

Nanocellulose isolation from *Amorpha fruticosa* by an enzyme-assisted pretreatment

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Abstract: Nanocellulose has many advantages, such as a wide range of sources of raw materials, renewability, biodegradability, high aspect ratio and large specific surface area. It can be potentially used in medicine, electronics, information technology, energy industry, aerospace and some other high-technological fields. For preparation of nanocellulose, it is particularly important to separate nanocellulose from raw materials by an environment-friendly method with environmental protection awareness. Consequently, we here report an effective and environmental friendly method to isolate nanocellulose from a shrub plant, i.e., *Amorpha fruticosa* Linn. Firstly, the plant fiber is pretreated with chemicals to remove lignin and hemicellulose; then the derived purified cellulose is pretreated with enzyme hydrolysis, followed by slight treatment of high-pressure homogenization. The results showed that with the assistance of enzyme pretreatment, effective isolation of nanocellulose could be achieved, resulting in materials with a uniform diameter distribution and an average value of about 10 nm. The aspect ratio of the derived nanocellulose is greater than 1000. Such results showed that the method was green and effective for nanocellulose isolation, and the derived biomaterial as a unique biocompatible and high-strength biomass nanomaterial could be used in biomedical, environmental protection and other fields.

Keywords: cellulase, pretreatment, nanocellulose, *Amorpha fruticosa* Linn

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1. Introduction

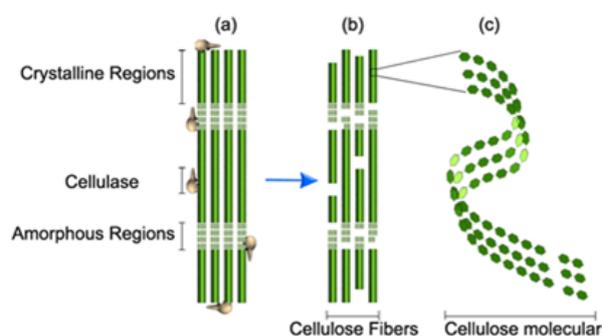
There is an increasing demand for new and innovative materials to meet the scientific and technological development and progress, and for the development of nano-materials for high-technological innovations (Dong et al., 2015; Dong et al., 2016; Dong et al., 2017). Nanocellulose is a new nano-material with one-dimension in approximately 1~100 nm in size. Nanocellulose has a large specific surface area (Moon et al., 2011), small scale effect, high chemical activity and additional characteristics, and the as-prepared products could be potentially used as sorbent materials (Wang et al., 2014), carried materials (Korhonen et al., 2011; Valo et al., 2013), catalytic materials (Lin and Dufresne, 2014), and even conductive materials (Olsson et al., 2010; Pääkkö et al., 2008). In addition, nanocellulose has high crystallinity, high Young's modulus, and high strength. The Young's modulus of nanocellulose is several times higher compared with ordinary cellulose. Therefore, nanocellulose can be po-

tentially used in reinforced composites (Abdul Khalil et al., 2012; Gawryla et al., 2009). In particular, nanocellulose is rich in hydroxyl groups, has self-assembly characteristic and is expected to be used as a smart material. In a nutshell, nanocellulose is a renewable, biodegradable and pure natural material, and has become a highly attractive research topic in material sciences.

At present, there are many methods for preparation of nanocellulose, including chemical, physical and biological methods. Chemical method is mainly used to prepare cellulose nanocrystals via acid hydrolysis, involving sulfuric acid and phosphoric acid (Dong et al., 1998; Martins et al., 2011; Nickerson and Habrle, 1947). The principle of acid hydrolysis is that the hydrogen ion breaks up the cellulose chain in the amorphous regions to leave the undegraded crystalline cellulose. Such chemical method is efficient and can maintain the original crystalline of cellulose, while the requirement of the equipment is harsh and pollution of the environment is inevitable. The physical methods used to prepare

nanocellulose can be divided into six types including high pressure homogeneous (Zimmermann et al., 2010; Zhuo et al., 2017), high-frequency ultrasound (Zhuo et al., 2017), micro fluidic (Zhuo et al., 2017), grinding (Abe et al., 2007; Zhao et al., 2007), electrostatic spinning (Gardner et al., 2008), and high-speed mixing (Uetani and Yano, 2010). Physical preparation is relatively simple, but consumes more energy. There are two methods for biological preparation of nanocellulose: bacterial production as a bottom-up strategy (Gardner et al., 2008; Tanpichai et al., 2012) and enzyme pretreatment as a top-down way (Hayashi et al., 2005; Janardhnan and Sain, 2007; 2011; Satyamurthy et al., 2011; Siqueira et al., 2010). In the bacterial method, bacteria directly produce nanoscale cellulose. Enzymatic hydrolysis is cellulose fibers hydrolyzed from macromolecular to micro-molecular sizes by cellulase. Compared to acid hydrolysis, enzyme hydrolysis is uniquely specific, more environmentally friendly and conditionally mild. Consequently, effective extraction method of nanocellulose is an environmentally protective method, which is favored by researchers. The method conforms well to the environmental protection concept, so it is an attractive way for the nanocellulose isolation.

There are many hypotheses about how cellulase works on cellulose. The generally accepted one is through synergistic mechanism (Sinnott, 1998; Thonart et al., 1980). The principle is that the endonuclease acts on the inside of the cellulosic polymer and then breaks down the non-crystalline part to produce new end groups. Then, the exonuclease hydrolyzed from the terminal at the end of the cellobiose, which hydrolyzes the cellobiose into glucose. As the progress of the reaction, the enhancement of the exogenous effect of the cellulase can not only hydrolyze the amorphous region of the cellulose but also the crystalline region of the cellulose. Therefore, it is possible to avoid the complete hydrolysis of cellulose by controlling the enzymatic hydrolysis conditions, so that it is more likely to have the reaction carried out in the amorphous region and to retain the crystallized regions. Based on the assumption, nanocellulose can be prepared effectively by the enzymatic pretreatment combined with a simple mechanical process (Figure 1). Theoretically, a wide range of sources of materials can be used for enzymatic hydrolysis of nanocellulose raw materials, such as bleached sulfite pulp, bagasse, bean dregs (Janardhnan and Sain, 2011; Pääkkö et al., 2007). Pääkkö et al. (2007) reported the preparation of nanocellulose fibers with diameters of 5-6 nm and 10-20 nm from bleached sulphite pulp by enzymatic hydrolysis combined with mechanical shear preparation. Wang et al. (2015) treated the bleached eucalyptus pulp by a beater assisted with a commercial cellulase endonuclease and a super-alloy GH5 type enzyme extracted from the bacteria, respectively, both of which were distributed in diameter range between 5-10 nm. They found that the diameter of nanocellulose treated with enzyme pretreatment combined with microfluidization was significantly smaller than that of nanocellulose derived by treatment of sole microfluidization. De Campos et al. (2013) explored the nanocellulose extraction from curaua



Note: (a) Purified cellulose; (b) enzyme pretreated cellulose; (c) cellulose molecular chain

Figure 1. Synergistic mechanism of cellulose degraded by enzyme.

and sugarcane bagasse as raw materials by enzyme pretreatment. Different ratios of endosulfanase, hemicellulase and pectinase-containing cellulase systems were used for the assisted hydrolysis, followed by ultrasonic treatment. The results proved the effectiveness of the enzyme pretreatment. Since cellulase is a protein that can be degraded, enzymatic hydrolysis is more environmentally friendly than chemical method, reaction-specific, condition-mild and energy-consumed. Therefore, the objective of this study was to fabricate nanometer fiber with high aspect ratio by enzymatic pretreatment method combined with slightly mechanical homogenization based on *Amorpha fruticosa* Linn., which has never been reported before.

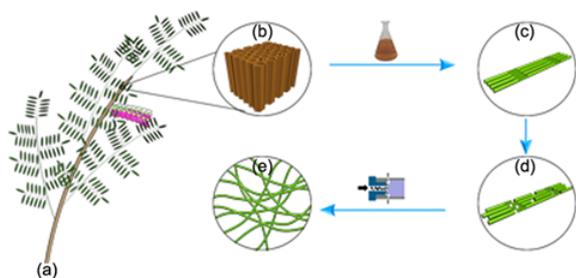
2. Materials and Methods

2.1. Materials

The *Amorpha fruticosa* Linn. material was taken from the outskirts of Taian and crushed to 120 mesh. Toluene, anhydrous ethanol, potassium hydroxide, analysis of pure, and glacial acetic acid were all purchased from Tianjin Kaitong Chemical Reagent Co., Ltd. in China. Sodium hypochlorite, purified analysis, was provided by the Damao Chemical Reagent Factory in China. Cellulase, enzyme activity 3 units/mg, was provided by the Beijing Solarbio Science & Technology Co., Ltd.

2.2. Preparation Process

Natural wood powders (2.5 g) were extracted with toluene-ethanol mixed solution (V/V=2:1) for 10 h to remove the extractives, then the lignin in wood powder was removed by 1% NaCl solution, then 5% KOH solution was used to remove hemicellulose, followed by 1% NaCl solution and successive 5% KOH solution to remove residual lignin and hemicellulose, and to finally obtain purified cellulose. The derived purified cellulose (0.5 g) was added to the buffer solution (pH=5, 25 mL), and the mixture was stirred. Meanwhile, cellulase solution (15 mL) was added to the mixture, which was placed in the water bath and stirred at constant temperature of 40°C for 12 h, 24 h and 36 h, respectively. The temperature was then slowly warmed up to 90°C for half an hour, to inactivate the enzyme, followed by filtration



Note: (a) *Amorpha fruticosa* Linn. plant; (b) the plant wood; (c) purified cellulose; (d) enzyme-pretreated cellulose; (e) nanocellulose.

Figure 2. Nanocellulose preparation process involved and the resulting finished product nanocellulose.

with a Buchner funnel for neutral condition. The enzyme-pretreated cellulose solution was diluted to 0.1% (w/v) concentration, and placed under high pressure of 500 bar for 1 min, the nano-cellulose was finally obtained (Figure 2).

2.3. Characterization and Analysis

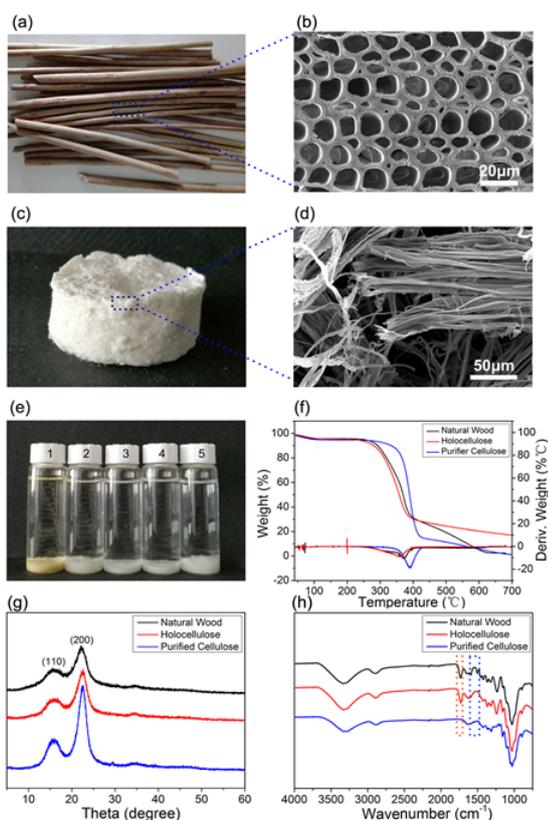
The microstructure of the sample was observed by the Field Emission Scanning Electron Microscopy (FE-SEM, Quanta200 FEI USA Inc). The operating parameters were high vacuum mode, operating voltage 12.5 kV, beam spot 5.0. The diameter of the nanofiber was measured by the Transmission Electron Microscopy (TEM, JEM-1400 JEOL USA Inc) and the Atomic Force Microscopy (AFM, NainoAFM, Nanosurf AG). The chemical components of the sample were measured by the Fourier Transform Infrared Spectroscopy (FTIR, Nicolet Magna 560, Thermo Nicolet Inc.). The crystallinity of sample was characterized by X-ray diffractometer (XRD, D/max 2200, Rigaku Corporation). The test parameters included the voltage of 40 kV, current of 30 mA, scanning angle of $10^{\circ}\sim 30^{\circ}$, and scanning speed of 4/min. The thermal stability of the sample was analyzed by means of thermogravimetric analyzer (TG, DTG-60AH, Shimadzu). The test parameters were heating speed of $10^{\circ}\text{C}/\text{min}$ and the temperature ranges of $35\sim 450^{\circ}\text{C}$.

3. Results and Discussion

Amorpha fruticosa is a kind of shrub plants with average stem diameter of about 1-2 cm and height of about 1 m (Figure 3a). It can be observed that the cell lumen size of *A. fruticosa* in diameter are almost uniform, i.e., about 5-15 μm (Figure 3b). As we know, the major components of *Amorpha fruticosa* plant are cellulose, lignin and hemicellulose, hemicellulose and lignin needed to be removed first to obtain purified cellulose for further isolation of nanocellulose (Zhuo et al., 2017). Consequently, wood flour was firstly extracted to remove the extraction components like resin acid, pigment, etc.. The color of the extracted wood flour was lighter than the original wood, but still brown (Figure 3e-1). Secondly, the wood powders were further extracted to remove lignin and present in white granular form (Figure 3e-2) (i.e., holocellulose). The light color of the derived powders was due to a little lignin residue in the

wood flour. Then, hemicellulose was treated for the first time, and the color of the derived wood solids basically remained non-changed, but gradually changed from granular form into powders (Figure 3e-3). The reason was that the lignin and hemicellulose were basically removed from the wood solids, so the powder could not maintain the original integrated form. The second removal of lignin (Figure 3e-4) and hemicellulose in sequence was to obtain highly purified cellulose (Figure 3e-5), and the finally purified cellulose color was pure white and the appearance was more fluffy. From the microscopic examination (Figure 3d), the surface of purified cellulose fiber was smooth and flat. The diameter distribution of single fiber was relatively uniform with an average diameter of about 3.5 μm . As the hemicellulose, cellulose and lignin in *Amorpha fruticosa* were treated with different reaction rates at the pyrolysis temperature range, the pretreated objectives exhibited different pyrolysis curves. Figure 3f shows that the wood flour begin to decompose at 230°C due to the first decomposition of hemicellulose component, and reach the maximum pyrolysis rate at about 375°C . The holocellulose was decomposed first at about 225°C , and the maximum pyrolysis rate was about 355°C . The initial decomposition temperature of the holocellulose was slightly lower than that of the wood flour, which might be due to the lignin more thermally stability than the hemicellulose. However, the purified cellulose began to decompose at a higher temperature of 350°C , and the quality dropped rapidly to reach a maximum degradation rate of about 390°C . This is because the cellulose contains a stable crystalline zone, which is not easily hydrolyzed than hemicellulose and lignin. It can be seen from Figure 3g that the diffraction peaks of the natural wood, the holocellulose and the purified cellulose are basically similar, and the two diffraction peaks appear at 16.2° and 22.3° , respectively, corresponding to the 110 and 200 crystal planes. These curves show that the cellulose aggregation depicted cellulose I crystal structure, suggesting that the crystal form of cellulose fiber has not yet been destroyed during the process of chemical pretreatment and mechanical processing. The crystallinities of the natural wood powder, holocellulose and purified cellulose were 54.64%, 64.01% and 79.67%, respectively. The crystallinity of the purified cellulose was the highest because the amorphous lignin and hemicellulose were gradually removed during the preparation of the purified cellulose. Figure 3h shows the changes in chemical composition during the preparation of purified cellulose. The peaks at 3337 cm^{-1} and 2897 cm^{-1} are respectively designated to the hydroxyl (OH) group in hydrogen bond and the C-H bond from the methyl and methylene groups. The peak at 1733 cm^{-1} shows the non-conjugated carbonyl (C=O) bond from hemicellulose, which exists only in wood powder and holocellulose, while the purified cellulose does not reveal this peak. It indicates that there is no hemicellulose in the purified cellulose, demonstrating the effectiveness of chemical pretreatment. Both the two absorption peaks at 1510 cm^{-1} and 1460 cm^{-1} represent the stretching vibration of the benzene ring skeleton structure in the

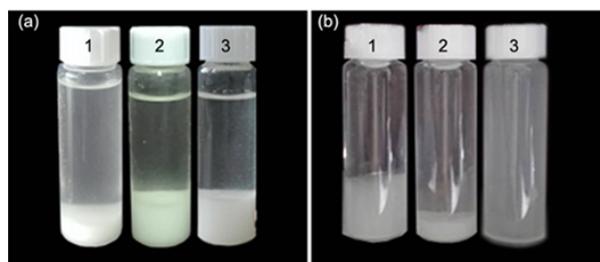
lignin and the $-CH_2$ deformation vibration on the skeleton. These two absorption peaks are only present in the wood flour, indicating that lignin has been effectively removed by purification pretreatment.



Note: (a) Digital photo of *Amorpha fruticosa* wood used; (b) SEM image of a cross section of the *Amorpha fruticosa* wood; (c) Digital photo of the purified cellulose; (d) SEM image of the purified cellulose; (e) Digital photo of the samples at various pretreatment stages: 1) wood flour after extraction, 2) wood powder after first removal of lignin (i.e., holocellulose), 3) wood powder after first removal of hemicellulose, 4) wood powder after second removal of lignin, 5) wood powder after second removal of hemicellulose (i.e., purified cellulose); (f) TG curves of the samples at various pretreatment stages; (g) XRD curves of the samples at various pretreatment stages; (h) FTIR spectra of the samples at various pretreatment stages.

Figure 3. Characterization of the samples at various pretreatment stages.

Figure 4a shows the purified cellulose suspensions after pretreatment for 12 h, 24 h, 36 h followed by ageing for 15 min. As compared with Figure 4a-2 the precipitation height of Figure 4a-1 is slightly lower and that of the Figure 4a-3 is almost unchanged. That is because the cellulase acts on the amorphous regions of the cellulose and decomposes the cellulose selectively and gradually. As the progression of time, cellulose was gradually degraded and became fluffy. However, as the reaction proceeded, cellulase gradually hydrolyzed the crystalline area of cellulose to form glucose. It is manifested that the cellulose precipitation gradually reduced in the reaction progress, and ultimately ceased at a certain time. It is found that the precipitation height did not show a significant change between at 24 h and 36 h, indicating that the cellulose amorphous area was decomposed at the end of 24 hours. Therefore, 24 h was selected as the best



Note: (a) the macroscopic morphology of the samples after enzyme pretreatment for 12 h (a-1), 24 h (a-2) and 36 h (a-3); (b) the macroscopic morphology of the samples suspensions after mechanical treatment (b-1), enzyme pretreatment (b-2), enzyme pretreatment combined with mechanical treatment (b-3), and after storage for one week.

Figure 4. The digital photos of samples obtained after treatment at different conditions.

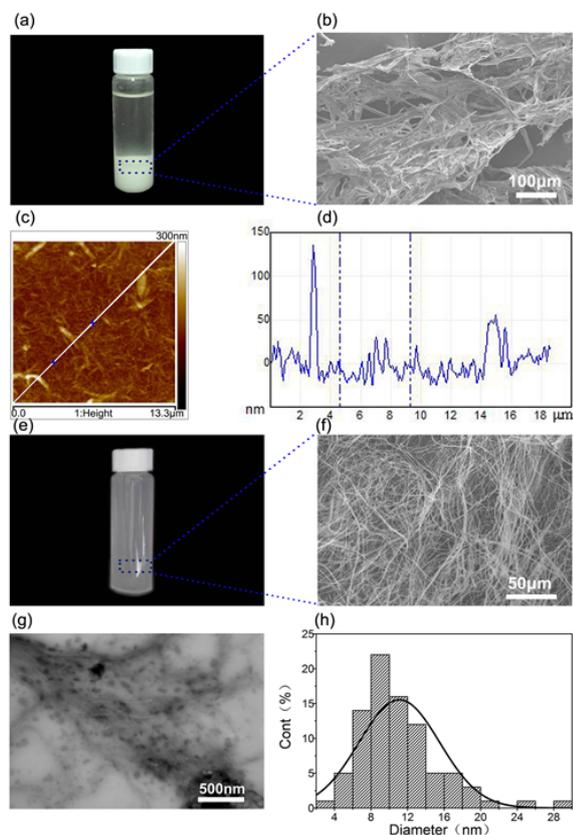
reaction time for cellulose treatment.

Figure 4b shows that purified cellulose was respectively treated by high pressure homogenization (500 bar, 1 min)(b-1), the enzyme hydrolysis (24 h)(b-2), and enzyme pretreatment (24 h) combined with high pressure homogenization (500 bar, 1 min)(b-3). The solution had obvious precipitation in the Figure 4b-1, Figure 4b-2, and the precipitation in Figure 4b-1 appeared more fluffy, while the precipitation of Figure b2 was more obvious. After pretreatment by the enzyme, Figure 4b-3 indicates that the suspension after further mechanical treatment was very stable without obvious precipitation, suggesting that the size of cellulose after only enzyme pretreatment or only mechanical treatment was still large, while the cellulose fibers prepared by enzyme pretreatment combined with mechanical treatment were theoretically reached nanometer scale as they were steadily suspended in the water even after one week.

Compared to the purified cellulose, the enzyme pretreated cellulose (Figure 5a) was more fluffy in morphology. From SEM observation (Figure 5b), the purified cellulose after the enzyme treatment almost kept the original shape with the form of flattened strip without obvious aggregation. The reason is that the cellulase destroys the non-crystalline zone of the cellulose and causes the cellulose to present multiple end. From the atomic force microscopy characterization (Figure 5c), the enzyme pretreated cellulose displays uniform dispersion with about 25 nm in diameter (Figure 5d).

After the enzymatic pretreatment for 24 hours, followed by high-pressure homogenization treatment of 500 bar for 1 min, the derived suspension presented no precipitation even after storage for one week (Figure 5e). The SEM observation shows that the derived nanocellulose presents in one-dimensional fiber with nanoscale diameter and microscale length (Figure 5f). The TEM characterization proves that the diameter of nanocellulose ranges between 2 nm and 30 nm, with an average value around 10 nm, and the aspect ratio greater than 1000 (Figure 5g). Therefore, the fineness of the obtained nanocellulose fiber diameter and the aspect ratio of nanocellulose fiber treated by the enzymatic pretreatment combined with the mechanical treatment were higher than the simple enzyme pretreated nanocellulose fiber.

Therefore, the nanocellulose fiber treated by the enzymatic pretreatment combined with the mechanical treatment is expected to be utilized in reinforcing material and flexible electronic devices.



Note: (a) Digital photo of enzymatic pretreatment of 24 h cellulose; (b) SEM image of the enzymatic hydrolysis of cellulose; (c) AFM image of the enzymatic hydrolysis of cellulose; (d) diameter distribution of AFM image of the enzymatic hydrolysis of cellulose; (e) digital photo of nanocellulose; (f) SEM image of the nanocellulose; (g) TEM image of the nanocellulose; (h) diameter distribution of TEM image of the nanocellulose.

Figure 5. The characterization of cellulose and nanocellulose.

4. Conclusion

Extraction of nanocellulose from biomass was achieved using enzyme-assisted pretreatment. The optimal time for enzyme-assisted pretreatment was 24 h. The diameter of the cellulose-pretreated cellulose was about 25 nm. After further homogenization treatment, the nanocellulose with the diameter of about 10 nm and the aspect ratio over 1000 was successfully isolated. This green method could be extended for isolation of nanocellulose from other biomass as sources. This biomass-based nanomaterial with high crystallinity and high aspect ratio is expected for utilization in the fields of reinforcing materials, flexible electronic devices and so on.

Author Contributions

Xiao Zhuo, Jie Wei, Jian-Feng Xu and Ru-Tan Pan conducted the experiment, and Xiao-Ying Dong, Ling Long, Yong-Feng Li designed the exploration. Gang Zhang and Yun-

Long Guo helped to draw figures. Xiao Zhuo, Jie Wei and Yong-Feng Li wrote the paper. Everybody comments on the final manuscript.

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