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Preparation and Characterization of Nano Crystalline Cellulose from Bamboo Fibers by Controlled Cellulase Hydrolysis

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Abstract

The extracellular cellulase enzyme produced by *Trichoderma reesei* was used to prepare Nanocrystalline Cellulose (NCC) by controlled hydrolysis of bamboo fibers. The morphology of the prepared bamboo cellulose nanocrystals was characterized by field emission scanning electron microscopy and the crystallinity was measured by X-ray diffraction. The degree of polymerization was tested by automatic viscosimeter. The surface charge in suspension was estimated by Zeta-potential. The results showed that all NCC from bamboo fibers presented a rod-like shape, an average diameter of 24.7 nm and length of 286 nm, with an aspect ratio of around 12. The zeta potential of cellulase hydrolyzed NCC was 4 times lower than that of NCC prepared by acid hydrolysis process.

Keywords: Nanocrystalline Cellulose; Bamboo Fibers; *Trichoderma Reesei*; Cellulase; Characterization

1 Introduction

Cellulose, the most abundant biopolymer on earth, is a homopolymer of β-1,4-D-glucose molecules linked in a linear chain [1]. Cellulose microfibrils can be found as intertwined microfibrils in the cell wall (containing 500–15 000 glucose units depending on it source) [2]. As well as these microfibrils, there exist Nano Crystalline Cellulose (NCC) (also composed by cellulose) which are elongated and flat, a few hundreds of nanometers long, 10-20 nm wide and a few nm thick [3]. The NCC, also called as cellulose nanowhiskers, cellulose crystallites or crystals in literatures, are mainly produced by concentrated sulfuric acid hydrolysis of Microcrystalline Cellulose (MCC) whereby

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the presence of amorphous region is completely hydrolyzed to yield highly crystalline NCC. These NCC have received increasing attention due to their extraordinary mechanical properties such as high Young's modulus and tensile strength [4]. The Young's modulus of NCC is as high as 134 GPa while the tensile strength of the crystal structure was estimated in the range of 0.8–10 GPa [5].

The filamentous fungus *Trichoderma reesei* is one of the most efficient producers of extracellular cellulose enzyme. Cellulases are produced as multi component enzyme system comprised usually of three components that act synergistically in the hydrolysis of cellulose: endoglucanases (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and cellobiase (β-glucosidase, EC 3.2.1.91). The extracellular cellulolytic system of *Trichoderma reesei* is composed of 60–80% of cellobiohydrolases, 20–36% of endoglucanases and 1% of β-glucosidases [6]. The first two components act directly on cellulose yielding mainly cellobiose, cellotriose or cellotetraose as the reaction products. The cellobiose is then hydrolyzed to glucose by cellobiase. Though endoglucanases and cellobiohydrolases degrade soluble cellodextrins and amorphous cellulose, cellobiohydrolases degrade crystalline cellulose more efficiently. The oligosaccharides formed during the cellulose hydrolysis are believed to play important roles in the natural cellulase induction. So solid cellulose itself is often used as both the substrate and the source of inducers in fermentation process for cellulose production [7]. Besides, it is commonly understood that bamboo is a plant widely growing all over the world, whose lignocellulosic fibers have a great industrial potential [8].

During the traditional preparation process of NCC, the hydrolyzing agent, sulfuric acid introduces bulky ester groups onto the hydroxyl groups and stabilizing the NCC in solution by preventing its agglomeration [9]. However, the use of sulfuric acid has a number of important drawbacks such as corrosivity, surface modification of cellulose and environmental incompatibility. Apart from use in composites, NCC finds applications in health care like personal hygiene products, biomedicines, cosmetics and so on. NCC in its pure form is safe and biocompatible, but the traditional acid hydrolysis process inserts sulfate groups on the surface of NCC.

Similarly, the sono-chemical assisted hydrolysis of cellulosic materials for the production of NCC is highly energy intensive due to the predominating hydrogen bonding between the cellulose microfibrils. This interfibrillar hydrogen bonding energy (∼20 MJ/kg mol) has to be overcome in order to hydrolyze the cellulose [10].

The surface modifications in traditional acid hydrolysis process pose bio-compatibility problem, fungal degraded cellulose retains its original chemical nature. Cellulase enzyme produced by various microbes, with its proven biotechnological advances in various fields, will be of immense use in the production of NCC. Meanwhile, as a biological catalyst, action of microbial enzymes reduces the energy requirement for cellulose hydrolysis. Earlier work showed a significant reduction in energy consumption during refining process when cellulose is subjected to fungal pretreatment [11, 12]. Therefore, the present study was attempted to produce NCC by controlled hydrolysis of bamboo fibers using the extracellular cellulase enzyme of fungus *Trichoderma reesei* under submerged fermentation process. The obtained NCC was then characterized by Field Emission Scanning Electron Microscopy (FESEM), X-ray Diffraction (XRD), Degree of Polymerization (DP) and Zeta-potential.

The main outcome of this work provided a feasible way of producing NCC from bamboo fibers through enzymatic hydrolysis. This aspect is new as it offers a novel route to reduce energy consumption and increase environmental compatibility in producing these NCC materials.

2 Experimental

2.1 Materials

Native Moso bamboo were purchased from bamboo farm in Anji, Zhejiang province, China. *Trichoderma reesei* (ATCC 13631) was obtained from China General Microbiological Culture Collection Center, Beijing, China. Analytical grades of hydrochloric acid, sulfuric acid, sodium hydroxide, acetic acid and sodium hypochlorite were purchased from Sigma-Aldrich.

2.2 Preparation of Bamboo Fibers

Moso bamboo was cut with a FRITSCH Pulverisette mill, until fine particulate fibers were obtained. The fibers were then treated with a 4.0 wt% NaOH solution at 80 °C for 90 min under mechanical stirring. This treatment, repeated three times, allowed purifying cellulose by removing other constituents like lignin, hemicellulose, resin and so on present in the fibers. After each treatment, fibers were filtered and washed with distilled water until the alkali was completely eliminated. A subsequent chlorine bleaching treatment was carried out. The solution used consisted of equal parts of acetate buffer, 1.7 wt% aqueous chlorite and distilled water. The bleaching treatment was performed at 80 °C for 180 min under mechanical stirring and repeated four times. After each treatment the fibers were filtered and washed with distilled water. Table 1 presents the main components (including cellulose, hemicellulose and lignin) of the moso bamboo and the obtained bamboo fibers. The methods for testing the content of cellulose, hemicellulose and lignin are based on the TAPPI T 222om-88 standard.

Table 1: Main components of the moso bamboo and the obtained bamboo fibers

Samples	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Moso bamboo	47.5	23.1	27.2
Bamboo fibers	91.3	1.2	4.6

2.3 Preparation of Bamboo MCC

The MCC was prepared from the bleached bamboo fibres by conventional hydrochloric acid hydrolysis (4.0 mol/L HCl). This resulted in MCC with wide size distribution in the micrometer range. To have uniform size distribution, the MCC was sieved through sieve and the size range of 50–70 μm was selected for further work. The SEM images of the bamboo MCC used for the following cellulase hydrolysis were presented in Fig. 1.

2.4 Preparation of NCC by Fermentation Treatment of the Bamboo MCC

The 24 h inoculum of the fungus *Trichoderma reesei* was prepared in potato dextrose broth by inoculation of spore suspension ($\sim 3\times10^6$ spores/ml). The optimized concentration (5.0%) of inoculum was added in Mandel's medium having MCC as the sole carbon source in 250 ml

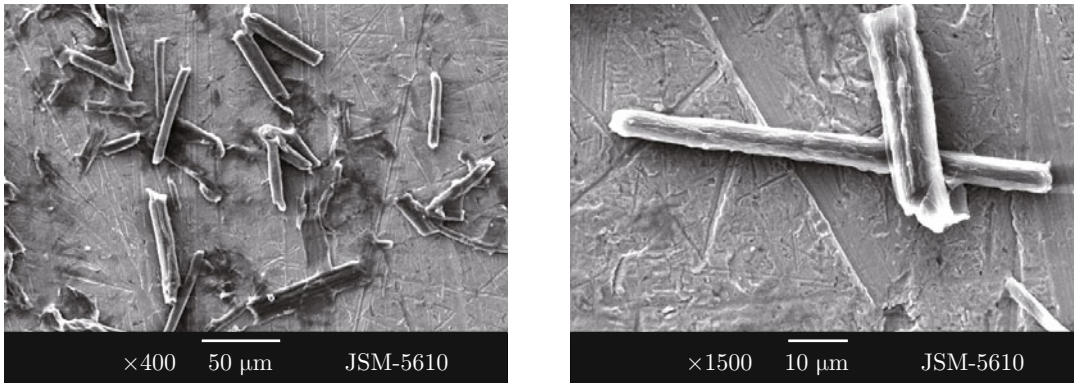


Fig. 1: SEM images of the prepared bamboo MCC used for the following cellulase hydrolysis

conical flask at 25 °C under shaking condition. After fermentation, the broth was centrifuged to remove particles larger than 1 μm [13]. The resultant supernatant was filtered through a 100 kDa ultrafiltration membrane by vacuum suction, and the NCC retained on the membrane was collected with a jet of ultrapure water and freeze-dried for further analyses. The cellulose content was determined by using the method as described by the literature [14]. Yield was calculated as the percent of cellulose content to that of initial MCC concentration.

For comparison, NCC was prepared by conventional process using 60 wt% of sulfuric acid hydrolysis of MCC at 45 °C for 1 h. The acid-hydrolyzed sample was washed with plenty of distilled water by repeating the centrifugation and dilution process until its pH was neutral, and then freeze-dried.

2.5 Cellulase Activity Analysis

The cellulase activity of the culture filtrate was determined by using the assay procedures described elsewhere [15]. The cellulase activity was measured by Filter Paper Assay (FPA), CMC assay and ρNPG assay. The reducing sugars were measured for FPA and CMC assay by Nelson Somogyi method [16], while ρ-nitrophenyl glucose was measured for ρNPG assay [17]. The activity is expressed as IU/ml of filtrate which corresponds to one μmol of glucose released per minute per ml.

2.6 FESEM Analysis

The morphology of the obtained NCC was checked using FESEM using a JEOL 6400F microscope operated with an accelerating voltage of 2 kV and a working distance of 4.4 mm. 50 μL sediment suspensions (0.01 wt%) was dropped onto clean silicon wafers followed by air-drying for 24 h and then sputtered with an approximately 6 nm layer of gold/palladium. The dimensions of the bamboo NCC were determined by using the UTHSCSA Image Tool with sample sizes of at least 250 fibers per SEM micrograph. Statistical analysis was performed using OriginPro 8, one-way analysis of variance.

2.7 XRD and DP Analysis

The diffractograms were recorded on a Rigaku diffractometer operating at 50 kV, 100 mA and

Cu Kα radiation. The samples were scanned in 2θ ranges varying from 5 to 40° (2° min^{-1}). The extent of crystallinity was estimated on the basis of areas under crystalline and amorphous peaks after appropriate baseline correction. DP was measured viscosimetrically in copper ethylenediamine solution and the obtained intrinsic viscosities were converted into the respective values of DP according to the method stated in the literature [18].

2.8 Zeta Potential Measurement

Potential charges which may be on the surface of NCC in pure water were measured with a Malvern 3000 Zetasizer. A NCC suspension (0.05 wt%), previously sonificated for 5 min, was prepared and analyzed to determine the zeta potential.

3 Results and Discussion

3.1 NCC Yield

The enzymatic hydrolysis of cellulose, particularly hydrogen bonded and ordered crystalline regions of MCC, is a very complex process. The extra cellular cellulase producing fungi *Trichoderma reesei* was chosen due to its low level of β-glucosidase enzyme, since our aim was to avoid complete hydrolysis to glucose. The use of MCC in fermentation process poses a problem of nutrient availability due to its insolubility. The exoglucanase component of cellulase enzyme is the active participant in hydrolyzing the crystalline region of MCC. So, we have optimized the process condition for increased production of exoglucanase by the *Trichoderma reesei*. This fungus, when grown using synthetic medium at 25 °C showed the highest exoglucanse activity.

During submerged fermentation, the FPA (0.61 IU/ml), CMCase (0.04 IU/ml) and β-glucosidase (60.36 IU/ml) activities were found to reach their maxima at 5 days of incubation after which there was a significant drop in their activities. The cellulose analysis by chemical method was used for the estimation of percent yield of NCC at different incubation periods. The yield of nanocellulose is given in Fig. 2. Till 3 days of fermentation, the yield was very low after which there was an exponential increase in the yield. The optimal yield of achieved NCC was 18% after 5 days fermentation. Then, the growth trend of NCC yield slowed down. The reason may be that the NCC yield pattern corresponds to the growth pattern of the fungus. After 5 days

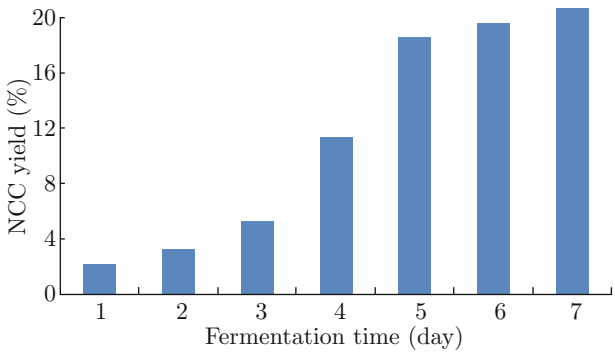


Fig. 2: Yield of bamboo NCC during the cellulase hydrolysis process

fermentation, the growth of the fungus decreased and stopped gradually. So it is not necessary to ferment after 5 days.

3.2 Morphology and Dimension

Fig. 3 shows the photographs and FESEM images of bamboo NCC prepared by acid hydrolysis (a) and cellulase hydrolysis (b). In both cases, the NCC particles had similar rod-like morphology. However, the NCC dispersion prepared by the acid hydrolysis had better stability than that prepared by the microbial hydrolysis, probably due to the fact that acid hydrolysis imparted to the NCC more anionic charges. This will be discussed later in more detail. The length and diameter of NCC were analyzed from the FESEM images using the UTHSCSA Image Tool and given in Table 2. All NCC prepared by cellulase hydrolysis presented an average diameter of 24.7 nm and length of 286 nm, with an aspect ratio of around 12. The NCC prepared by acid hydrolysis was narrow and sharper leading to high aspect ratio while that of cellulase hydrolysis prepared NCC had low aspect ratio. This resultant shape could be due to differences in the mode of action by acid and fungus. Since acid hydrolysis is completely a surface phenomenon, its action is influenced purely by crystallite size and shaking condition resulting in high aspect ratio. In case of cellulase hydrolysis, apart from shaking condition, two other parameters that influence the resultant shape of NCC are the effect of cellulose binding domain secreted by the fungus in loosening up the crystalline structure and the penetration of mycelium into the MCC. This result is supported by reduction in crystallinity as analyzed by XRD.

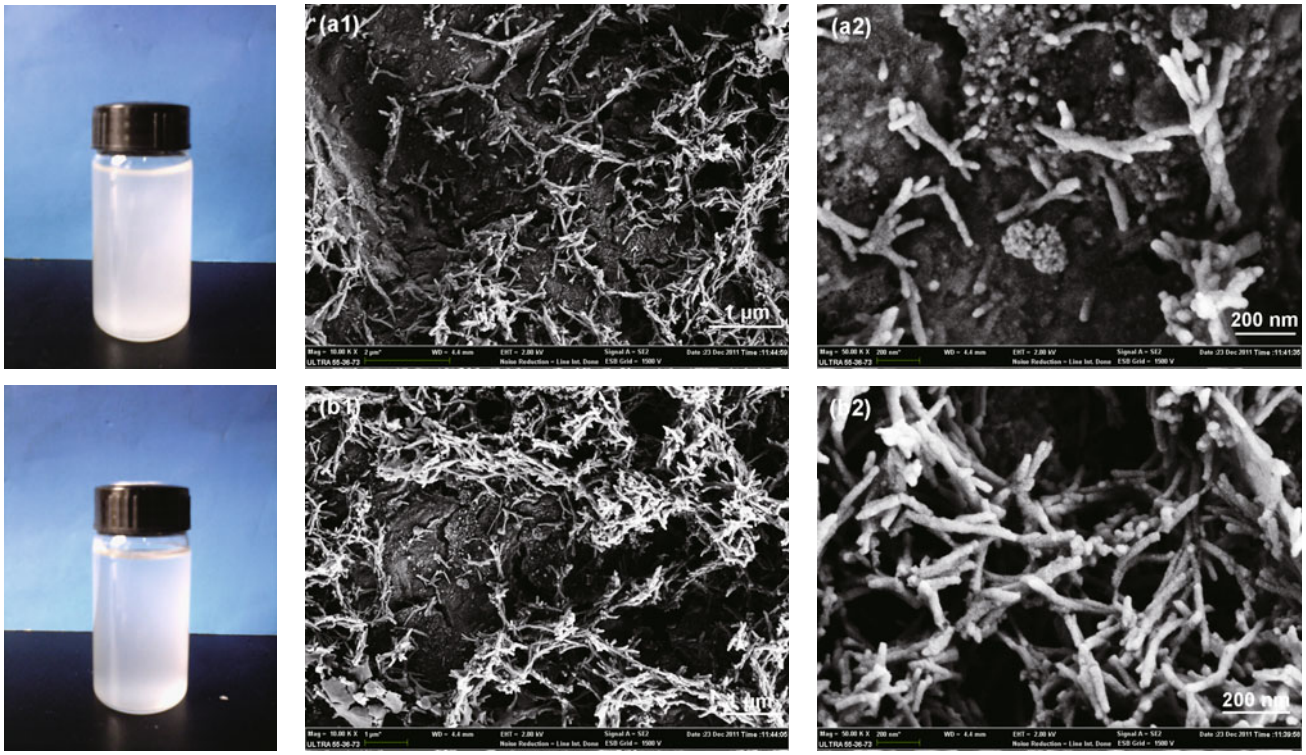


Fig. 3: Photographs of the NCC dispersion and FESEM images of the freeze-dried NCC particles prepared by acid hydrolysis (a) and microbial hydrolysis (b)

Table 2: Crystallinity, DP, morphology and dimension analysis of the obtained bamboo NCC

Samples	Crystallinity (%)	DP	Morphology	Dimension		
				Length (nm)	Diameter (nm)	Aspect ratio (L/D)
MCC	80.2	392.4	–	50–70 μm (by sieving process)		
NCC from acid hydrolysis	78.8	174.1	Rod-like	205	14.0	15
NCC from cellulase hydrolysis	72.0	211.5	Rod-like	286	24.7	12

3.3 Crystallinity and DP

The XRD analysis of bamboo MCC and NCC was done to determine the crystallinity, which is the important aspect to understand the moisture sorption, swelling ability and accessibility to cellulase enzyme. Microfibrils are arranged into lattices within the cell wall. This result in a highly crystalline structure that is insoluble in water and resistant to reagents. However, areas of the lattice contain unstructured regions that are caused by the presence of amorphous cellulose, or which arise as a result of small crystalline units being imperfectly packed together. There is a marginal reduction in crystallinity in NCC prepared by acid hydrolysis while significant reduction (∼8%) was noticed in case of cellulase hydrolysis prepared NCC, which might be caused by the penetration of fungal mycelium into MCC and the hydrolysis of crystalline region of MCC.

Table 2 shows the crystallinity and DP values of the obtained NCC. In the acid hydrolysis process, the DP of the obtained NCC reduced a half (to 174.1) compare with the DP of the original MCC (392.4). However during the cellulase hydrolysis process, the DP of NCC reduced gradually from fermentation day 1 to day 5, at 371.0, 350.4, 314.1, 259.7 and 211.5 respectively. Since the initial cellulose hydrolysis did not significantly reduce the DP, the yield of NCC was low during that period as shown in Fig. 2. With increased yield of NCC, the DP of the obtained NCC dropped rapidly especially from fermentation day 3 to day 5.

3.4 Colloid Stability

The physical mechanism that is used to stabilize most aqueous nanoparticles systems is electrostatic repulsion. The colloidal particles of interest are charged, resulting in their mutual repulsion at extended distances. Zeta potential is derived from measuring the mobility distribution of a dispersion of charged particles as they are subjected to an electric field. Fig. 4 shows the Zeta potential of the bamboo NCC suspensions prepared by acid hydrolysis and cellulase hydrolysis. The average zeta potential was −40.8 mV and −11.4 mV for the NCC prepared by acid hydrolysis and by microbial hydrolysis, respectively. It is known that the higher negative charge density of the NCC prepared by the acid hydrolysis was caused by the attachment of the sulfate groups to the NCC surface. These sulfate groups on the NCC may have negative effects on its biocompatibility. On the other hand, the NCC prepared by cellulase hydrolysis obtained the biocompatibility but flocculation appeared frequently.

4 Conclusions

NCC was successfully prepared from bamboo MCC by fermentation treatment with the fungi of

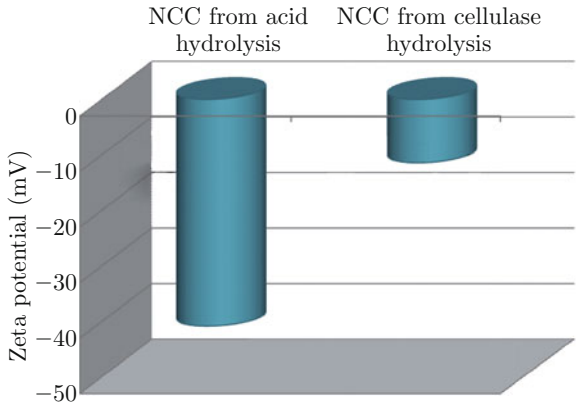


Fig. 4: Zeta potential of the bamboo NCC suspensions prepared respectively by acid hydrolysis and cellulase hydrolysis

Trichoderma reesei. After 5 days of fermentation, the NCC yield was about 18%. The NCC obtained by this method had lower crystallinity than that prepared by the conventional acid hydrolysis method, probably due to the penetration of the fungi into the ordered regions of the MCC during the incubation process. The reinforcing effect of NCC in polymer composites is mainly due to very strong and rigid three-dimensional network of hydrogen-bonded whiskers. The traditional method of acid hydrolysis resulted in sulfation on the surface of NCC. While the surface chemistry of NCC prepared by cellulase hydrolysis remains unaltered. This in turn will improve its performance as nanofillers in composites. Also this enhances the bio-compatibility of NCC and its scope in biomedical applications and pharmaceuticals.

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