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CONTROLLED ENZYMOLYSIS PREPARATION OF NANOCRYSTALLINE CELLULOSE FROM PRETREATED COTTON FIBERS

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Natural cotton fibers were pretreated with DMSO, NaOH, or ultrasonic waves and hydrolyzed by cellulase (*Trichoderma vride* G) to prepare nanocrystalline cellulose (NCC). The as-prepared samples were characterized by TEM, DLS, XRD, and FT-IR. The influences of the pretreatment on the yield and morphology of NCC were investigated. The alterations of the crystalline phase and chemical structure of NCC were also measured during the enzymolysis process. The experimental results proved that the pretreated cotton fibers could be hydrolyzed by the *Trichoderma* cellulase to prepare a nanosized strip (10 to 40 nm $\times$ 70 to 280 nm) and grainy (20 nm or 6 nm) crystalline cellulose, in which the different morphologies resulted from the different manners of pretreatment. The unaltered crystalline phase and chemical composition of NCC prepared by enzymolysis of pretreated cotton fibers were measured. The highest yield of NCC reached was 32.4%.

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INTRODUCTION

Rapid economic and social developments are leading to an increase in consumption of non-renewable resources, which will be consumed at a faster rate, resulting in the total depletion of some resources. So, it is a significant strategy for the sustainable development of the human society to research and exploit renewable resources and their derivatives. Cellulose is the most abundant and cheapest natural polymer that can be used for developing derivative products and functional materials. Nanocrystalline cellulose (NCC) is an important product, which can be used for a variety of applications, such as papermaking (Manfredi *et al.* 2011), nanocomposites (Bras *et al.* 2011; Hubbe *et al.* 2008; Zhou *et al.* 2011; Samir *et al.* 2004; Qu *et al.* 2010; George *et al.* 2011), birefringent film (Beck *et al.* 2011; Cranston and Gray 2008), and template synthesis (Shin and Exarhos 2007; Zhou *et al.* 2007).

The classic preparation method for NCC employs inorganic acid to bring about hydrolysis (Rosa *et al.* 2010; Zhang *et al.* 2007; Lu and Hsieh 2010; Liu *et al.* 2011; Bai *et al.* 2009). In this process, sulfuric acid or the mixed acid is used as the acid catalyst of the hydrolysis of cellulose or microcrystalline cellulose to prepare NCC. The nanosized cellulose can also be obtained through mechanical treatment of cellulose by use of a high-

pressure homogenizer (Lee *et al.* 2009), cavitation milling (Pinjari and Pandit 2010), or acid hydrolysis (or steam explosion) coupled with high shear homogenization (Kaushik and Singh 2011; Liu *et al.* 2011). Recently, TEMPO(2,2,6,6-tetramethyl-piperidine-N-oxyl)-mediated oxidation (Fujisawa *et al.* 2011; Iwamoto *et al.* 2010; Qin *et al.* 2011) and ionic liquids (Tsioptsias and Panayiotou 2008; Ma *et al.* 2011; Deng *et al.* 2009) have also been used for the preparation of nanosized cellulose.

The alternative method is the controlled enzymolysis of cellulose; this may be considered to be a green chemistry process having high developmental potential due to its mild reaction conditions and the renewable nature of cellulase. It is known that a complete cellulase system consists of three classes of enzymes. Of these, the cello-biohydrolases (CBH, EC 3.2.1.91) cleave cellobiosyl units from the ends of cellulose chains. The endoglucanases (EG, EC 3.2.1.4) cleave internal glucosidic bonds. Beta-glucosidase (EC 3.2.1.21) cleaves glucose units from cello-oligosaccharides. An enzymatic process to hydrolyze cellulosic materials can be accomplished through a series of reactions with these various enzymes. The cellulase not only gets into the amorphous domain of cellulose, but also acts on the crystalline domain because of the function of cello-biohydrolases (CBH). But the cellobiose formed in the reaction process can absorb on the activity centre of CBH and prevent the thorough enzymolysis (Zhao *et al.* 2003). This effect can be favorable for the controlled enzymolysis preparation of NCC. There are few reports on enzymolysis preparation methods of NCC. Hayashi *et al.* (2005) used cellulase to hydrolyze the microcrystalline cellulose of cladophorales to obtain the crystalline cellulose with nanometer scale. Satyamurthy *et al.* (2011) have prepared the cellulose nanowhiskers through the controlled microbial hydrolysis of microcrystalline cellulose with the cellulolytic fungus *Trichoderma reesei*; in this case 22% yield of NCC was obtained.

The present study focuses on natural cotton fibers pretreated with DMSO, NaOH, or ultrasonic waves, then hydrolyzed by the cellulase of *Trichoderma Viride G* to prepare NCC. The aim of doing pretreatment to the cotton cellulose is to increase the accessibility of the material to cellulase. The cellulase of *Trichoderma Vride G* has been studied intensively, produced commercially, and applied widely (Li *et al.* 1965; Hayashi *et al.* 1998; Zhou *et al.* 2008; van Wyk and Mohulatsi 2003). At present, most of the commercial cellulases are made from fungi, especially *Trichoderma, Aspergillus*, and *Penicillium* species, among which the cellulase from the *Trichoderma* has the highest activity and anti-metabolic repression ability. At the same time, the *Trichoderma* fungi are safe due to their non-toxic nature and can grow in diverse environments, from which the cellulase can be extracted easily. Therefore, in the research, the *Trichoderma Viride G* was chosen.

The as-prepared NCC samples were characterized by TEM, DLS, XRD, and FT-IR. The influences of the reaction conditions and pretreatment ways on the yield and morphology of NCC have been investigated. The changes of crystalline phases and chemical compositions during the enzymolysis were determined.

EXPERIMENTAL

Materials

The cotton fiber was provided by Anhui Xuelong Chemical Fiber Company. The cellulase (*Trichoderma Viride G*, FPA enzyme activity: 10685 u/g) was provided by Shanghai Kaiyang Biological Co., Ltd. The other reagents were of analytical grade.

Methods

Pretreatment of cotton fibers

DMSO and NaOH pretreatment: The cotton pulp was pretreated with DMSO or NaOH solution (5 mol/L) at 75 ^{o}C for 4 h and then washed with distilled water to a pH of 7.

Ultrasonic pretreatment: The cotton pulp was pretreated mechanically with an ultrasonic cell crusher (300 W, Sonics, USA) for 200 s with 5 intervals, then filtrated and used for the enzymolysis reactant.

Microscopic observation of the pretreated and non-pretreated cotton fibers

The pretreated sample was diluted to 0.05%-0.1% with distilled water. Then 2 to 3 drops of diluted solution were placed on the surface of a thin glass slide and covered with another glass slide. The microscopic observation was carried out with an optical microscope (BX51 Olympus, Japan).

Preparation of NCC suspension by the controlled enzymolysis method

Influence of reaction conditions on the yield of NCC: The dosage influence of cellulase on the yield of NCC was evaluated with 1.0 g of the ultrasonic treated cotton fibers. This was mixed with 30 mL of the buffer solution (HAc+NaAc, pH = 4.8) and different dosages of cellulase, then vibrated for 2 days at 45 ^{o}C. The dosages of cellulase were set as 10 FPIU/g, 13 FPIU/g, 16 FPIU/g, and 19 FPIU/g.

Influence of reaction times on the yield of NCC: 1.0 g of the ultrasonic treated cotton fibers was mixed with 30 mL of the buffer solution and 13 FPIU/g cellulase, then vibrated for the set time at 45 ^{o}C. The reaction times were set as 1 day, 2 days, and 3 days.

Influence of temperatures on the yield of NCC: 1.0 g of the ultrasonic treated cotton fibers was mixed with 30 mL of the buffer solution and 13 FPIU/g cellulase. This was vibrated for 2 days at the constant temperature, which was respectively set as 45 ^{o}C, 48 ^{o}C, 50 ^{o}C, 52 ^{o}C, and 55 ^{o}C. NCC suspension samples were obtained by centrifugation of the reaction product.

Influence of the manner of pretreatment on the yield of NCC

1.0 g of the cotton cellulose pretreated in different ways, 30.0 mL of buffer solution, and 13 FIPU/g cellulase were mixed into a conical flask, which then was put into the vibrating case at 45 ^{o}C for 2 days. NCC samples were obtained by centrifugation of the reaction mixtures. The supernatant NCC colloidal suspensions were dried in a vacuum drying apparatus to obtain the powder samples, which were used for analysis of XRD and FTIR.

Characterizations of NCC

A JEM-2010HR transmission electron microscope (JAPAN) was used to carry out the TEM examination. ZEN3600 laser scattering instrument (England) was used for the particle size distribution analysis. The X-ray diffraction spectrum was measured with the D/max-IIIA X-ray diffractometer (Japan), using dried powders as the analytic sample. The relative crystallinity was calculated from Formula (1) (Ludueña *et al.* 2011). Infrared spectrum was analyzed by the FTIR-7600 Fourier transform infrared spectrometry (Australia) with dried powdered sample in the range of 4,000 to 400 cm^{-1}. Pellets were prepared from the mixtures of the samples and KBr (1:100 in weight).

$$X_C = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \tag{1}$$

where X_c (%) is the relative crystallinity, I_{002} is the diffraction intensity of the (002) crystal face, and I_{am} is the diffraction intensity of amorphous domain.

Determination of the reducing sugar content and the yield of NCC

The reducing sugar content in the sample was determined by the 3,5-dinitro-salicylic acid (DNS) method. The absorbance of solution was measured at 550 nm. According to the standard curves, the reducing sugar content of the measured solution could be obtained. The total reducing sugar content was calculated with Formula (2),

$$W_1 = m \times \frac{V}{V_m} \tag{2}$$

where W_1 is the total reducing sugar content; m is the reducing sugar content of the measured solution; V is the total volume; and V_m is the volume of the measured solution. The yield of NCC could be obtained from Formula (3),

$$P_{NCC} = \frac{W - W_1 - W_2}{W} \times 100\% \tag{3}$$
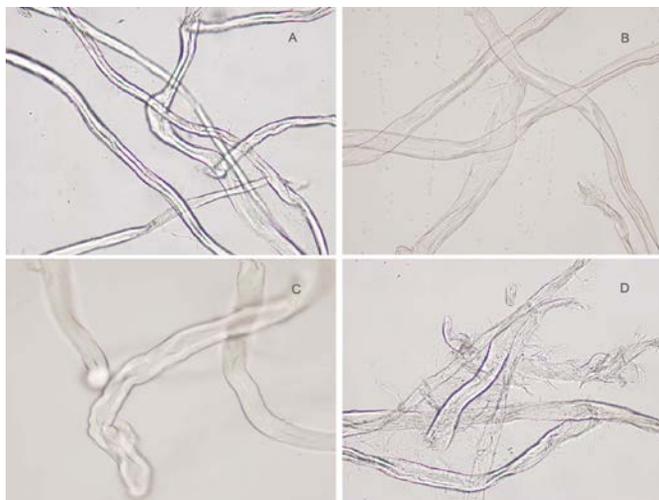
where P_{NCC} is the yield of NCC, W is the weight of the cotton fiber, W_1 is the total reducing sugar content, and W_2 is the weight of the non-reacted cellulose.

RESULTS AND DISCUSSION

Influences of Pretreatment Procedure on the Structure of Cotton Fibers

Figures 1A through 1D are photomicrographs of the untreated cotton fibers, DMSO-, NaOH-, and ultrasonic-pretreated cotton fibers, respectively. Obviously, DMSO and NaOH treated fibers swelled and increased in coarseness, but they were not broomed (did not branch off). In particular, the NaOH-pretreated sample displayed a larger increase in coarseness, plasticity appearance, and weak transparency, which indicated

that crystallinity decreased. The reasons may be that a part of the amorphous domain was partially destroyed. As a result of the breakdown of the amorphous structures, the hydrogen bonding and the crosslinking density of groups in the molecular structure were weakened so that large numbers of previously unexposed groups were made accessible as a result of the breakdown of the crystalline structures. The ultrasonic pretreated cotton fibers (Fig.1D) displayed obvious brooming and sub-wire. These effects were attributed to the ultrasonic vibrations splitting the amorphous domains and partially crystalline domains having defects.
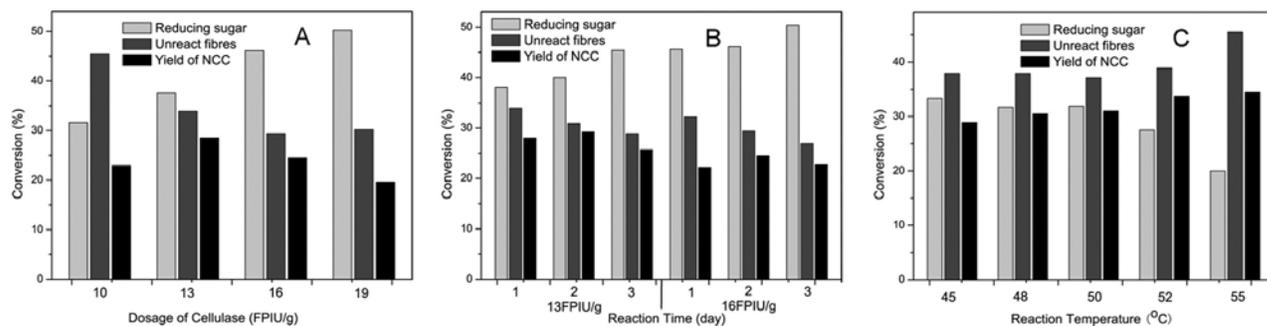

Fig. 1. Photomicrographs of un-pretreated and pretreated cotton fibers. A: untreated; B: DMSO pretreated; C: NaOH pretreated; D: Ultrasonic pretreated

Preparation of NCC
Influence of reaction conditions on the yield of NCC

In order to study the controlled enzymolysis process of the cotton, the component contents of the reaction mixture were determined with different reaction conditions. Results were obtained for the contents of reducing sugar, unreacted fibers, and yield of NCC. Figure 2A-C displays the influences of cellulase, time, and temperature on the enzymolysis products. From Fig. 2A, it is easy to understand that the reducing sugar content increased with the increase of cellulase dosage, but the highest yield of NCC corresponded to 13 FPIU/g cellulase dosages.

Fig. 2. Influences of reaction conditions on the yield of NCC. A: Dosages of cellulase; B: Reaction times; C: Reaction temperatures

The extension of reaction time was favorable to the formation of reducing sugar, as seen in Fig. 2B. In the research, 2 days were set to achieve the high yield of NCC and inhibit the excessive enzymolysis. Based on the yield of reducing sugar, the favorable activity temperature of the cellulase from *Trichoderma Viride G* was about 45, which was demonstrated in Fig. 2C. By contrast, higher temperatures gave rise to higher yield of NCC. This could be because the formation of NCC was feasible to dynamic control.

Influence of the manner of pretreatment on the yield of NCC

Figure 3 shows the influences of the different manners of pretreatment on the enzymolysis reaction. When comparing the pretreated cotton fibers with the nontreated sample, the percentage of unreacted fiber decreased, and the yields of NCC and the reducing sugar contents increased. In particular, for the NaOH pretreated sample, the percentage of the remaining fiber decreased from 66% to 31%, and the yield of NCC increased from 14% to 32.4%. The reason was that the NaOH-pretreated fibers swelled obviously, the middle order domain and low-ordered regions in the cellulose were dissolved out, and also the higher-ordered regions were converted into middle-ordered or low-ordered regions. As a consequence, the degree of polymerization decreased and the surface areas and accessibility increased; both inter- and intra-molecular hydrogen bonds were broken by the higher sodium hydroxide concentrations (5 mol/L), which were favorable to the enzymolysis (Goswami *et al.* 2009). The microscopic examination (Fig. 1) partially confirmed the influence of NaOH on the structure of cotton fibers. On the other hand, the alkali could remove the remaining lignin and hemicellulose in the cotton pulp, which inhibited the enzymolysis reaction (Yoshida *et al.* 2008).
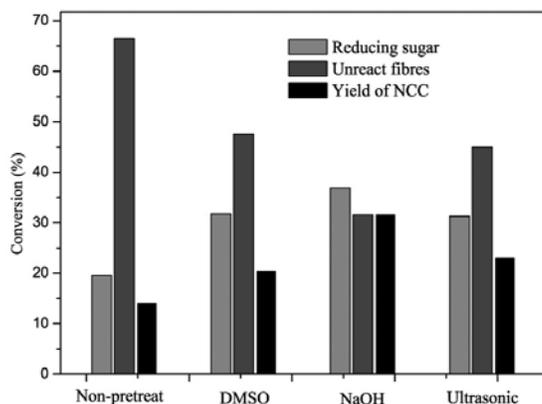

Fig. 3. Influence of pretreatment ways on the yield of NCC
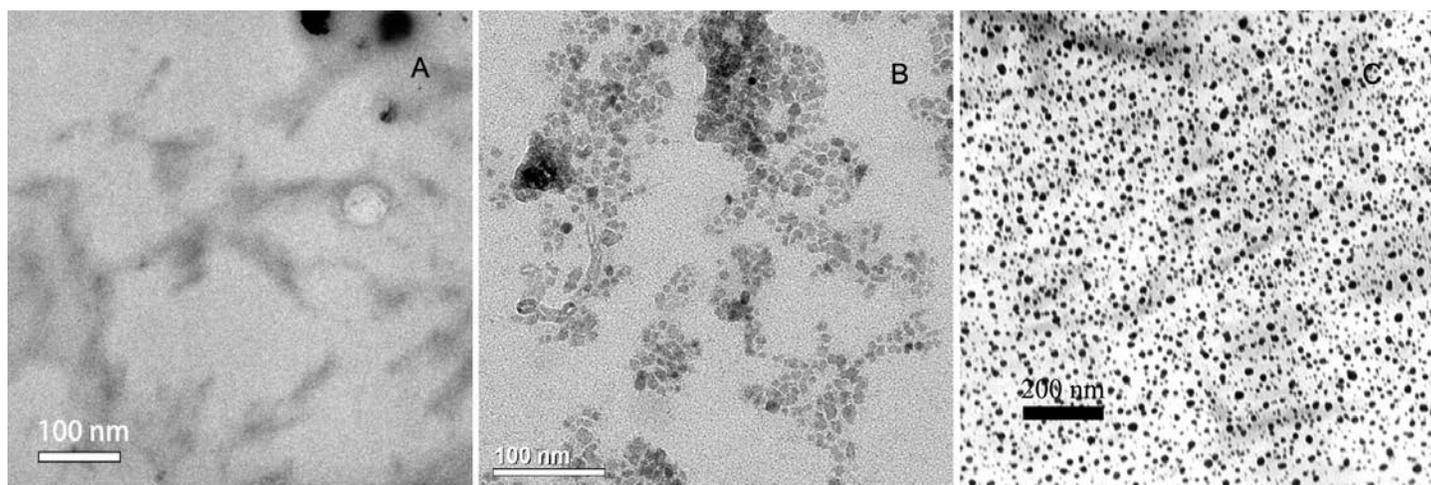
Characterizations of NCC
Morphology analysis of NCC

When the cotton fibers were pretreated in different ways, various changes of the structures would take place, which resulted in the different morphologies of the formed NCC particles. Figure 4 shows the TEM photos of NCC from the cotton fibers that had been pretreated in different ways. The NCC prepared from the DMSO pretreated fibers was in the form of rod-like grains with length of 70 to 280 nm and width of 10 to 40 nm, as shown in Fig. 4A. The NCC from NaOH and ultrasonic pretreated cotton fibers were

spherical grains with mean size about 20 nm and 6 nm, as shown in Fig. 4B and 4C, respectively.
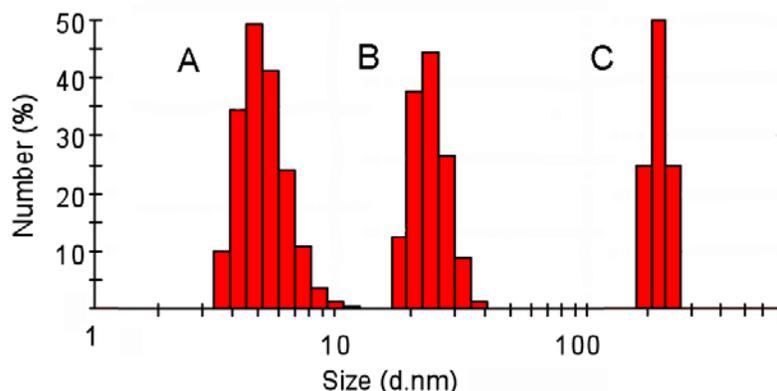
DMSO is a solvent that can solubilize lignin and hemicellulose on the surface of microfibers. It can diffuse into the cotton fibers along the axial direction of the cellulose molecular chain without causing horizontal damage, resulting in the swelling of celluloses, which enhances the enzymolysis of cellulose to form the strip nanosized particles. By contrast, NaOH or ultrasonic pretreatment can destroy the crosslinking of cellulose chains at the amorphous domains and the crystalline domains with defects, which may be the reason for the globularity of NCC obtained from NaOH or ultrasonic pretreated cotton fibers.

Fig. 4. TEM photographs of NCC prepared from pretreated cotton fibers. A: DMSO pretreated; B: NaOH pretreated; C: Ultrasonic pretreated

Particle size distribution analysis of NCC

The mean size of NCC obtained from ultrasonic pretreated sample was 5 to 6 nm in the number graph (Fig. 5A) and volume distribution graph (see Appendix), which was in agreement with the TEM observations, but the light intensity distribution graph (see Appendix) revealed the existence of bigger particles. The NCC obtained from the NaOH-pretreated fibers displayed two groups of peaks in the volume distribution and light intensity distribution graphs (see Appendix), which indicated that there were two kinds of particles in the system; one's average size was 25 nm (bigger than that in TEM image); the other was 250 nm, which were the aggregates. But in the number distribution graph (Fig. 5B), there was only one peak, demonstrating that the number of the aggregates was so small that it could be neglected. The laser scattering analysis displayed the NCC prepared from the DMSO pretreated fibers the size about 250 nm, hydrodynamic radius, which was the axial direction length, in fact. There weren't any other peaks in the distribution graphs of the number, volume, and light intensity (Fig. 5C and also see Appendix), which indicates that NCC particles were monodispersed with little flocculation.
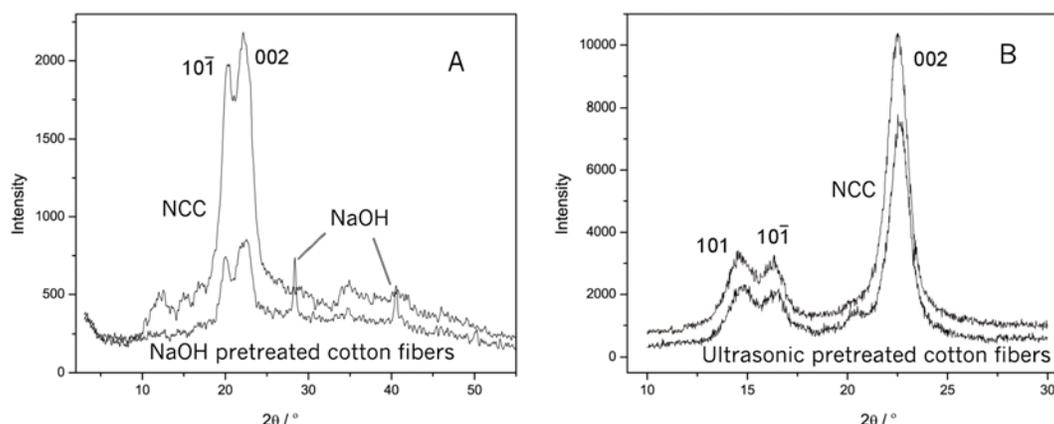
Fig. 5. Particle size distributions of NCC from pretreated cotton fibers. A: Ultrasonic pretreated; B: NaOH pretreated; C: DMSO pretreated

XRD spectra of NCC

The pretreatments with NaOH refer to the chemical process, in which the forming and breakage of the chemical bonds happened, and the DMSO and ultrasonic wave pretreatments are similar physical processes. In the study, the samples from NaOH and the ultrasonic wave pretreatments were used for the XRD and FTIR investigations. The XRD patterns of the NCC prepared from the NaOH and ultrasonic pretreated celluloses are shown in Fig. 6A and 6B, respectively. The patterns of pretreated celluloses were used as the contrast. When the cellulose was mixed into NaOH solution of 20% concentration, the amorphous and some of the crystalline domains would be infiltrated by NaOH, some of which remained in the NaOH-pretreated cotton fibers and contributed the sharper peaks in the XRD. The natural cellulose would be turned into hydrated cellulose, transforming the cellulose I into the cellulose II (Liu *et al.* 2011), which was maintained in the NCC after enzymolysis. The characteristic peaks at the (101) and (002) planes (2θ = 20.4º and 22.4º) became sharper and showed an increase of crystallinity, which was 78.1 according to Formula (1); this was higher than the crystallinity index of NCC from enzymolysis without pretreatment (Janardhnan and Sain 2011). The obvious reason was that the enzymolysis destroyed the amorphous domain in the cellulose structure. Because of the complexity of the cellulose structure, there existed some amorphous components in the crystalline region and between them, so the crystallinity of NCC could not reach 100%.
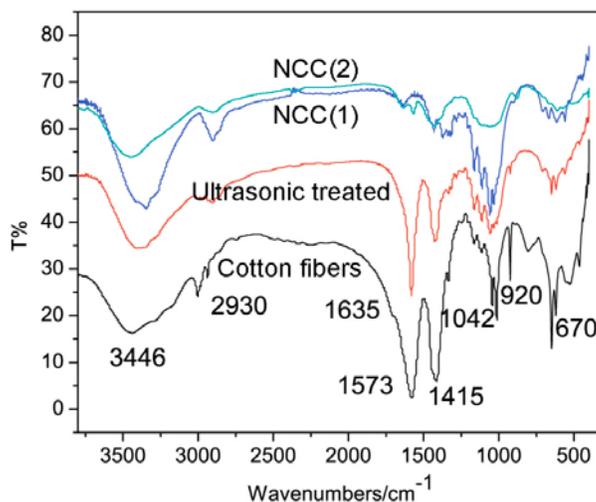
Both X-ray diffraction diagrams of cellulose powder and nanocrystals (Fig. 6B) showed three cellulose I characteristic peaks at 2θ = 14.7º, 16.4º, and 22.6º (Liu *et al.* 2011). The 22.6º peak of the (002) plane of cellulose nanocrystals became sharper, indicating higher perfection of the crystal lattice in the (002) plane than original cellulose. The peak for the (101) plane (2θ = 14.7º) also became more intense and separated from the 101(2θ = 16.4º peak for cellulose nanocrystals. The enzymolysis process of fibers with ultrasonic pretreatment did not change the crystalline phase of cellulose. So, NCC will maintain the crystalline phase of the original cellulose prior to pretreatment.

Fig. 6. XRD spectra of NCC and pretreated cotton fibers. A: NaOH pretreated cotton fibers and corresponding NCC; B: Ultrasonic pretreated cotton fibers and corresponding NCC

FT-IR spectra of NCC

Figure 7 shows the FT-IR spectra of natural cellulose (cotton fibers), ultrasonic treated cellulose, and NCC obtained from ultrasonic and NaOH pretreated celluloses. The broad bands in the 3650–3000 cm^{-1} region are attributed to O–H stretching vibrations, and the peaks at 2930 cm^{-1} correspond to C–H stretching vibrations. The 1635 cm^{-1} and 1573 cm^{-1} peaks are associated with the H–O–H stretching vibration of absorbed water and the aromatic stretching vibration of remaining lignin in the cotton (Qiu and Chen, 2008), respectively. The HCH and OCH in-plane bending vibrations gave rise to a band at 1415cm^{-1}, and the bands at 1042 cm^{-1} were caused by the stretching vibration absorption of C-O (Rosa *et al.* 2010). The absorption bands at 921cm^{-1} were the result of vibration absorption of glycosidic bond, and the C–OH out-of-plane bending mode around 670 cm^{-1} (Lu and Hsieh 2010). These peaks match well in both the spectra, and no significant difference could be observed, suggesting an unaltered chemistry of NCC prepared by enzymolysis of ultrasonic and NaOH pretreated cotton fibers.

Fig. 7. FT-IR spectra of NCC and cotton fibers. NCC(1): Prepared from ultrasonic pretreated cotton fibers; NCC(2): Prepared from NaOH pretreated cotton fibers

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CONCLUSIONS

1. The research demonstrated that in the case of cotton fibers pre-treated with DMSO, NaOH, or ultrasonic waves there was an increase of swelling and brooming, which was favorable to enzymolysis to prepare NCC.

2. The NCC prepared from the DMSO-pretreated fiber consisted of rod-like grains with 70-280 nm length and 10-40 nm width. Those from NaOH and ultrasonic pretreated cotton fibers were spherical particles with size 20 nm and 6 nm (TEM), respectively. The highest yield of NCC can reach up 32.4%.

3. The XRD and FTIR spectra indicated that the enzymolysis process of cotton fibers pretreated with ultrasonic or NaOH could not alter the crystalline phase and chemical composition.

4. In this paper, original work was reported for the enzymolysis of pretreated natural fibers with *Trichoderma cellulase* to prepare NCC, which is a method with outstanding advantages in terms of green and sustainable development. In the future, some important studies need to be completed, such as the purification of products and appropriate immobilization of cellulase.

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