solvents for dissolving cellulose (Maki-Arvela, Anugwom, Virtanen, Sjoholm, & Mikkola, 2010; Swatloski, Spear, Holbrey, & Rogers, 2002). Complete dissolution of biomass, such as corn stover and switchgrass, was reported to achieve high digestibility of cellulose at 160 °C for 3 h, and glucose recovery after hydrolysis was over 90% (Li et al., 2011). After dissolution, cellulose could be regenerated by adding water to IL, and the natural structure of cellulose in biomass, cellulose I, was transformed to cellulose II accompanying a change of crystallinity (Wu et al., 2011). A recent study of IL pretreatment showed that the enhanced digestibility of glucan and xylan could be achieved by using partial dissolution at mild conditions (Xu, Shi, & Wang, 2012b).

Notably, the effects of some methods are material-dependent, which means the effectiveness of certain treatment could vary for different materials (Kumar, Barrett, Delwiche, & Stroeve, 2009). Furthermore, although the general goal of pretreatment is to break up the structure of plant cell walls for downstream processing at a low cost, pretreatment is still variable and objective dependent. For example, acid pretreatment under severe conditions may remove and break down hemicellulose (Schell, Farmer, Newman, & McMillan, 2003), which is preferred for glucose–ethanol conversion but is not cost-effective for co-fermentation with both glucose and xylose because of an additional neutralization step.

In summary, all of these pretreatment methods are effective in increasing enzymatic digestibility by either removing or degrading amorphous parts (lignin and hemicellulose) or redistributing lignin. Great efforts have been made to understand the detailed structural change of cellulose, and the structural effects of crystalline cellulose on enzymatic hydrolysis. As we can conclude from Table 2, most of those pretreatment methods cause significant mass loss. Even for AFEX, a washing step is often necessary for removing surface deposits (Balan et al., 2009). Thus, an X-ray scattering test without balancing the mass loss provides limited information.

3. Wide-angle X-ray scattering (WAXS) and small-angle X-ray scattering (SAXS)

WAXS and SAXS are powerful tools to probe the structure of polymer (Chu & Hsiao, 2001; Jakob, Fengel, Tschegg, & Fratzl, 1995). A detailed comparison of the scattering techniques, light, neutrons, and X-rays, can be found elsewhere (Lindner & Zemb, 2002) and is out of the scope of this review. Generally, SAXS covers the range 2–200 nm and occurs at low scattering angles from 1 to 10° 2θ (Chu & Hsiao, 2001) whereas WAXS covers the angular range from 5 to 60° 2θ, and is used to probe mesoscale dimensions; and therefore, SAXS and WAXS are used for studying polymer structure in length scale and at molecular level, respectively (Guinier, Fournet, Walker, & Vineyard, 1956). X-ray crystallography could be used to investigate polymer structure at an atomic scale. For biomass study, it might not be practical to obtain a single crystal of cellulose; instead fiber scattering and powder scattering have been employed to characterize crystallographic structure, crystallinity, crystal size, and preferred orientation (Lucas et al., 2010). The basic components of X-ray system are radiation system, collimation system, and detection system. The tunability of X-ray wavelength made X-ray technique a powerful tool for structural study of polymer materials. Sample preparation typically is simple, and samples can be studied nondestructively (Jakob, Fratzl, & Tschegg, 1994), but for powder scattering, the preparation of randomly oriented powder is necessary for avoiding preferred orientation. The fundamental relationship describing WAXS follows Bragg's law (Eq. (1)):

$$n\lambda = 2d \sin\theta \tag{1}$$

where n is an integer, λ is the wavelength of radiation source, d is the spacing between the planes in the atomic lattice, and θ is Bragg angle (the angle between the incident ray and the scattering planes).

Notably, the relative unit, Bragg angle (θ), was dominantly used in biomass study. Since the wavelength may vary depending on radiation source (e.g. Cu Kα 0.154 nm, Cr Kα 0.229 nm), the absolute unit (s, nm^{-1}, or q, nm^{-1}) is recommended for comparing the X-ray results from different radiation source (Eq. (2)).

$$q = 2\pi \times s \tag{2a}$$

$$s = \frac{2 \sin\theta}{\lambda} \tag{2b}$$

Synchrotron radiation, with the benefit of a high ratio of signal to noise with an intense light source, has been employed in much research (Chen et al., 2007; Chu & Hsiao, 2001). With synchrotron X-ray, the small beam divergence of incident X-ray and the high-energy source make it possible to perform advanced experiments. Also, synchrotron collects most data in less than one minute because of its high energy source, allowing in situ investigation of biomass structural changes. The use of simultaneous SAXS/WAXS for polymer study began in the early 1990s (Bark, Zachmann, Alamo, & Mandelkern, 1992). Then, the importance of understanding the phase transformation during polymer processing by this technology was proposed (Bras & Ryan, 1998). Fig. 1 shows the setup of simultaneous SAXS/WAXS at the X27C beamline at National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY.

In addition to WAXS and SAXS, other advanced photon techniques have been developed, such as ultra-small-angle X-ray scattering (USAXS) and grazing-incidence small-angle X-ray scattering (GISAXS), which are available in many synchrotron light sources such as Argonne National Laboratory in the U.S. and European Synchrotron Radiation Facility in France. USAXS techniques are able to probe structures with dimensions approaching ca. 10 μm and therefore are complementary to a range of microscopy techniques available (Blazek & Gilbert, 2011). A combination of WAXS, SAXS, USAXS and dynamic and static light scattering techniques may allow probing of an extremely broad range of length scales (de Souza Lima & Borsali, 2004).

Our previous study using synchrotron SAXS/WAXS showed that different parts of biomass had different 2-D patterns (Xu, Shi, & Wang, 2012a). For instance, Fig. 1A shows a 2D isotropic pattern from the inner pith of corn stover, which could be transformed to a 1D curve for studying crystallinity, crystal form and size. For samples containing oriented cellulose (e.g., the rind of corn stalk), cellulose crystals generate reflections in 2D pattern (Fig. 1B), which provides much more information about 3D structure order. SAXS studies also suggested different structures at nanometer scale. Recent study also showed significant differences of enzymatic digestibility among different parts of corn stover (Zeng et al., 2011).

4. Current study of biomass structure using X-ray scattering

The study of cellulose structure has a long history (Liang & Marchessault, 1959; Sisson, 1935). The goals of such study

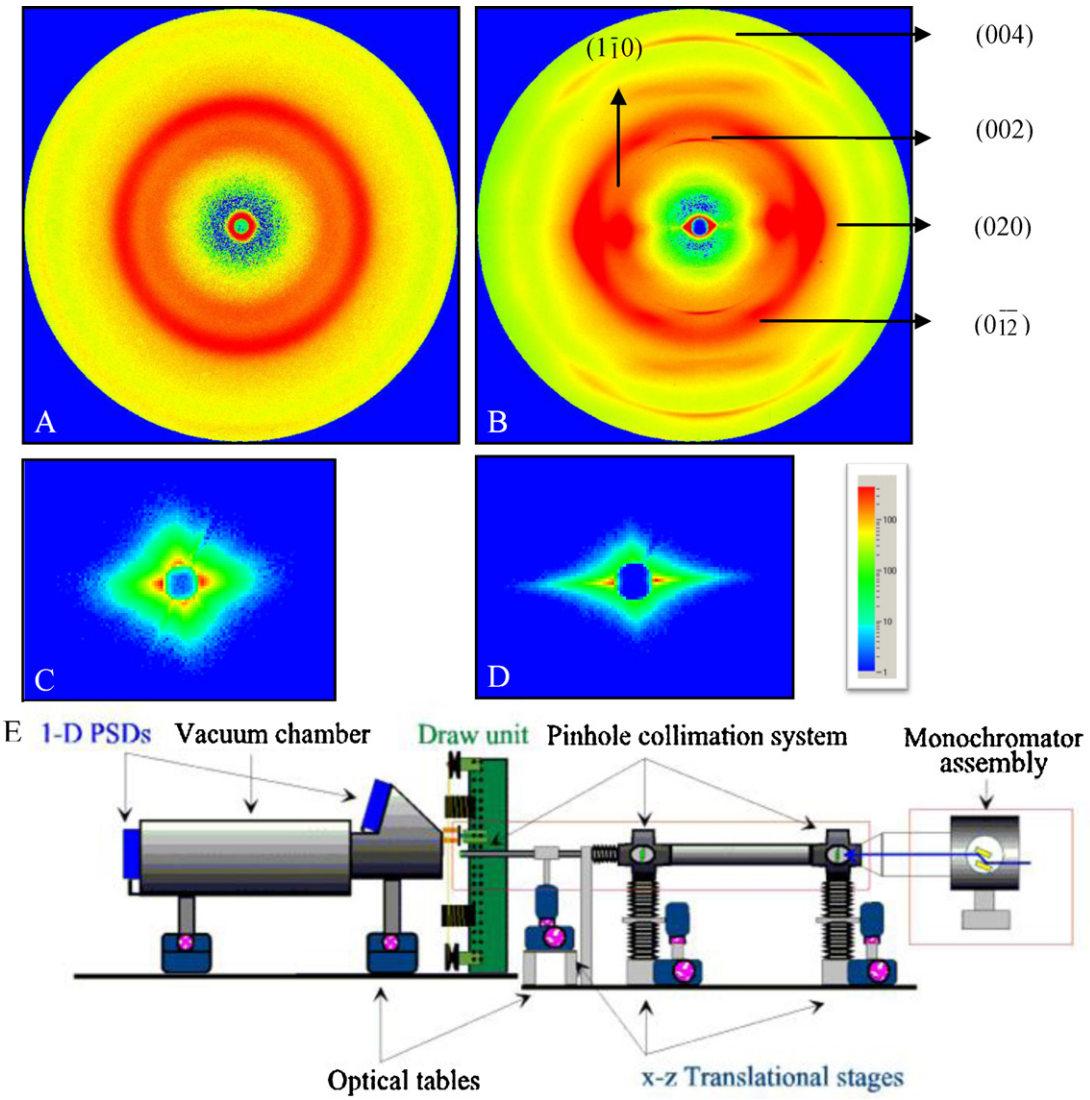

Fig. 1. SAXS/WAXS 2-D pattern of corn stover (WAXS: inner pith (A) and outer rind (B); SAXS: inner pith (C) and outer rind (D)) and schematics of simultaneous WAXS and SAXS setup (E, at the X27C beamline, National Synchrotron Light Source, Brookhaven National Laboratory, NY).
Image courtesy of Drs. LixiaRong and Benjamin S. Hsiao, Stony Brook University, Stony Brook, NY.

include deducing O—H bond orientation (Cael, Gardner, Koenig, & Blackwell, 1975) and providing molecular models of cellulose in both crystalline and noncrystalline regions. Since Sisson and Clark first developed a method to study crystallite orientation in cellulose fiber in 1933 (Sisson & Clark, 1933), a number of methods have been proposed to study the structure of cellulose (Hindeleh & Johnson, 1978) with structure parameters, including crystallinity and orientation factor, crystal size (Table 3). It is assumed that the scattering profile ($h(2\theta)$) of X-ray is the convolution of the instrument function ($g(2\theta)$) and the pure specimen function ($f(2\theta)$) (Alexander, 1954, 1969) (Eq. (3)).

$$h(2\theta) = g(2\theta) \otimes f(2\theta) \quad (3)$$

Although the contribution of instrument scattering, compared with that of specimen scattering, to the total scattering profile is limited, it nevertheless cannot be neglected. A profile fitting approach was proposed using a physically based model to generate line profile of specimen scattering for further analysis (Cheary & Coelho, 1992). This approach, however, still has the drawback that the analysis of different specimen profiles from one sample cannot yield a consistent result for every hkl line. For cellulose study, a two-phase model was used for scattering analysis; the model assumed that cellulose microfibril consists of alternating regions of crystalline (ordered) and amorphous (disordered) cellulose (Newman & Hemmingson, 1994). Many biomass applications or assumptions were deduced from this model (Heux, Dinand, & Vignon, 1999). In addition, early molecular models of cellulose were developed from algae with monoclinic and triclinic unit cell, which were identified as two forms, Iβ and Iα, respectively (Sugiyama et al., 1991). The molecular structures of the two forms, which were considered to coexist in plant cell wall, have been well elaborated with synchrotron radiation (Nishiyama, Langan, & Chanzy, 2002; Nishiyama, Sugiyama, Chanzy, & Langan, 2003).

For biomass study, a number of techniques are available for structural determination, in which WAXS has been used frequently in studying the crystalline structure of cellulose including crystalline orientation, crystallinity, conformation and domain size (Hermans & Weidinger, 1949; Sisson, 1935). Studies on biomass using X-ray scattering technique were frequently reported. For example, X-ray and FTIR were used together to study the crystalline structure of cellulose with sodium hydroxide and carbon dioxide treatment (Oh et al., 2005). Liu et al. (2005) reported structure and morphology of cellulose in wheat straw by means of WAXS. However, the structural study of biomass using X-ray scattering at

Table 3
Methods of crystalline structure studies.

Year	Materials used	Method description	References
Crystallinity calculation			
1915	Not mentioned	Crystallinity is calculated from scattering intensity. A full set of atomic positions for a whole crystal is needed for the calculation	Thygesen et al. (2005)
1959	Cotton cellulose	**Segal's method**: Crystallinity (crystallinity index) estimation using the equation: $CrI = \frac{(I_{002}-I_{am})}{I_{002}} \times 100$ I_{002}: The maximum intensity of the 002 lattice diffraction at an s value of 2.51 nm^{-1} (Fig. 2A), or 22.5 degree 2θ when a Cu Kα radiation is used; I_{am}: The intensity of amorphous scatter at an s value of 2.03 nm^{-1} (Fig. 2A), or 18 degree 2θ when a Cu Kα radiation is used	Segal et al. (1959)
1961	Polypropylene	**Ruland's method**: The scattering of amorphous part obtained by measuring amorphous compound is subtracted from whole scattering. Crystallinity calculation using equation: $x_{cr} = \frac{\int_0^\infty s^2 I_{cr} ds}{\int_0^\infty s^2 I ds} \times \frac{\int_0^\infty s^2 \overline{f^2} ds}{\int_0^\infty s^2 \overline{f^2} D ds}$ See reference for detail parameter definition	Ruland (1961), Vonk (1973)
1967	Different types of chemicals	**Rietveld's method**: A least-square fitting procedure was provided to fit background, crystal part and amorphous part. This method was then modified and improved by others. A best fit for certain sample needs 17 refined parameters. Crystallinity was calculated as the ratio of crystalline scattering to whole scattering	Madsen and Hill (1988), McCusker et al. (1999), Rietveld (1967, 1969)
1973	PET powder	A new density-related equation was provided to calculate crystallinity: $x = \frac{Q_c(Q-Q_a)}{Q(Q_c-Q_a)}$ where Q, Q_c and Q_a are the density of whole sample, crystalline part and amorphous part, respectively	Chung and Scott (1973)
1990	PET film	A fitting model was provided to determine the crystallinity with the equation: $I_B(s) = (1-X_c)I_{am}(s) + X_c \langle f(s)^2 \rangle [1-\exp(-ks^2)]$ See reference for detail parameter definition	Polizzi et al. (1990)
1991	Cotton fiber	A computer program "PEAK" was provided to process experiment data with different functions, the equation for total crystallinity (α) and partial crystallinity (α_i) used is: $\alpha = \frac{F_c}{F_c+F_a}$; $\alpha_i = \frac{F_i}{\sum F_i + F_a}$; where F presents peak area	Majdanac et al. (1991)
2010	Cellulose (Avicel PH 101, fibrous)	A statistical method, which calculates crystallinity using principle component regression with normalized X-ray data, was developed by Bansal et al.	Bansal et al. (2010)
Crystal size calculation			
1918	Not mentioned	A method was developed by P. Scherrer for calculation of apparent crystal size $\tau = \frac{K\lambda}{\beta \cos\theta}$ where τ is crystal size, K is the shape factor, β is half width of crystalline peak, λ is the wavelength of radiation source and θ is Bragg angle	Langford and Wilson (1978)
Orientation study			
1933	Cellulose fiber	Orientation quantity with the assumption of the distribution of the crystallites is proportional to that of intensity around the 002 scattering ring	Sisson and Clark (1933)
1939	Cotton fiber	Methods discussion of Sisson's statement	Berkley (1939)
1946	Cellulose fiber	Orientation parameter calculation using crystallite orientation distribution profile (CODP) of the (002) X-ray scattering: $f = 1 - \frac{3}{2}\left(\overline{\sin^2 \alpha_m}\right)$ where $\overline{\sin^2 \alpha_m} = \frac{\int_0^{\pi/2} I \sin^2 \alpha_{hkl} \cos \alpha_{hkl} d\alpha_{hkl}}{\int_0^{\pi/2} I \cos \alpha_{hkl} d\alpha_{hkl}}$	Hermans et al. (1946)
1959	Cotton cellulose	Methods comparison of crystalline index (%). Correlation method: $CrI = \frac{\sum_{2\theta} X_{2\theta} Y_{2\theta} - \left(\sum_{2\theta} X_{2\theta} \sum_{2\theta} Y_{2\theta}/N\right)}{\sum_{2\theta} X_{2\theta}^2 - \left[\left(\sum_{2\theta} X_{2\theta}\right)^2/N\right]} \times 100$ where N is the total number of pairs of observations; and integral method. $CrI = \frac{\sum_{2\theta} |U-A|_{2\theta}}{\sum_{2\theta} |C-A|_{2\theta}} \times 100$	Wakelin et al. (1959)
1987	Cotton	Modified Herman's orientation calculation using the crystallite using CODP of (002), (101) and (10$\bar{1}$) other than only (002)	Warrier et al. (1987)

nanometer scale is in its infancy because of various cross-linkages and twists among cellulose, hemicellulose, and lignin (Langan et al., 2011). Currently, the X-ray scattering study of biomass structural changes during processing is focusing on the change of biomass crystallinity.

4.1. Crystallinity

The fraction of crystalline parts is defined as crystallinity (or the degree of crystallinity). Other terms, such as crystallinity index, were also used to describe the ordered structure of cellulose (Park, Baker, Himmel, Parilla, & Johnson, 2010). Besides X-ray scattering technique, solid-state NMR is also used to distinguish between crystalline (ordered) and non-crystalline (disordered) cellulose (Liitiä et al., 2003) through different methods such as studying the differences in proton rotating-frame relaxation time constants $T_{1\rho}$(H) (Teeäär, Serimaa, & Paakkarl, 1987), the peak position, width, intensity, and multiplicity (Earl & VanderHart, 1981), and the differences in ^{13}C spin–lattice relaxation time constants T_1(C) (Harris et al., 2012; Horii, Hirai, & Kitamaru, 1987). FTIR is not an absolute measurement technique but rather provides relative values for crystallinity determination (Park et al., 2010). Kataoka and Kondo (1998) reported an infrared (IR) crystallinity index that is calculated as the intensity ratio between IR absorption at 1427 and 895 cm^{-1}.

The IR crystallinity index has a relationship with X-ray crystallinity, but it does not represent the percentage of crystalline content. A recent report showed that the lateral order index ($\alpha_{1429/893}$) and hydrogen-bond intensity ($\alpha_{3336/1336}$) could only be used for qualitative analysis of crystallinity change (Kljun et al., 2011).

The results from different approaches are usually different even for the same sample because the principle of method determines the difference in sensitivity. For instance, WAXS is considered sensitive to the long-range order of semicrystalline polymer (Alexander, 1969), whereas short-range sub-crystalline order can be probed by NMR and infrared techniques (Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008). Hermans and Weidinger (1948) suggested that there is an intermediate states existing between the ordered and disordered regions, named mesomorphous. The crystalline cellulose was then divided into "crystal-interior" which is well ordered and "crystal-surface" cellulose which has both well-ordered and poorly ordered cellulose (Newman & Hemmingson, 1994). The X-ray diffraction peak would only be contributed by crystallites above a certain size (Majdanac, Poleti, & Teodorovic, 1991), whereas the NMR parameter includes only all crystal-interior without crystal-surface because crystal surface of cellulose does not contribute those sharp peaks in NMR measurement (Newman, Ha, & Melton, 1994).

4.1.1. Methods comparison by X-ray scattering

Different methods for analysis of X-ray scattering patterns have been developed to calculate crystallinity depending on how to separate or model crystalline part and amorphous parts (Table 3). One of the most frequently used methods is the Segal's method (Segal, Creely, Martin, & Conrad, 1959). The intensity of crystalline and amorphous peaks was considered an important factor in analyzing X-ray data in the mid-20th century (Goppel, 1949; Krimm & Tobolsky, 1951). Segal's method, which designated the term "crystallinity index (CrI)" to represent the relative degree of crystallinity, could be used easily to obtain general information about crystalline parts. The value of CrI is calculated using the equation in Table 3. Notably, the value of crystallinity index does not truly represent the percentage of crystalline parts in whole mass; a detailed discussion is provided in the next section. The 1D curve integrated from 2D pattern could be analyzed easily to generate those factors needed in Segal's equation (Fig. 2A). Thus, this method has been used frequently for study of crystallinity change in which crystallinity calculation is based on whole mass rather than on the cellulose in biomass.

Instead of studying peak intensity, scientists started using full scattering pattern to obtain crystalline information in the 1960s (Ruland, 1961). It is assumed that Bragg peaks are sharp and splittable, which make it possible to construct crystalline peak that is partially diffused with amorphous scattering. Sample crystallinity could be calculated by the two-phase concept as the ratio of the area under crystalline peaks to the total scattering area (Table 3). Amorphous sample (e.g. lignin) is usually necessary for scattering deduction in Ruland's method (Fig. 2B). Thus, the results are highly dependent on the amorphous materials selected, which make it challenging to analyze samples from different sources.

Another approach, peak fitting procedure (Rietveld, 1967), was developed to refine and model those peaks with assumed functions such as Gaussian functions (Fig. 2C) (Hult, Iversen, & Sugiyama, 2003). Fundamentally understanding the knowledge of crystal structure is required for this refining process. Since this method accounts for the full convolution of cellulose and provides consistent results, it has been employed frequently in semicrystalline polymer studies (Bish & Howard, 1988; McCusker, Von Dreele, Cox, Louer, & Scardi, 1999; Stephens, 1999).

Comparisons of different methods on various biomass materials have been reported (Park et al., 2010; Thygesen, Oddershede,

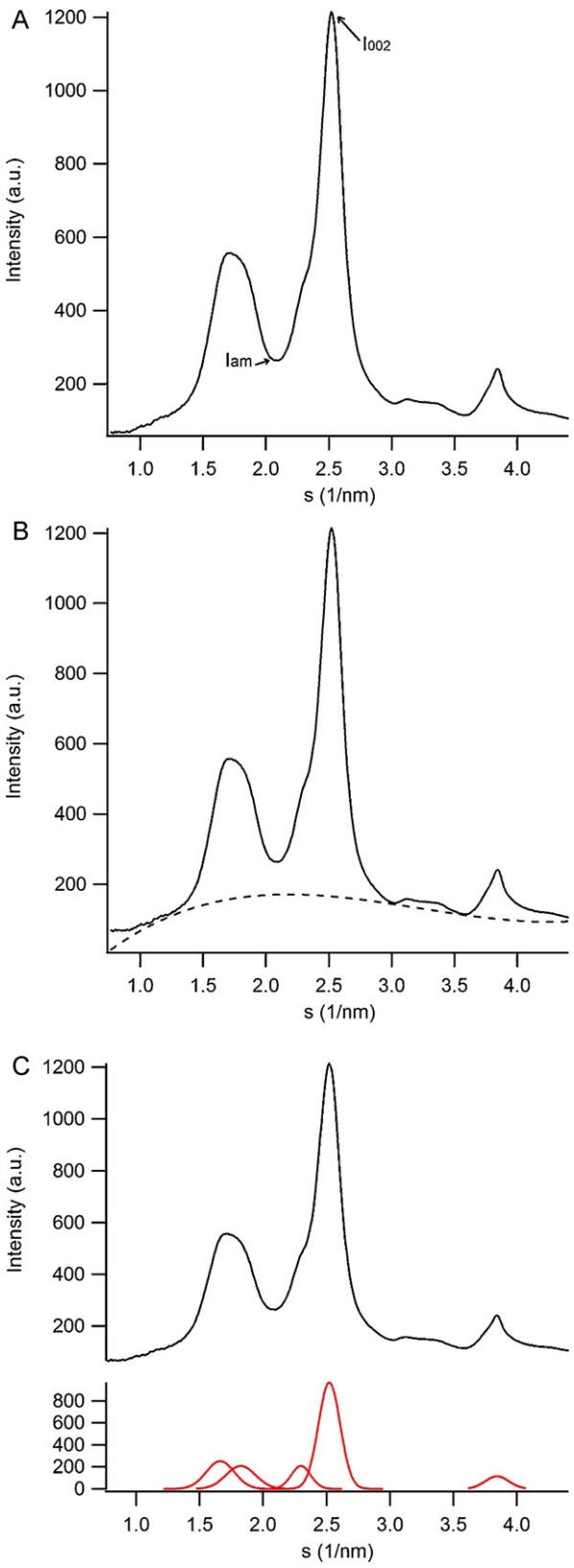

Fig. 2. Methods of crystallinity calculation from WAXS (patterns generated from synchrotron radiation using cellulose, Avicel PH101). (A) Segal's method, crystallinity obtained from ratio of peak intensities; (B) Ruland's method, amorphous scatter is subtracted from whole scatter and (C) peak deconvolution method, peak fitting with functions to deconvolve overlapped peaks.

Table 4
Comparison of the results from different crystallinity calculations.

Material	Segal's	Ruland's	Rietveld's	Cellulose content (%)
Corn stover	47	39	37	33
Norway spruce	47	56	33	49
Hemp fibers	77	49	60	63
Filter paper	83	72	57	84
Avicel cellulose	62	67	41	87

Source: Adapted from Thygesen et al., 2005.

Lilholt, Thomsen, & Ståhl, 2005). The results of crystallinity vary significantly depending on methods used (Table 4). In the instance of corn stover, the value from Segal's method is higher than the value from any other methods. One explanation is that the overestimation of crystallinity index was considered a result of preferred orientation (Thygesen et al., 2005). Underestimated peak intensity I_{AM} was believed to be another reason (Park et al., 2010). The value of crystallinity index (47 g/100 g) was found to be even higher than the cellulose percentage (about 33% in the study). Since no solid evidence proves that other biomass components besides cellulose show crystalline characteristics with WAXS, the unrealistic results suggest that the results from Segal's method are not able to reflect the degree of biomass crystallinity but instead provide a parameter for comparison. Therefore, Rietveld's method was considered preferable based on the comparative study. Although other methods for crystallinity calculation can be found in history (Chung & Scott, 1973; Majdanac et al., 1991; Polizzi, Fagherazzi, Benedetti, Battagliarin, & Asano, 1990), the issue of how to model and separate crystal peaks effectively using different functions remains. Gaussian function was found to fit well at the angle (2θ) range of 0–60 (Chen, Jakes, & Foreman, 2004), whereas pseudo-Voigt function fits well at a higher angle range (Enzo, Fagherazzi, Benedetti, & Polizzi, 1988; Madsen & Hill, 1988). The fact is that none of these functions could model scattering pattern in the entire angle range perfectly (Howard & Preston, 1989). Meanwhile, for natural cellulose, including the cellulose in plant cell walls, the two-phase model should be modified, because evidence shows that amorphous cellulose is related to the number of cellulose chains on microfibril surface (Newman, 1999).

It is interesting to note that different X-ray scattering methods have also been used to calculate the degree of crystallinity of starch, another important biopolymer (Lopez-Rubio et al., 2008). The degree of crystallinity values of a wide range of native starches calculated by a peak fitting procedure are similar to the double helix content as measured by ^{13}C solid-state NMR but higher than the crystallinity measured by the two-phase concept, dividing the area under the crystalline peaks by the total area under the diffractogram.

4.1.2. Application of crystallinity in the study of biomass

As concerns about energy and environmental issues have intensified, biomass conversion has garnered unprecedented attention, and crystallinity studies by using WAXS have been used more frequently than ever before (Cao & Tan, 2005; Liu et al., 2009; Sangseethong, Meunier-Goddik, Tantasucharit, Liaw, & Penner, 2007; Zhu, O'Dwyer, Chang, Granda, & Holtzapple, 2008). Different calculation methods and biomass were used to study the detailed structure and its change during processing. Computer software was used to model the scattering peaks from data generated by X-ray instrument (Chen et al., 2005). Debates about the effect of crystallinity, however, still exist. One problem is that crystallinity results are significantly different even for the same type of biomass (Table 5). The variable location of biomass is one of the reasons for the variation. Another reason is that instrumental difference affects the results (Garvey, Parker, & Simon, 2005). Also, it is possible to introduce artifacts when separating crystalline and amorphous parts of scattering for calculating crystallinity. Thus, it is less possible to compare the results from different methods; we suggest that attention should be paid to how the crystalline structure changes during processing.

Table 5
Summary of biomass crystallinity.

Biomass	Crystallinity (%)	References
Corn stover	50.3	Kumar et al. (2009)
	43	Kim and Holtzapple (2006)
	~60	Kim et al. (2003)
	50.3	Davison et al. (2005)
Switchgrass	26.2	Li et al. (2010)
	51.6	Kumar and Gupta (2009)
Hard wood	71.6	Thompson et al. (1992)
	73.2	Grethlein (1985)
	CrI 77.0% (Segal's), 66.0% (Rietveld's)	Ramos et al. (1993)
Wheat straw	69.6	Gharpuray et al. (1983)
	~80	Christakopoulos et al. (1991)
	35	Puri (1984)
Sweet sorghum bagasse	32.6	Theerarattananoon et al. (2010)
Rice straw	77.0	Wei and Cheng (1985)
Coastal Bermuda grass	50.2	Lee et al. (2010)
Cotton linter	74, 63, and 32	Bertran and Dale (1985)
Solka Floc cellulose	74.2	Fan et al. (1980)

Note: Segal's method was employed if not specified.

Mukherjee and Woods (1953) reported an X-ray study of cellulose degradation with sulfuric acid treatment. This is probably the earliest study about the effects of acid treatment on cellulose degradation. In the 1970s, many studies about cellulose were reported (Stipanovic & Sarko, 1976), but studies of biomass utilization were rare. At the end of the 1970s, because of the economic crisis brought on by a fossil fuel shortage, the demand for renewable energy stimulated the studies of lignocellulosic biomass conversion. Systematic study of the structural effect on enzyme hydrolysis was first reported by researchers at Kansas State University, USA (Fan et al., 1980; Fan, Lee, & Beardmore, 1981). A linear relationship was found between crystallinity and the rate of enzyme hydrolysis, and initial hydrolysis rate decreased as crystallinity increased. Since then, the debate about which structural parameter is determinant for enzyme hydrolysis has never stopped because of limited methods of measurement and discrepancies in 3D structure among those materials. Some researchers agreed with Fan's opinions (Chang & Holtzapple, 2000; Gharpuray, Lee, & Fan, 1983; Sinitsyn et al., 1991). For instance, Focher, Marzetti, Sarto, Beltrame, and Carniti (1984) reported the effects of substrate structure on enzyme hydrolysis in which the decrease of crystallinity was one of the reasons explaining an increase of hydrolysis rate. Other researchers, however, believed the increased hydrolysis yield to be a combined result of other factors, such as increase in SSA and decrease in particle size. For instance, Puri (1984), by comparing the results after different pretreatment methods (CO_2 explosion, alkali explosion, etc.), concluded that the rate of enzymatic saccharification is related to particle size, SSA, and DP, instead of crystallinity. After two years, with different materials (straw and bagasse) and different pretreatments (alkali explosion), he and his co-worker reported the similar conclusions that the percentage crystallinity of cellulose (calculated as the ratio of crystalline cellulose to the sum of fibrous and dissolved materials) did not change during pretreatment (Puri & Pearce, 1986), and the percentage crystallinity was not the main determinant for enzymatic saccharification of biomass. As found

Table 6
Mass crystallinity changes during pretreatments. This table is not meant to be exhaustive.

Pretreatments	Biomass	Crystallinity (%) change		References
		Before	After	
Diluted sulfuric acid	Corn stover	50.3	52.5	Kumar et al. (2009)
	Switchgrass	26.2	39.1	Li et al. (2010)
	Hardwood	73.2	76.1	Grethlein (1985)
	Hardwood	71.6	82.3	Thompson et al. (1992)
	Sweet sorghum bagasse	32.6	56.1	Theerarattananoon et al. (2010)
Ammonia fiber explosion (AFEX)	Coastal Bermuda grass	50.2	52.2–59.5	Lee et al. (2010)
	Rice straw	40.7	18.7–42.9	Gollapalli et al. (2002)
	Corn stover	50.3	36.3	Davison et al. (2005)
Liquid hot water	Hardwood	71.6	85.8	Thompson et al. (1992)
Subcritical water	Switchgrass	51.6	64.3	Kumar and Gupta (2009)
Steam explosion	Wood	Increased about 1.5 times		Tanahashi et al. (1983)
CO_2 explosion	Wheat straw	35	56	Puri (1984)
Lime	Corn stover	43	60	Kim and Holtzapple (2006)
Hydrogen peroxide	Rice straw	77.0	90.7	Wei and Cheng (1985)
Zinc chloride	Textile cotton wastes	Decreased[a]		Focher et al. (1984)
Ammonia recycled percolation	Corn stover	~60	~75	Kim et al. (2003)
1-Ethyl-3-methylimidazolium acetate	Switchgrass	26.2	2.6	Li et al. (2010)
Ethylene glycol	Wheat straw	69.6	55.0	Gharpuray et al. (1983)
Buffer	Cotton linter	74, 63, and 32	76, 64 and 45	Bertran and Dale (1985)
Ball milling	Wheat straw	~80	26.3	Christakopoulos et al. (1991)

Segal's method was employed if not specified.
[a] Calculation of crystallinity is a use of peak comparison.

in Puri's study, the biomass used contained multiple components, including hemicellulose and lignin, which differed from the substrate (at least 99.5% cellulose) used in Fan's study. As a result, the crystallinity calculation was based on cellulose itself for Fan's study, whereas the calculation was based on total mass before pretreatment for Puri's study. Puri provided some explanations on why the efficiency of enzymatic hydrolysis increased after pretreatment (e.g. changes of the surface area of substrate), but it was still not concluded whether or not crystallinity is a determinant on enzymatic hydrolysis because the percentage crystallinity only represents the amount of crystalline cellulose, not a full picture of cellulose (e.g., amorphous cellulose).

The effect of enzymatic hydrolysis on crystallinity also was investigated during this period. In a study of purified cellulose samples with different crystallinity, crystallinity was found to increase after hydrolysis as a result of crystallization of amorphous cellulose (Bertran & Dale, 1985). Ramos, Nazhad, and Saddler (1993) also reported the effect of enzyme hydrolysis on crystallinity, in which mechanism of "peeling-off" was initiated to suggest that either DP or crystallinity cannot be used to predict enzyme hydrolysis of cellulose, especially structurally complicated cellulose in biomass.

4.1.3. Biomass crystallinity or cellulose crystallinity?

Table 6 summarizes the effects of various pretreatment methods on biomass crystallinity. Segal's method is dominantly used because of its ease of use. Ball milling, a physical pretreatment used for production of amorphous cellulose (Paakkari, Serimaa, & Fink, 1989), decreases biomass crystallinity significantly. However, biomass crystallinity increased after most thermal/chemical pretreatment methods except AFEX and some ionic liquid treatments. This is not consistent with Fan's conclusion that low crystallinity is preferred for enzymatic hydrolysis (Fan et al., 1980). Removing or degrading lignin and hemicelluloses during pretreatments enhances the enzyme hydrolysis of cellulose but it is not clear if the increased crystallinity itself is desirable. Another question is whether amorphous cellulose is crystallized during pretreatment, causing the increase in biomass crystallinity. To answers these questions, we need to use the term crystallinty correctly. In understanding the procedure of the listed pretreatments (Table 2) and biomass characteristics through Section 2 of this review, the previous studies of biomass processing using biomass crystallinity provided little information about cellulose structure. Although the effects of pretreatment methods vary, one of the most significant contributions of pretreatment is the removal or redistribution of amorphous components, including lignin, hemicellulose, and extractives (Zeng et al., 2007). Thus, biomass crystallinity could be affected by the removal of those non-cellulose components, which makes it difficult to compare crystalline structure of treated and untreated biomass and to build a relationship between biomass crystallinity and enzymatic hydrolysis.

According to the summarized research papers about X-ray study of biomass in this review, we suggest using cellulose crystallinity (a percentage of crystalline component in cellulose), which is calculated by balancing mass crystallinity (a percentage of crystalline cellulose in whole biomass) with cellulose content (Eq. (4)).

$$CCr\% = \frac{Cr}{C} \times 100 \quad (4)$$

where CCr is cellulose crystallinity, C is cellulose percentage in biomass, and Cr is mass crystallinity calculated from scattering pattern of biomass.

We suggest that, for crystallinity study of biomass, attention should be paid to cellulose rather than whole biomass, and the scattering methods (e.g., Rietveld's method), instead of the intensity methods, for crystallinity (Cr) calculation are preferred. Therefore, it is possible to address the questions: How does cellulose crystallinity contribute to enzymatic hydrolysis of biomass? Is the amorphous cellulose in biomass crystallized in some pretreatments (Xu et al., in press)? Otherwise, contradictions persist when the "crystallinity" concept was not used correctly, and makes researchers consider crystallinity less important (Mansfield et al., 1999) or even negligible in the structural study of biomass.

4.2. Orientation distribution

Orientation distribution of polymer, another important parameter, could be related to certain mechanical or material properties. An oriented system with cylindrical rotational symmetry, such as the cellulose in biomass, could provide 3D structural information in a single 2D image (Burger, Hsiao, & Chu, 2010). Fig. 1A shows

the 2D pattern from a perpendicular beam through a biomass chip, in which one can find anisotropic characteristic. The Herman's orientation factor has been used to study orientation distribution for more than 60 years (Hermans, Hermans, Vermaas, & Weidinger, 1946). Modified methods also have been developed to study different materials (Warrier, Munshi, & Chidambareswaran, 1987). The classic method is powerful in studying different types of orientations, including parallel, perpendicular and oblique orientation. It was found that the orientation parameters could be genetic in origin and independent of growth conditions in a cotton study (Moharir, Van Langenhove, Van Nimmen, Louwagie, & Kiekens, 1999). Crystal orientation, together with crystallinity and crystal size, also was reported to study the characteristic of regenerated cellulose fibers (Chen et al., 2007). Also, it was proved, by studying wood sample, that no correlation exists between fiber orientation and fiber dimensions (Eklund, Säll, & Linder, 2003; Sarén, Serimaa, & Tolonen, 2006). However, for powder samples with random packing, studying the orientation of whole mass is difficult, which is one reason why current research on preferred orientation of lignocellulosic biomass during processing is rare. Thus, we recommended beginning the orientation study of biomass with unground samples.

4.3. Crystal size

Crystal (crystalline) size, unlike particle size, is contingent on the method used. Scherrer's equation (Table 3), which was developed almost 100 years ago, is the classic method for crystallite size calculation (Langford & Wilson, 1978). The factor of full width at half maximum could be obtained after peak analysis. Since many factors may affect the peak width in WAXS pattern and sometimes the peak deconvolution approach may introduce artifacts, this method is an estimate, especially for a crystalline complex with different sizes. The Scherrer constant, K, should be used cautiously because it depends on how the width and the crystal shape are determined. A K-value of 0.89, which works well for spherical crystals without cubic symmetry (Langford & Wilson, 1978), was employed in most of the research related to crystal size. Although this method is an estimate, it nevertheless was used frequently in polymer studies (Marchisio, 2009; Shivakumara & Bellakki, 2009). For instance, a study on cellulose fibril in wood using both WAXS and SAXS showed the crystal size of cellulose in sprucewood is close to 2.2 nm (Jakob et al., 1995). The crystalline structure information of cellulose in corn stalk has been reported, and the crystal size was found as 3.8 nm (Reddy & Yang, 2005). In a study of the enzymatic hydrolysis of pretreated wood samples, both crystallinity and crystal size of peroxide-treated samples decreased gradually during hydrolysis, but those of full bleached samples did not change significantly (Ramos et al., 1993). In contrast, crystal size, as well as crystallinity index, was found to increase after cellulase hydrolysis in study of the effect of enzymatic hydrolysis on biomass (softwood, hardwood and linter) structure (Cao & Tan, 2005). For sizes changes during biomass pretreatment, however, the study was limited. One example is that the crystal size of wood increased from about 3 to 6.3 nm after steam explosion (Tanahashi, Takada, Higuchi, & Hanai, 1983). More fundamental study of crystalline structure of biomass is suggested.

4.4. SAXS study

The native structure of biomass is complicated and inhomogeneous, which make it necessary to use various complementary approaches. It is well known that the structure information can be accessible with SAXS at both length scales larger than the separation between crystal planes and on the spacing between the regular stacks of lamellae (Chu & Hsiao, 2001). SAXS has been employed successfully for the quantification of the lamellar architecture of semicrystalline growth rings in other biopolymers such as native starch granules (Blazek & Gilbert, 2011). An understanding of biomass nano-structure is helpful to clarify the differences of numerous biomass in nano-structure. Together with WAXS, SAXS could explain how biomass structure is influenced by thermochemical or biological processing; thus, desirable processing and design could be selected.

The characteristics of polymer on a larger scale are usually more complicated and less organized than that at an atomic scale, which makes the data analysis of SAXS challenging. One approach assumed multiple structural levels, including a Guinier and a power-law region. A unified equation has been developed for analysis of SAXS data (Beaucage, 1995). The equation defines multiple levels, and each level may contain a Guinier region describing an average structural size and a power-law region describing the mass or surface fractal. The unified model is given by:

$$I(q) = G_i \exp\left(\frac{-q^2 R_{gi}^2}{3}\right) + B_i \exp\left(\frac{-q^2 R_{g(i-1)}^2}{3}\right) \times \left\{\frac{[erf(qR_{g_i}/6^{1/2})]^3}{q}\right\}^{P_i} \quad (5)$$

where i represents the structural levels, G_i is the exponential prefactor, R_{gi} is the radius of gyration, B_i is a constant prefactor specific to the type of power-law scattering, P_i, and the magnitude of the scattering vector is defined as $q = 4\pi \sin\theta/\lambda$ (θ is half of the scattering angle).

Small-angle X-ray scattering has been applied to the study of polymer ultrastructure and structural changes during thermochemical processing (Chu & Wang, 1988; Crawshaw & Cameron, 2000; Ivan Krakovský, 1997; Waigh, Donald, Heidelbach, Riekel, & Gidley, 1999). For example, the diameter of cellulose fibrils was reported as 2.5 nm in sprucewood (*Picea abies*) with SAXS study (Jakob et al., 1994). The spiral angles of the fibrils in earlywood and latewood were found to be significantly different (Jakob et al., 1995; Reiterer, Jakob, Stanzl-Tschegg, & Fratzl, 1998). In biomass pyrolysis, SAXS was used to understand the nano-structural evolution during decomposition (Paris, Zollfrank, & Zickler, 2005; Smith, MacDonald, Ellis, Obrovac, & Dahn, 2012). Our recent study using SAXS showed that the increase of microvoids in sorghum biomass during sulfuric acid treatment contributes to the increase in total surface area of biomass, which enhances biomass digestibility to enzyme (Xu et al., in press). Studies on individual components, including cellulose and lignin, were also reported frequently (Astley & Donald, 2001; Canetti, Bertini, De Chirico, & Audisio, 2006; Nishiyama et al., 2002; Vickers, Briggs, Ibbett, Payne, & Smith, 2001). For instance, lignin from pine wood was reported to have a specific surface area of 34 m^2/g and to show surface fractal characteristic (Vainio et al., 2004).

Small-angle X-ray scattering is also a powerful tool to study pore structure change during processing (Richards et al., 2000; Röder & Sixta, 2004). Most of the structure information obtained, including sample composition, crystallinity, and SSA is from dried (air-dried or freeze-dried) samples. Questions about how biomass structure changes during drying process and how water plays a role in biomass processing remain unanswered. After some pretreatments (e.g. acid pretreatment), drying process causes irreversible cellulose pore closure (McMillan, 1994), which eventually decreases enzymatic digestibility. This phenomenon is termed "hornification" which refers to the formation of irreversible or partially reversible hydrogen bonding in cellulosic material during water removal (Fernandes Diniz, Gil, & Castro, 2004). Covalent lactone bridges were thought to establish in lignocellulosic structure after water removal. By studying the scattering pattern from voids in

fiber with SAXS, pore size changes have been interpreted successfully (Crawshaw & Cameron, 2000). In addition, water's ability to increase SSA of cellulose has been reported (Ardizzone et al., 1999; Zografi, Kontny, Yang, & Brenner, 1984). Crystallinity increases with water absorption because of crystallization of amorphous cellulose as a result of water's hydrogen bonding capacity (Howsmon & Marchessault, 1959; Wadehra & Manley, 1965). Thus, a real-time study of biomass structural change with simultaneous WAXS and SAXS is suggested to investigate crystal structure and orientation during biomass processing (Chu & Hsiao, 2001; Hsiao, Yang, Somani, Avila-Orta, & Zhu, 2005). In this case, a customized sample reactor for in situ scattering study is needed for investigation.

Small-angle neutron scattering (SANS) is another scattering technique that involves scattering by the atomic nuclei instead of electrons (Blazek & Gilbert, 2011). Interesting results about biomass structural changes at the nanometer scale during diluted acid pretreatment with small-angle neutron scattering have been reported recently (Pingali et al., 2010). Although a complete structural model is not provided, the results are important for strategic development of biomass pretreatment. Thus, SAXS and SANS could be ideal complementary techniques in study of biomass structure.

5. Conclusions

With the demands of reducing carbon dioxide emission and transitioning energy sources, study of cellulose and biomass polymer has become imperative. The study of lignocellulosic biomass calls for understanding the structure of different cellulose materials as well as structural changes during processing. Since lignocellulosic biomass, unlike pure cellulose, is usually a complex of amorphous and crystalline parts that are twisted together, structural investigation is a challenge. Multiple structural techniques have been used in biomass structure study; WAXS and SAXS provide valuable information about molecular structure. We summarized various methods for crystalline structural study and compared the structural changes of biomass during processing. We concluded that current structure studies are limited to some basic concepts such as crystallinity and SSA. Biomass crystallinity, which has been used frequently in biomass study, provides limited value because biomass contains multiple components. Cellulose crystallinity is suggested for future biomass study. Advanced methods for composition analysis and X-ray scattering analysis are needed for further investigation of biomass structure. Meanwhile, detailed investigation of structural features and structural changes during biomass processing is extremely important for revealing the complicated bio-physico-chemical mechanisms and modeling the different biochemical reactions. Cost-oriented process design could be based on prediction models. Full-scale study of X-ray scattering, including WAXS and SAXS, is supposed to be well employed in study of biomass structural changes. The naturally oriented cellulose structure and its effect on biomass processing should gain attention. With the new generation of photon technology, X-ray is definitely a reliable source for the study of real-time structural change and mutant or transgenic plants. Synchrotron light source facility, combined with X-ray, could make possible unveiling of the molecular assembly of lignocellulosic biomass.

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