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Review

A critical review on analysis in pretreatment of lignocelluloses: Degree of polymerization, adsorption/desorption, and accessibility

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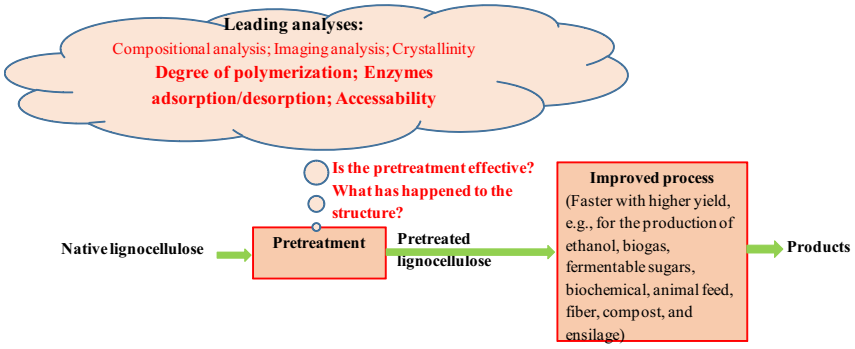
HIGHLIGHTS

- Change in the cellulose degree of polymerization is an important factor in pretreatment.
- Adsorption/desorption and accessibility of the enzymes are also important features.
- Methods, strengths and weakness, and some details of the analysis are reviewed.
- The methods are helpful to understand and improve the pretreatment techniques.
- It is impossible to investigate the effects of just one factor in the pretreatments.

GRAPHICAL ABSTRACT



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ABSTRACT

The pretreatment of lignocelluloses results in changes in the different properties of these materials. In a recent review (Karimi and Taherzadeh, 2016), the details of compositional, imaging, and crystallinity analyses of lignocelluloses were reviewed and critically discussed. Changes in the cellulose degree of polymerization, accessibility, and enzyme adsorption/desorption by pretreatments are also among the effective parameters. This paper deals with the measurement techniques, modifications, and relation to bioconversions, as well as the challenges of these three properties. These analyses are very helpful to investigate the pretreatment processes; however, the pretreatments are very complicated and challenging processes. It is not easily possible to study the effects of only one of these parameters and even to find which one is the dominant one. Moreover, it is not possible to accurately predict the changes in the bioconversion yield using these methods.

1. Introduction

Lignocelluloses are a very complicated matrix of different polymers and chemicals, affecting the yield of their bioconversions (e.g., Karimi and Pandey, 2014). When the hydrolytic enzymes or microorganisms attack the lignocellulose's carbohydrates, various factors affect their effectiveness. In a recent review (Karimi and Taherzadeh, 2016) some of the factors, including those that affect the analytical results of the composition of lignocelluloses, imaging, and crystallinity, were reviewed and critically discussed. This article is dedicated to the remaining factors.

The length of the carbohydrate chains is among the factors that highly affect the hydrolysis. Among the carbohydrates, the major

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constituent, cellulose chains are typically larger and more difficult to hydrolyze. The larger the cellulose chains, the lower the hydrolysis yields; thus, the degree of polymerization of the cellulose is known to be a critical factor in the hydrolysis (Karimi et al., 2013). On the other hand, the carbohydrate polymers need to be accessible for the hydrolysis. Besides accessibility, the enzyme or microorganisms should be adsorbed on the polymer chains, and after the hydrolysis they should be desorbed to start the hydrolysis of the other chains. These factors are known as determining factors in the bioconversion of the lignocelluloses (Kumar and Wyman, 2009, 2013). These analyses help to give different interpretations in the pretreatment processes; however, there are some challenges with the analysis and in correlating these factors to the lignocelluloses bioconversions. This review paper introduces the different analytical methods, their strengths and weaknesses, modifications, and its possibility to be used for correlating with the pretreatment efficiency.

2. Degree of polymerization (DP) of cellulose

The DP of cellulose, the number of glucose in the molecule, is known as a key parameter contributing to the enzymatic hydrolysis of the lignocelluloses (Hallac and Ragauskas, 2011) (Fig. 1). Long cellulose chains contain more hydrogen bonds and are difficult to hydrolyze (Zhang and Lynd, 2005). Furthermore, the DP reduction by a pretreatment process is accompanied by the formation of more cellulose ends available to the exoglucanase (Yang et al., 2011). In addition, the shorter cellulose chains are more reactive to the enzymes, as the shorter chains containing lower hydrogen bonding have weaker networks and are easier for the enzymes to access (Hallac and Ragauskas, 2011). Thus, it can be expected to increase the susceptibility of the cellulose to hydrolysis by decreasing the cellulose DP (Zhang and Lynd, 2005). Nonetheless, the information that is available on the effects of the DP on hydrolysis is still limited. Moreover, no clear relation has been detected between the DP and hydrolysis (Yang et al., 2011). For instance, Sinitsyn et al. (1991) decreased the DP of pure cellulose and bagasse by more than fivefold using the irradiation pretreatment, in which the other properties including the crystallinity were not altered. They found higher synergy between some types of endoglucanase and cellobiohydrolases at a lower cellulose DP. However, no significant change in the enzymatic hydrolysis yield was observed by changing the DP.

In the pretreatments that reduce the cellulose DP, e.g., dilute acid hydrolysis, chains with different sizes are formed, including soluble and insoluble cellulosic polymers and oligomers. Insoluble cellulose molecules have a DP from 100 to 20,000, while the soluble cellulose has a DP in the range of 2–12. Cellulose chains with a DP of less than 6 are soluble and those in the range of 6–12 are slightly soluble (Zhang and Lynd, 2004).

The activity of β-glucosidase is reduced by low DP cellulose chains (Lee and Fan, 1980). This can be one of the negative effects of the pretreatment and the DP reduction on the enzymatic hydrolysis. Generally, limited information is available regarding the effects of the different cellulose with different degrees of polymerization on the enzymatic hydrolysis. Moreover, the effects of the DP on a number of other parameters and properties, e.g., the cellulase adsorption and desorption (Yang et al., 2011).

2.1. DP measurement methods

Viscometric technique is the most widely used method for the cellulose DP measurement (Hubbell and Ragauskas, 2010). It is probably the easiest and cheapest way. It needs dissolution in a cellulose solvent, such as cuproethylenediamine, measuring the intrinsic viscosity, e.g., by using a capillary viscosimeter and calculating the DP by applying a correlation such as the Immergut formula (Pala et al., 2007).

A modified and more accurate viscometric method for the DP measurement was recently presented by Kumar et al. (2009). In this method, the lignocellulose powder is dissolved in the copper diethylene amine under continuous nitrogen flushing. The cellulose DP is then determined using the intrinsic viscosity measurement, according to the ASTM standard D1795. The effect of the hemicellulosic polymers on the viscosity is also excluded, resulting in a better approximation of the cellulose DP. This method is suitable for comparing the effects of the pretreatment of lignocelluloses and resulted in more reasonable values, although the absolute values are not as accurate as the more advanced methods. In spite of its simplicity, this method is suggested for the comparison of the lignocellulosic materials. Even the comparisons of the hydrolysis and the DP of the different lignocelluloses measured by viscometry make sense. For example, the DP of wheat straw, rice straw, poplar, and aspen measured by viscometry was 2660, 1820, 3500, and 4581, respectively (Hallac and Ragauskas, 2011). It corresponds to the general agreement that agricultural residues are more amenable to hydrolysis than wood residues.

The DP can also be calculated by the molecular weight distributions of the derivatized cellulose (e.g., tricarbanilate cellulose). The number- and weight-average degree of polymerization (DPn and DPw, respectively) can be obtained by gel permeation chromatography (GPC) and be simply related to the DP (Hubbell and Ragauskas, 2010). The lack of availability of the cellulose tricarbanilate, as a standard, is one of the drawbacks; however, the GPC results offer detailed information, suitable for following the cellulase mode of action on the cellulose (Pala et al., 2007).

Most of the methods used for the DP measurement of cellulose are based on the cellulose dissolution. The dissolution can be obtained using either metal complex solutions (e.g., cuam, cuen, and cadoxen) or preparing easily soluble derivatives of the cellulose (e.g., by nitration and tricarbanilation). After dissolution,

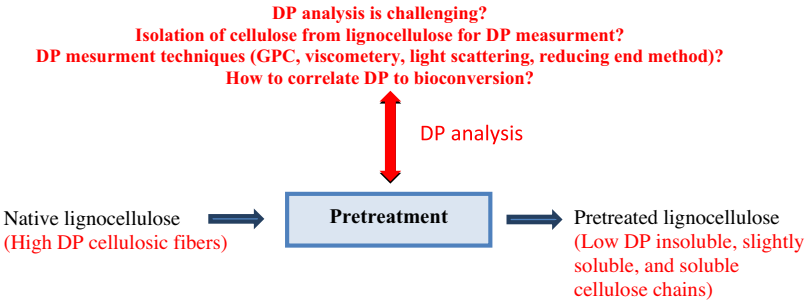

Fig. 1. Changes in cellulose degree of polymerization (DP) by pretreatment and challenges.

besides the viscometry and GPC, the DP can also be measured by membrane osmometry, vapor pressure oscomometry, cryoscopy, ebullioscopy, and chromatography, although they are time consuming and need a number of preparations (Zhang and Lynd, 2004).

An alternative approach to measuring the number-average DP is to divide the number of glycosyl residues by the number of chain ends, by measuring the available reducing end sugar assays, e.g., 3,5-dinitrosalicylic acid (DNS), Nelson–Somogyi, or 2,2′-bicinchoninate (BCA) methods. A rapid procedure presented by Zhang and Lynd (2005) can be used to determine the DP of the pure cellulose using the ratio of the glucosyl monomer concentration measured by the phenol-sulfuric acid method) to the reducing-end concentration measured by a modified BCA method. BCA was modified in order to avoid breaking the glucosidic bond cleavage. However, this method has limitation for real biomass, as compounds such as protein react with the BCA reagent and may overthrow the numbers. Light scattering techniques can also be used for determination of the cellulose molecular weight and DP without the cellulose derivatization. The typical system uses the size exclusion chromatography coupled with a multi-angle laser light scattering. However, the method is suitable for pure cellulose and less for typical celluloses containing an appreciated level of lignin, as a result of the difficulties with dissolution (Yanagisawa and Isogai, 2007).

For the DP measurement of the lignocelluloses, the cellulose in the biomass must be isolated. Nevertheless, it is difficult or let us say impossible to separate the cellulose without changing its structure (Hallac and Ragauskas, 2011). Because of the restrictions in the measurement and interference with other effective factors, it has been stated in most studies that "more investigations are necessary" (Kumar and Wyman, 2013). Browning (1967) presented a chemical method for the isolation of the cellulose from the lignocellulose, during which the length of the cellulose chains is not significantly changed. However, the DP change in this purification method depends on the species and the composition of the lignocelluloses (Hallac and Ragauskas, 2011).

The main problem in investigating the effects of the cellulose DP change following pretreatment is simultaneous changes in a number of properties, including changes in the lignin and hemicellulose content, lignin–carbohydrate complexes, accessibility, and crystallinity (Shafiei et al., 2015).

3. Cellulase adsorption/desorption

The structure of the cellulases is adapted to enter the insoluble substrates. The hydrolysis of cellulose starts with the adsorption of the enzymes onto the cellulose molecules. Thus, the enzymes adsorption/desorption is one of the main properties of a lignocellulose, affecting the rate of hydrolysis. After hydrolysis of a cellulose segment, the cellulases should be desorbed to start the hydrolysis of a new segment or move to the next parts. Thus, desorption is also an important factor affecting the hydrolysis. Nonspecific adsorption of the cellulase, e.g., on lignin, can play a major limiting role (Kumar and Wyman, 2009, 2008). Instead of cellulose, cellulases are adsorbed on lignin, making fewer enzymes available for the hydrolysis. Adsorption/desorption of the cellulases is suggested, as properties of lignocelluloses matched well with the hydrolysis (Fig. 2). A number of properties, e.g., crystallinity, accessible surface area, particle size, and lignin and hemicellulose content, are involved in the cellulase adsorption/desorption. In other words, cellulase adsorption/desorption is a suitable representative for a number of affecting parameters.

Physical adsorption of the cellulases on the cellulose reaches the equilibrium state within a very short time (e.g., 30–90 min), as compared to the time required for the hydrolysis (e.g., 72 h). Several equilibrium models and dynamic models have described the adsorption experimental data (Zhang and Lynd, 2004). Among the applied models, the Langmuir isotherm model fits well with the equilibrium data in most of the cases, even in the presence of lignin (Suvajittanont et al., 2000). Several modifications to the Langmuir isotherm model have also been presented (Zhang and Lynd, 2004). Despite the well-fitting data, there are several assumptions regarding the Langmuir equation that may not comply with the cellulase–cellulose system, including the partial irreversible adsorption (particularly on the high lignin content binding sites), cellulase component interactions, multiple types of adsorption sites on the cellulose, and enzyme entrapment in the cellulose pores. Furthermore, cellulase is a mixture of different enzymes, and each enzyme has different Langmuir constants (Zhang and Lynd, 2004). In spite of these drawbacks, the adsorption equilibrium constants have a strong correlation with the yield and rate of the hydrolysis (Lynd et al., 2002), which supports the hypothesis that the hydrolysis is primarily controlled by the cellulase adsorption onto the cellulose surface (Kumar and Wyman, 2009).

3.1. Experimental procedure for adsorption and desorption

The adsorption/desorption experiments are easy to perform. However, to avoid the hydrolysis of cellulose, they should be carried out at 4 °C (Lee et al., 1982). The cellulase adsorption/desorption analysis can be performed in a 1–10 ml media similar to the hydrolysis experiment (i.e., 50 mM sodium citrate buffer, pH 4.8) for 10 min to 2 h with vigorous stirring or shaking, gentle shaking for 6 h, or for 12 h without shaking (Gao et al., 2014; Kumar and Wyman, 2008; Lee et al., 1982; Zhu et al., 2009). For direct measurement of the absorbed cellulases, the biomass may be washed with water to remove the non-adsorbed enzymes (Zhu et al., 2009) or without washing (Kumar and Wyman, 2008). Furthermore, a very small amount of pretreated or untreated sample, e.g., 0.05 g, is necessary (Noori and Karimi, 2016).

Free sugars may be removed before the adsorption analysis. Besides inhibiting the enzymatic hydrolysis, the free sugars, e.g., glucose and particularly cellobiose, can severely and negatively affect the cellulase adsorption (Kumar and Wyman, 2008). This should be considered for the lignocelluloses containing high free sugars, e.g., sweet sorghum.

Desorption can be conducted easily by dilution with water, citrate buffer, or caustic water and equilibrating for 10 min to 2 h (Hu, 2009; Kumar and Wyman, 2009). A typical way is dilution with an equal amount of citrate buffer (50 mM, pH 4.8) and equilibrating at 4 °C for 2 h. A number of other solutions, e.g., citrate buffer at a higher pH, detergents, salts, and polyhydric alcohols were also used to improve the cellulase desorption. Among them, high concentrated ethylene glycol (EG) (e.g., 72–75%) is among the most suitable solution for the effective desorption (Hong et al., 2007; Zhu et al., 2009). In order to compare the desorption results between the untreated and the pretreated substrates or different lignocelluloses, desorption in a citrate buffer similar to the one used in the hydrolysis gives reasonable results (Kumar and Wyman, 2008).

3.2. Analysis of the enzyme for the cellulase adsorption/desorption

The cellulases adsorbed on the cellulose can be determined based on the difference of the initial cellulase and the free cellulase. The concentration of the free enzymes can be measured by traditional methods, e.g., Lowry, Biuret, bicinchoninic acid (BCA), and Bradford. When some free sugars are present in the biomass, the proteins can be precipitated by the acetone and re-dissolved

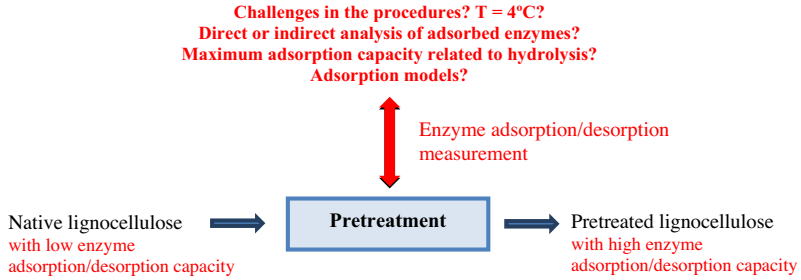

Fig. 2. Enzyme adsorption/desorption and pretreatment.

in the citrate buffer before the analysis (Lee et al., 1982). However, the enzymes adsorbed on/in the cellulose cannot be directly measured by these methods. The Kjeldahl method, a favorable method for determining the nitrogen and protein content in many industrial products particularly in food and fertilizer industries, was used for the direct measuring of the protein adsorbed on the biomass and shown to be not suitable for this purpose (Kumar and Wyman, 2008). Different direct or indirect measurement methods are available to measure the adsorbed proteins, as summarized here:

3.2.1. Indirect measurements

Three different methods for indirect measurement are dilution, protein precipitation, and radioactive labeling. They are based on the following equation:

$$\text{Bound cellulase on cellulose} = \text{Total cellulase loaded} - \text{Free cellulase}$$

The adsorbed cellulases can be washed with a large volume of a buffer, and the protein mass concentration is assayed by the traditional protein assayed methods. The major challenge is that of very low concentrations of free enzyme, resulting in inaccuracy in the protein assay. Moreover, the method is not applicable to lignocelluloses as a result of interference with lignin and hemicelluloses (Zhu et al., 2009). Another method is measurement of the released cellulases using activity measurement. This method is very time consuming, sensitive, and accompanied by changes in the composition of the cellulases (Zhang et al., 2006).

One of the major disadvantages of the indirect methods is that the accuracy of the methods cannot be checked by the material balances. Sensitivity to the free sugars, lignin, chemicals, salts, color, and other components are also among the problems (Kumar and Wyman, 2008).

3.2.2. Direct measurements

Different direct measurement methods are presented. Using radio- or fluorescent-labeled cellulases, high-throughput fast protein liquid chromatography (HT-FPLC), nitrogen element analysis, and hydrolysis of the absorbed enzymes to the amino acids and their analysis, are among the methods.

Kumar and Wyman (2008) presented a direct method for measuring the cellulase absorbed on the cellulose. This method is based on the nitrogen element analysis, using a CHNS/O analyzer, which is accurate, but costly. Since the lignocelluloses contain nitrogen components, the nitrogen in the biomass should also be measured and subtracted. The nitrogen content measured is then converted into the corresponding protein content using a nitrogen factor (NF), which requires some more measurements to give an accurate value. The NF for cellulases and xylanase is similar or greater than that of plants' protein (NF = 6.25).

A simple method is also presented based on the hydrolysis of the absorbed enzymes to the amino acids and their analysis. The hydrolysis of the cellulases can be performed by autoclaving with 10 M of NaOH. Without interfering with the free sugars, cellulose, hemicellulose, and lignin, the ninhydrin assay can be used to quantify the total amount of amino acids released from the hydrolyzed cellulase. The proteins in the untreated and the pretreated samples can be measured by the ninhydrin assay and considered in the calculations (Zhu et al., 2009). Radio-labeled cellulases have also been successfully used to study the cellulase adsorption/desorption as well as investigate the reversibility of the adsorption/desorption (Palonen et al., 2004). However, this method is very expensive and needs enzyme purification and labeling.

3.3. Adsorption parameters for comparison and challenges

The Langmuir model has two parameters, maximum adsorption capacity (σ, or specified adsorption capacity) and single adsorption equilibrium constant (K_d). They can be used to compare the kinetic properties of the various cellulase–cellulose systems, e.g., pretreated and untreated lignocelluloses. Maximum adsorption capacity can be related the binding capacity of biomass. The adsorption equilibrium constant is related to the inverse of the binding affinity (liters per gram of cellulase). A strong direct relation was reported between the maximum adsorption capacity and the enzymatic hydrolysis (Gao et al., 2014).

One of the main fundamental problems with the adsorption/desorption analysis is performing the adsorption analysis at 4 °C, avoiding the hydrolysis of the cellulose. Conversely, the temperature at which the hydrolysis is performed is 37–50 °C. Both adsorption and desorption are highly affected by temperature. At 4 °C, the rate of adsorption on the cellulose and lignin is more or less the same, whereas the cellulase adsorption capacity of the lignin is less than one-tenth of that of cellulose. On the other hand, at 50 °C, the adsorption of the cellulase on the cellulose is much faster (reaching an equilibrium in 1 h with a peak at 0.25 h), while the cellulase adsorption on the lignin is much slower and took more than 12 h to reach equilibrium (Zheng et al., 2013). Therefore, the adsorption/desorption data obtained at 4 °C should not always be expected to match well with the enzymatic hydrolysis data at the high temperatures (e.g., 37–50 °C).

4. Carbohydrates accessibility

One of the main objectives of almost all pretreatments is to increase the cellulose accessibility for the hydrolytic microorganisms or enzymes (Fig. 3). Even the main goal of the lignin/hemicellulose removal is to increase this accessibility (Shafiei et al., 2015; Taherzadeh and Karimi, 2008). Cellulose accessibility was reported to be the most important factor affecting the digestibility of the lignocelluloses, among the delignification, destruction of the hydrogen bonds, breakdown of intramolecular hydrogen bonds,

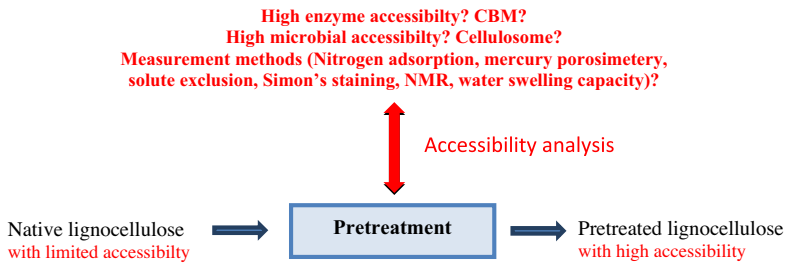

Fig. 3. Accessibility and pretreatment.

cellulose crystallinity, and lignin and hemicellulose content (Huang et al., 2010).

4.1. Accessible surface required for the microbial digestion

In anaerobic digestion for the biomethane or biohydrogen production, four sequential phases are conducted with different microorganisms. The first stage, which is the limiting stage in the case of recalcitrant lignocelluloses, is hydrolysis by hydrolytic bacteria (Kabir et al., 2015). The sizes of these bacteria are in the range of between 0.2 and 20 μm, despite their different shapes. Therefore, the biomass substrate should have enough pores, with at least 0.2–20 μm to be digested by these bacteria.

The cellulolytic bacteria are truly efficient hydrolyzers of the cellulose/hemicellulose, more efficient than the free enzyme systems (Shoham et al., 1999). As they reach the cellulose surfaces, the bacteria produce extracellular multi-enzyme complexes, so called cellulosomes, localized at the interface between the cell and the cellulose/hemicellulose, and they degrade the cellulose and the hemicellulose to soluble products that are then directly absorbed (Shoham et al., 1999). These intricate enzymes are designed for efficient hydrolysis of the carbohydrates. Cellulosome is a very complex system and composed of different functional domains and subunits, interacting with each other as well as the cellulose/hemicellulose (Shoham et al., 1999; Dykstra et al., 2014; Salehi Jouzani and Taherzadeh, 2015).

One of the cellulosome subunits is a large glycoprotein, which is non-catalytic, called scaffolding, containing different types of cellulases and hemicellulases, with the optimized ratio and order of the component (Schwarz, 2001). It adheres to cellulose/hemicellulose by cellulose/carbohydrate-binding domains. Cellulosome is typically attached to the cell wall of the bacteria; however, it can also be present freely in the solution (Bayer et al., 2004).

The initial stage in the degradation of the lignocellulose is the physical binding of the entire microorganism to the cellulose substrate, as the cellulosome formation is induced and occurs at the cell-substrate interface (Shoham et al., 1999); thus, the direct physical contact between the microorganisms and lignocellulosic surfaces is a requirement to start the hydrolysis. Therefore, lignocelluloses with only exterior surfaces are not suitable for the microbial digestion, due to the low accessible surface area for the microbial digestion. This can be simply observed by a 60-day anaerobic digestion of a piece of wood, resulting in no considerable weight loss or change in its appearance. This discussion is also true for the improvement of the digestibility of the lignocelluloses, e.g., rice straw, for the animal feed production as well as for the composting of the wood wastes.

4.2. Accessible surface required for enzymatic hydrolysis

Cellulase and hemicellulases cocktails can be produced by different microorganisms, mainly fungi and bacteria. Different types of these enzymes are purified and commercially available with reasonable prices. Hydrolysis with cellulase involves three stages (Taherzadeh and Karimi, 2007): (a) enzyme adsorption to the cellulose surface, (b) hydrolysis of the cellulose, and (c) desorption of the adsorbed enzymes into the liquid. Thus, similar to the microbial hydrolysis, direct physical contact of the enzymes and cellulose is necessary; and, accessible surface area for the enzymes is among the most effective factors affecting the rate and yield of the cellulose/hemicellulose enzymatic hydrolysis.

Cellulases are composed of a core and a long tail, like a tadpole, including a catalytic domain, linker, and a carbohydrate-binding module (CBM), which is significantly varied by the strains produced. As a simple approximate, it can be considered as an ellipsoid core with around 6 nm diameter and approximate length of 20 nm (Srisodsuk et al., 1993). For diffusion of a molecule to a pore, the size of diffusing component cannot be equal to the pore size, as the wall confinement plays an important role. For instance, the maximum accessible particle size is 1.31 nm for a 3 nm cylindrical pore. Thus, considering the wall confinements, pores with 40–60 nm width limit the cellulases diffusion to a high degree (called critical width). Practically, even 200 nm may also limit the enzymes diffusion to a certain extent, because of the confinement as well as the pore clogging effects by previously bound enzymes, irreversible enzymes adsorption, jamming, and protein aggregates (Bubner et al., 2012). On the other hand, the most severe inhibitor of the cellobiohydrolase is the cellobiose, resulting in the feedback inhibition of the cellulases. When the pores are very narrow, the β-glucosidase, the cellulase enzyme that is mainly responsible for the cellobiose hydrolysis, cannot accompany the cellobiohydrolases in the pores. Thus, synergistic action of the different types of cellulases, which is the requirement for efficient and fast hydrolysis of cellulose, needs some more spaces (Bubner et al., 2012).

4.3. Accessible surface of the lignocelluloses

Lignocellulosic materials have external and internal surfaces, where the total accessible surface area is the sum of these areas. External surface depends on the size and shape, and its area can be increased by size reduction, that is, a typical physical pretreatment of the lignocelluloses. On the other hand, the internal surfaces of a lignocellulose depend on the pore sizes and distributions. Lignocelluloses have a very small internal surface, especially after drying. A suitable pretreatment should significantly increase the internal surfaces (Shafiei et al., 2015).

Accessible surface area is considered as an important factor for the digestibility of a lignocellulose. The accessible surface area is gradually increased by the enzymatic hydrolysis, as a result of the partial cellulose and hemicellulose removal. Thus, a more porous biomass is formed at the latter stages of the hydrolysis. However, hydrolysis is usually much faster at the beginning of the enzymatic hydrolysis and considerably slower at the latter stages, despite the availability of higher surfaces. This indicates that the surface area is not the main controlling factor for the hydrolysis. Easier hydrolysis of available amorphous cellulose is related to

the faster hydrolysis of the biomass at the first stages and the slower rate related to the hydrolysis of the higher crystallinity regions, deactivation of enzymes, and increasing the concentration of the lignin. Consequently, the accessible surface area is not the only factor and should be considered in addition to the other effective factors affecting the hydrolysis (Khodaverdi et al., 2012).

4.4. Analytical methods for accessible surface area measurement

Nitrogen adsorption, mercury porosimetry, and solute exclusion are among the methods used for accessible surface area measurement. Pores are generally classified as micropores (>2 nm diameter), mesopores (2–50 nm diameter), and macropores (>50 nm diameter) according to their sizes (Sing et al., 2008). Main limiting features of lignocelluloses to enzymatic hydrolysis is the lack of accessibility, which can be related to the pore sizes. It should be noted that different methods give considerably different results, as the principles of the measurement are different. As an example, solute exclusion analysis showed a mean pore diameter of 3 nm for pine Kraft fibers, whereas this diameter was reported as 13 nm as detected by NMR (Suurnakki et al., 1997). However, for comparison purposes, all of them may make sense.

4.4.1. Nitrogen adsorption

Long ago, Grace and Maass (1931) presented a method for the sorption of the different vapors on wood and cellulose, which was then further used for the measurement of the total surface area and the pore size distribution of the wood. Volume, specific surface area, and the size distribution of the lignocelluloses pores can be determined by this method (Thode et al., 1958). The Brunauer–Emmett–Teller (BET) equation can be used for the calculation of the specific surface area, while the pore size distribution is determined using the isothermal data of either the adsorption or desorption. Details of the calculations are presented by Barrett et al. (1951).

One of the advantages of using the nitrogen adsorption method is that it measures only the open pores, but not the closed interior pores, as the cylindrical pore model is assumed. However, the pore network can affect the desorption isotherm, used in the determination of the pore size distribution. For obtaining the desorption data, the pressure is reduced for the evaporation of the liquid; however, the liquid is not uniformly evaporated, as the liquid in the narrower channels remain filled. The evaporation can also result in changes in the pore distribution. Moreover, the distribution of the pores and their sizes may be significantly changed during the treatment with the liquid nitrogen at a reduced temperature (e.g., at −196 °C) (Westermarck, 2000).

4.4.2. Mercury porosimetry (MP)

Washburn (1921) introduced MP as a suitable method for determining the pore size distribution of porous materials, which has subsequently been used to analyze the pores in cement, catalysts, and adsorbents. This method is also used for the analysis of the pores in the lignocelluloses. Similar to nitrogen adsorption, surface tension, capillary forces, and pressure are the basis of the method. The method was first used for the determination of the macropore-size distribution in the range of 85,000–200 Å pore diameter (Drake, 1949). MP was further improved to perform at low and high pressures. Depending on the equipment used, low-pressure can be suitable for the determination of the macropores (14–200 μm) and high-pressure for the mesopores and macropores (3 nm to −14 μm) (Tantasucharit, 1995). Commercial types of MP are now available for the determination of different powder properties, including total pore volume, incremental volume, differential volume, log-differential volume, total pore surface area, mean pore diameter, incremental area, pore size distribution, and percentage of porosity. The MP method can analyze a wider range of pore sizes in a shorter time than nitrogen adsorption; however, nitrogen adsorption is more widely used than MP (Allen, 1997).

Pores with 0.3–300 nm diameter (mesopores and macropores) can be analyzed by nitrogen adsorption, while mercury porosimetry may be suitable for pores with 14–200 μm (macropores). However, the results from both of these methods are more or less the same (Conner et al., 1986).

MP measurement is rather fast and easy. First, all the gas in the sample is evacuated, and then mercury is forced to enter the samples under a vacuum and pressure process to obtain intrusion-extrusion curves. The curves are then used for the calculation of the pore structure. The total introduced volume of mercury at the highest pressure is considered as the total pore volume. The area above the intrusion curve is used as the total pore surface area. Based on the assumption of the cylindrical shape, the mean pore diameter and volume pore size distribution can be calculated (Ritter and Erich, 1948).

One of the disadvantages of MP is the compression and damage to the sample during the high pressures forcing the mercury to enter the pores. Another problem is the overestimation of the volume for the smallest pores (Auvinet and Bouvard, 1989). The major drawback of the nitrogen adsorption and mercury porosimetry methods is measuring the available surface area using the molecules that are very much smaller than the hydrolytic enzymes and the microorganisms. Another disadvantage is the need to use a dried sample, while the internal structure is changed during the drying process (Tantasucharit, 1995).

4.4.3. Solute exclusion (SE)

Stone and Scallan (1967) developed the SE method, which was further established for the estimation of the accessible surface area for an enzyme as well as the pore volume distribution of the lignocelluloses (Tarkow et al., 1966). An experimental procedure complete with caveats was then presented by Van Dyke (1972). This method is quite simple and does not need special equipment; only a refractometer or polarimeter is necessary. In this method, the lignocellulose is added to a known weight of indicators, called “probes”, dissolved in water and mixed. Three scenarios are possible: (1) the concentration of the probes will not change, when all the pores are accessible to the probes; (2) the probes will be slightly less diluted than in the first scenario, when larger probe molecules are used; (3) the inaccessible water equals the total water of swelling or fiber saturation point, when the probe molecules are very large and cannot enter the pores.

A series of probes with different sizes can be used. The probes should not be uncharged and physically or chemically adsorbed onto the pores, should have a narrow molecular weight distribution, and be available over a wide range of molecular weights. Probes should be spherical and have a known size in the solution. Different probes are suggested for this method. Among them, polyethylene glycol as well as dextrans and related sugars are the most applied ones (Tantasucharit, 1995).

In this method, it is assumed that the concentration of the probes in the accessible pores is equal to that in the solution out of the pores. Moreover, when the probe diameter is less than the pore diameter, complete penetration of the probe is assumed (Lin et al., 1985). Furthermore, the pores are assumed to be parallel slits with a similar width between the multiple lamellae (Stone and Scallan, 1968; Tantasucharit, 1995), which is questionable.

The main advantage of this method is the possibility of performing the analysis in the same solution media as used in the enzymatic hydrolysis. In spite of the assumptions, promising results and a reasonable relation between the rate of porosity by the SE and the enzymatic hydrolysis have been observed. For instance, the accessible surface area of the cotton linters to

molecules with 40 Å sizes was compared with the initial rate of the enzymatic hydrolysis, and a linear relationship was observed (Stone et al., 1969). Moreover, a linear correlation was found between the accessible area of the hard- and soft wood to the molecules with 51 Å diameters, and the initial enzymatic hydrolysis was observed (Grethelin, 1985). However, this method is very time consuming, involving several solution preparation, weighting, agitation for several hours, settling without agitation, centrifugation, optical measurement, and need for special care and attention to obtain accurate results.

4.4.4. Simons' stain (SS)

Simon (1950) developed a method for the study of the interior structure of the fibers fibrillation and mechanical damage using two sensitive color probes. He used a mixed solution containing the two probes, a blue dye with a small molecular size and low affinity for cellulose, and an orange dye with a large molecular size and high affinity for cellulose. According to this method, the damaged fibers were stained orange, observable under an optical microscope. The original method is very time consuming and has some limitations. As the method has the possibility to indicate the large and small pores of the fibers, it was further improved in order to be used for the measurement of the interior and exterior surface area of the lignocelluloses, with a relatively high sensitivity to changes in the cellulose accessibility (Chandra et al., 2008; Goshadrou et al., 2013).

The blue probe used has a 1 nm molecular diameter and a well-defined chemical formula, entering all the pores larger than 1 nm. However, the orange probe, obtained by condensation of 5-nitro-o-toluenesulfonic acid in an alkali solution, has a molecular diameter range of 5–36 nm. The orange dye has a higher affinity to the hydroxyl groups on the lignocellulosic surface. The ratio of these adsorbed probes (orange to blue dyes; O/B ratio) is used to analyze the amount of large and small pores and subsequently as an indication of the cellulose accessibility to the hydrolytic enzymes. Furthermore, it was shown that lower enzyme loading is required for efficient hydrolysis when the substrate has a higher O/B ratio (Arantes and Saddler, 2011; Chandra et al., 2008).

One of the modifications to this method was conducted when it was found that the high molecular weight fraction of the orange dye used by Simon had a high affinity to cellulose and that the low molecular weight part had a similar affinity to the cellulose as the blue dye. Then, the low molecular weight part of the orange dye was removed by ultrafiltration. The accessible area was then well correlated to the enzymatic hydrolysis of the lignocelluloses (Esteghlalian et al., 2001).

The method is rather simple and inexpensive. It needs some chemicals, a simple filtration set up, a spectrophotometer, and an optical microscope; all are available in almost all laboratories. However, the method is rather time consuming, even after the modification in which the incubation time was reduced from around 50 h to around 6 h and needs different sample and dye preparations. Moreover, the method is suitable for comparing the pore sizes of the untreated and pretreated lignocelluloses. However, the accuracy of the method is not as high as the NMR methods. The Simons' staining analysis is also very sensitive to the pore inlet size. As indicated by the NMR analyses, there are some large pores in the lignocelluloses with small entrances (ink-bottle shaped pores), in which the dyes cannot enter and detect them. In addition, some of the pretreatment methods result in increased pore tortuosity within the biomass that negatively affects the Simon's staining evaluations (Meng et al., 2013).

4.4.5. Nuclear magnetic resonance (NMR)

NMR-based methods are powerful methods for the analysis of accessible surface areas of the lignocelluloses. NMR cryoporometry (NMRC) is among the best methods for detailed study of the pore size distribution in the lignocelluloses. The method is a perturbative method that is independent of the spin interactions and offers direct measurements of the pore sizes and volume. The pores on the scale of 2 nm to 2 μm can be analyzed. The ability to analyze the wet samples are among the main advantages of this method, as drying which is necessary for most methods, e.g., nitrogen adsorption, can significantly change the pore sizes and the volumes (Östlund et al., 2010).

Cryoporometry is based on the fact that small crystals of a liquid molecule in the pores melt at a lower temperature than molecules in the bulk of the liquid. A liquid is charged to the pores, cooled to frozen, and warmed to melted. NMR is used to quantify the liquid inside the pores that has melted as a function of the temperature. The pore sizes are inversely proportional to the liquid melting point depression. The method can be used to compare the pore sizes obtained by pretreatments and compare it with the untreated samples. A clear image of the pore size changes can be observed (Meng et al., 2013).

Besides NMRC, different thermoporometry and cryoporometry methods are also presented for the analysis of accessible surface area and pore size distributions of the lignocellulose. All of them have the same basis of melting a small region of a solid at a lower temperature than the bulk biomass. The detection of the melting can be done by differential scanning calorimetry (DSC), neutron scattering (ND), and NMR.

Other NMR based methods, e.g., NMR relaxometry and NMR diffusometry, are also suggested to investigate the details of the changes in accessibility upon pretreatment of the lignocelluloses. Here, the probe molecule is water. The nature and strength of the association-adsorbed water localized within the pores is directly related to the ultrastructural and chemical state of the lignocelluloses (Felby et al., 2008; Menon et al., 1987). The main disadvantages of the NMR based methods are long experiment time and requirement for expensive instrument and complicated setup (Meng et al., 2013).

4.4.6. Water retention value (WRV)

If water cannot enter the pores, then it is for certain that no enzyme can enter. Thus, WRV, also known as water swelling capacity, is used as an indication of the accessible interior surface area and the suitability of the lignocelluloses to enzymatic hydrolysis (Noori and Karimi, 2016). This property of a biomass, which is the ability to swell in the presence of water, can be measured rapidly and easily, in most of the laboratories (Jeihanipour et al., 2010; Shafiei et al., 2014). Ogiwara and Arai (1968) tried to find relationships between the enzymatic hydrolysis and the WRV of different cellulose fibers. They reported a linear relationship between the WRV and the hydrolysis rate of untreated and pretreated substrates with different chemical compositions and crystallinity. However, they observed that the slope of the regression line was changed with the pretreatment method. It is interesting that the results of this simple and inexpensive method correlated well with the improvement observed in the enzymatic hydrolysis by pretreatment. Its results are comparable to the results of more advanced methods, e.g., NMR and Simons' staining (Chandra et al., 2009; Östlund et al., 2010).

Recently, Ju et al. (2013) reported that higher a xylan content increases the swelling, while lignin decreased the WRV. An increase in WRV corresponded to the increase in the efficiency of the enzymatic hydrolysis. Being able to use the same media as used for the enzymatic hydrolysis is also another advantage of the method. However, in some cases, the WRV showed limited sensitivity to be correlated to the enzymatic hydrolysis results changed by pretreatment (Chandra et al., 2009).

5. Concluding remarks

Degree of polymerization is a parameter that can be related to the bioconversion of the lignocelluloses. However, there are some challenges in the DP measurement methods. Prior to the DP measurements, the cellulose should be isolated from the lignocelluloses structure. The isolation results in some changes in the DP. Moreover, it is difficult or may be impossible with the current knowledge to investigate the effects of the DP individually. It is also very difficult to investigate the relative importance of the different changes that have occurred due to the pretreatment.

Cellulase adsorption/desorption is a property that is related to the bioconversion of the lignocelluloses. Reliable methods are available for its measurement; however, special attention should be paid to the measurement of the enzyme absorbed on the biomass. One of the major drawbacks of the cellulase adsorption/desorption analysis is when conducting the measurement at 4 °C, to avoid the hydrolysis of the cellulose. This temperature is far from the hydrolysis temperature (>37 °C). Nevertheless, the enzyme adsorption is highly dependent on the temperature. The kinetics of the cellulase adsorption and desorption on the cellulose and lignin are very different at 4 °C compared to that at high temperatures (e.g., 37–50 °C).

Substrate accessibility is another important property of the lignocelluloses, which is a desirable parameter in all pretreatments. External surface can be increased by the size reduction and changing of the particle shapes. Increase in the internal surface of the lignocelluloses is followed by typical chemical and biological pretreatments. It can be measured by different methods, including nitrogen adsorption, mercury porosimetry, solute exclusion, Simon's staining, NMR, and water swelling capacity; however, all methods have their specific drawbacks. Water swelling capacity is among the simplest methods, with its changes typically corresponding to the changes in the hydrolysis.

Overall, it is not possible to exactly predict the effectiveness of a pretreatment with any of the mentioned analysis. However, it is possible to find the reason and the mechanisms for the changes in the bioconversions by the above-mentioned methods, analyze the changes, and try to improve the effectiveness of the pretreatments.

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