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PREPARATION AND CHARACTERIZATION OF SPHERICAL NANOSIZED CELLULOSE BY ENZYMATIC HYDROLYSIS OF PULP FIBERS

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ABSTRACT

In this work, the pulp fibers were enzymolyzed to prepare the nanosized cellulose (NC). The as-prepared samples were characterized by optical microscopy, electron microscopy, and Raman spectra. The experimental results indicated that enzymatic hydrolysis of pulp fibers could produce the spherical NC with a mean particle size of about 30 nm, which had the excellent monodispersity and uniformity. When the concentration of complex enzymes was 20 u/mL (cellulase : xylanase = 9 : 1), the yield of NC was 13.6%. The single cellulase was used, even if the concentration and time reached up to 200 u/mL, only a mixture of strip and granular flocculation were obtained. The positive synergistic effect between xylanase and cellulase could be due to the enzymolysis of hemicellulose located on the cellulose microfibers to be favorable of cutting and splitting of the microfibers by the endoglucanase in cellulase. Otherwise, the additive copper sulfate could decrease the formation of reducing sugar effectively.

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Keywords: Wood fiber, Complex enzyme, Nanosized cellulose, Enzymatic hydrolysis.

1 INTRODUCTION

With the development of human society, all of the fossil energy and mineral resources stored in the earth will be depleted gradually. Therefore, it is an urgency to study the manufacturing and applications of derivatives from the renewable materials in order to reduce the heavy dependence on consumable resources. The wood fiber is the most abundant renewable resource on the earth, and its derivative, the nanosized cellulose, has the different morphological characteristics and the respective specialty, which can be divided into the nanofibrillated cellulose, nanosized whiskers, and nanosized particles. Those nanosized celluloses have many potential applications, including environmental management [1], drug delivery [2], solar energy conversion [3], human tissue engineering [4], flexibility electronic devices [5], and composite materials [6]. The preparing methods of nanosized celluloses include acid catalyzed hydrolysis [7]–[8], mechanical method [9], TEMPO (2,2,6,6-tetramethylepiperidin-1-oxy) oxidative method [10]–[11], ionic liquid method [12], binding method (binding of oxidative or enzymatic hydrolysis and mechanical) [13]. These processes have their own characteristics, but also some shortcomings. The acid hydrolysis process will produce a large amount of acid-containing wastewater, the mechanical methods consume too much energy, while the oxidation and ionic liquid method need very expensive reagent. Therefore, in addition to continuing to solve the existing obstacles, the study of new ways to prepare nanosized cellulose is also necessary [14].

The enzymolysis of cellulases can perform the deconstruction or cutting-up process of fibers at the mild reaction conditions. The catalytic enzyme is also a kind of renewable material. It is widely recognized that the enzymolysis of cellulases is just a formation reaction of glucose molecules from cellulose, which limits the researchers' thoughts that few people try to use the enzymatic hydrolysis to prepare the nanosized cellulose. In fact, there are the intermediate with nanometer scale during the enzymolysis of cellulose, by means of which the enzymatic hydrolysis preparation of nanosized cellulose may be realized.

The cellulase is a kind of multi-components mixture, including exo-β-1,4-glucanase (exoglucanase, CBH), endo-β-1,4-glucanase (endoglucanase, EG), and β-1,4-glucanase. The study [15] has shown that enzymolysis process can be divided into three parts: (1) the endoglucanase acts on the amorphous zone of cellulose fibers, hydrolyzing the glucosidic bonds randomly, and truncating the cellulose macromolecules to produce new polysaccharide chain ends;

(2) exoglucanase acts on the reducing or non-reducing end of the cellulose polysaccharide chain, hydrolyzing the glucosidic bond, and cleaving the cellulose from the end in cellobiose; (3) with endoglucanase and exoglucanase, produced soluble oligo-glucose is finally degraded to glucose by glucosidase. According to the enzymolysis mechanism, we only need to strengthen the process (1) while inhibiting the process (2) and (3), so as to realize the manufacture of nanosized cellulose by the enzymolysis of cellulose. Meyabadi and Dadashian have obtained spherical cellulose nanoparticles with 40–90 nm from the enzymatic hydrolysis of cotton fibers, in which the reaction time was 7 days [16]. Based on 60 min ultrasonic treatment every 12 h and 5 days hydrolysis time, the rod-shaped NC with width of less than 10 nm and length of 50–80 nm have been prepared by Cui and his co-workers [17].

We have reported that the pretreated chemically natural cotton fibers have been hydrolyzed by cellulase (Trichoderma Vride G) to prepare the nanosized cellulose [18], in which the reaction time was several days. In present article, the complex enzymes contained cellulose and xylanase was going to be used in the enzymolysis preparation the spherical nanosized cellulose with a mean particle size of about 30 nm, in which the pulp fiber (bleached Kraft eucalyptus pulp) was used as the source of cellulose, and the reaction time was shortened into tens of minutes.

2 EXPERIMENTAL

2.1 Materials

The pulp fiber was bleached Kraft eucalyptus pulp, while was refined in a PFI mill (Mark VI No. 621, Hamjern Maskin A/S Hamar Company, Norway) to a beating degree 40° SR. Cellulase (Aspergillus Niger, enzyme activity: 1.1010^5 u/g) and xylanase (Trichoderma Viride G, enzyme activity: 1.07×10^5 u/g) were supplied by Shandong Xindeli Biotechnology Co., Ltd, and the other reagents were of analytical grade.

2.2 Swelling treatment of fiber and characterization

2.2.1 Swelling treatment of fiber

2.0 g of pulp fiber and 40 ml of glycerin solution (50% concentration) were added into a beaker, and the mixture was stirred at room temperature for 3 h. After the swelling process was completed, the fiber was filtered out, washed with distilled water several times, and immersed in distilled water for using in the subsequent experiments.

2.2.2 Observation of pulp fibers

Observation of Glycerin Treated Fibers with Optical Microscope: The pretreated sample was diluted with distilled water to a concentration of 0.05% to 0.10%, and then 2 or 3 drops of pulp fiber solution were taken on a thin glass slide, and pressed with another glass slide, absorbing spilled water with filter paper. The swollen effect and surface characteristics were observed under a light microscope (Model BX51, manufactured by OLYMPUS).

Observation of Glycerin Treated Fibers with SEM: The pre-treated fiber sample was dried naturally and stuck to a double-sided conductive adhesive tape on the sample holder and gold-plated. SEM (model JEOL JSM-7600F, manufactured by JEOL Ltd.) was finished to examine the swelling effect and appearance characteristics of the pulp fiber.

2.2.3 Determination of fiber coarseness

100 ml of the sample solution with fiber concentration 2×10^{-3}% (w%) was used for determination of fiber coarseness at FS 300 model fiber analysis instrument (Metso, Finland).

2.2.4 Analysis of Raman spectra

Raman spectrum of fiber sample was done on the Lab RAM Aramis Raman spectrum instrument (HJY France). Pretreated sample was prepared according to the experimental 2.2.1 and untreated sample was the eucalyptus pulp with PFI grinding treatment and beating degree 40° SR.

2.3 Preparation and SEM observation of nanosized cellulose

2.3.1 Preparation of nanosized cellulose

2.0 g of pretreated pulp fibers were added into a beaker, and mixed with a certain amount of cellulase or complex enzyme solution. Then, the beaker was placed in a constant temperature water bath for stirring treatment at a set temperature. After the reaction was finished, the reaction mixtures were treated by pumping filtration with 1–3 μm pore size filter paper, the residue was washed and dried under vacuum state to determine the amount of residual fibers. The filtrate was filtered under vacuum through a 0.22 μm microfiltration membrane again, the filter cake was washed with water and carefully transferred to a beaker with a small amount of water to obtain the nanosized cellulose sample. The amount of reducing sugar was determined by DNS method after the microfiltration separation. The yield of nanosized cellulose was calculated according to equation (1).

$$Y\% = 1 - Y_1 - Y_2 \quad (1)$$

where, Y = the yield of nanosized cellulose (w%); $Y_1 = W_1/2$: the yield of reducing sugar (w%), W_1 reducing sugar amount; $Y_2 = W_2/2$: the yield of unreacted fiber (w%), W_2 unreacted fiber amount.

2.3.1.1 Preparation of nanosized cellulose from pulp fibers by enzymatic hydrolysis of cellulase

Influence of enzyme concentrations: the cellulase concentrations ranged from 20 u/mL to 200 u/mL, the reaction time was 5 h and the reaction temperature was 50 °C.

The effect of reaction temperatures: the enzyme concentration was 200 u/mL, the reaction time is 5 h, and the reaction temperature ranged from 25 °C to 60 °C.

Influence of reaction time: the reaction times ranged from 1 to 12 h, the enzyme concentration was 200 u/mL, and the reaction temperature was 50 °C.

2.3.1.2 Preparation of nanosized cellulose by enzymolysis of cellulase-xylanase complex

Influence of xylanase concentration: the cellulase concentration was fixed at 10 u/mL, changing the xylanase concentration from 2 to 14 u/ml, the reaction time was 5 h, and the temperature was 50 °C.

Influence of reaction temperature: the reaction temperature was changed from 25 °C to 60 °C, the concentration of complex enzyme was 20 u/mL (cellulase : xylanase = 9 : 1), and the time was 5 h.

Influence of reaction time: the reaction times was extending from 1 h to 12 h, the concentration of complex enzyme was 20 u/mL (cellulose : xylanase = 9 : 1), and the temperature was 50 °C.

2.3.2 SEM Observation of Nanosized Cellulose

SEM sample from the enzymolysis of single cellulase was obtained from following reaction conditions: the enzyme concentration was 200 u/ml, the reaction temperature was 50 °C, and the time was 5 h. SEM sample from the enzymolysis of complex enzymes corresponded to the reaction conditions were that the enzyme concentration was 20 u/ml (cellulase : xylanase = 9 : 1), the reaction temperature was 50 °C, and the time was 5 h. The as-prepared nanosized cellulose solution was dropped onto a slide glass. After natural drying (70% RH), the sample was fixed at the sample holder with double-sided conductive adhesive tape and gold-plated. The morphology and particle size of the samples were

observed with a scanning electron microscope (model: JEOL JSM–7600F, manufactured by JEOL Ltd.).

3 RESULTS AND DISCUSSION

3.1 Pretreatment and characterization of pulp fibers

The swelling of fibers could increase the accessibility of enzymatic hydrolysis reaction, and the general methods was the alkali immersing or the ultrasonic treatment [18]. In the study, the polyol glycerol was used as pretreatment agent. Figure 1(b) and 1(d) shows the morphologies of the pretreated pulp fibers under an optical microscope and the scanning electron microscope respectively,

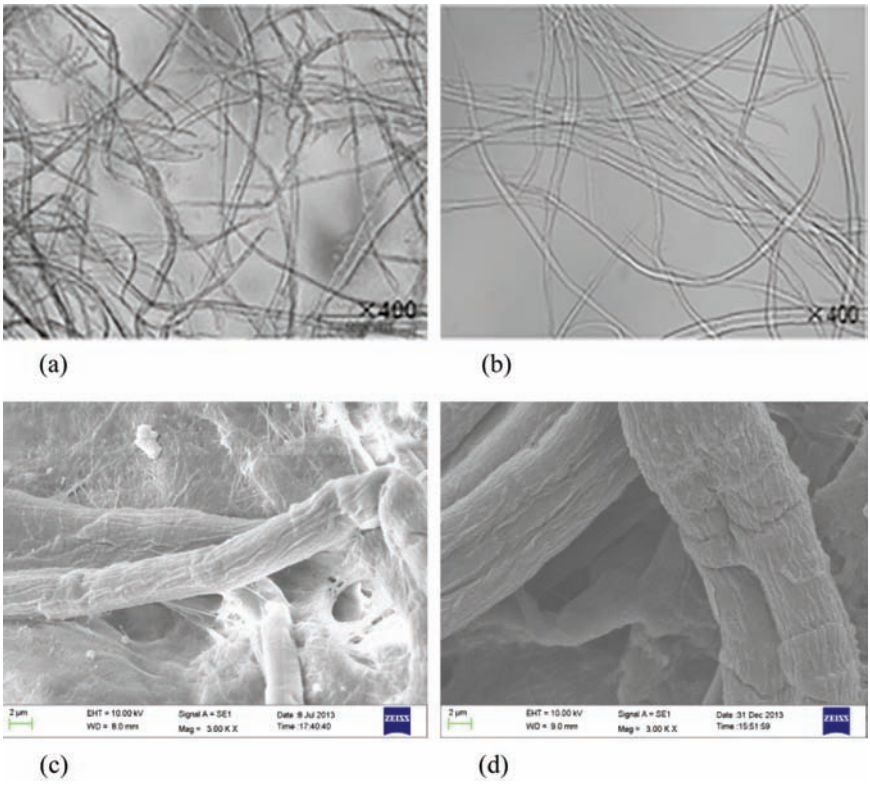

Figure 1. Morphology of Pulp fibers. (a) un-treated (Optical microscopy); (b) treated (Optical microscopy); (c) un-treated (SEM); (d) treated (SEM).

Figure 1(a) and 1(c) corresponding to the untreated pulp fibers. From those figures, it can be seen that the pretreated cellulose has thinner wall and smoother surface, and the fiber diameter increased significantly.

The reasons were surmised principally due to the formation of hydrogen bonds between glycerol molecules and molecular chains of the cellulose, which partly instead of the hydrogen bonds among the microfibers, the solvent molecules were embedded into the amorphous zone, and possibly even the crystallization zone, to make the fiber swell. The coarseness measurement of the fiber also confirmed that the pretreatment of glycerol could effectively swell the lignocellulosic fibers. As shown in Table 1, the diameter of the pretreated fiber was 223.7% of the original fiber.

The effects of swelling on the hydrogen band in the structures of pulp fibers can be reflected in Raman spectrum. Figure 2 showed the Raman spectra of the pulp fibers and the swollen ones. They had the same shape of absorption waves and peak position, except for the peak intensity. The peak of the swollen fiber at 3344 cm^{-1}, the stretching vibration of hydrogen bands [19], was evidently weaker than one of the original fiber, which showed the intramolecular hydrogen bond was partly destroyed, which may owe to the infiltration and substitution effect of the swelling agent glycerol.

Table 1. Fiber coarseness

Kinds	*Fibrils*	*PFI fibers*	*Swelling fibers*
Coarseness (mg/m)	0.076	0.093	0.17

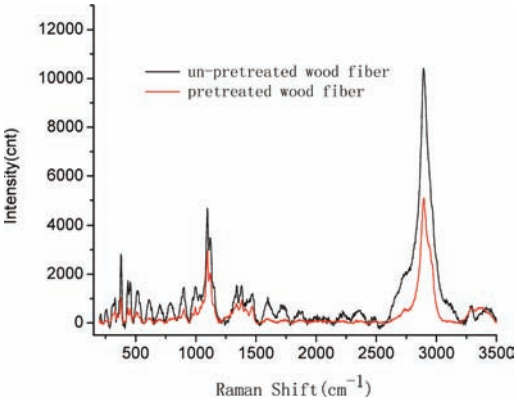

Figure 2. The Raman spectrum of the sample pretreated/un-pretreated by $CH_2(OH)CH(OH)CH_2(OH)$.

3.2 Preparation of nanosized cellulose from pulp fibers by enzymatic hydrolysis of cellulase

3.2.1 Influence of enzyme concentration

The nanosized cellulose had been prepared according to the describing in the experimental. The cellulase concentrations ranged from 20 u/mL to 200 u/mL, the experimental results were shown in Figure 3(a). It showed that with the increase of cellulase concentration, the yield of nanosized cellulose increased, but the content of reducing sugar also increased, which meaned the introduction of more by-product. When the cellulase concentration reached 200 u/mL, the yield of nanosized cellulose was 20.17%, reducing sugar content was 17.09%. Obviously, it was not a nice strategy to raise the yield of nanosized cellulose by increasing the cellulase concentration due to the byproduct reducing sugar content was too high, unless it was also the desirable product.

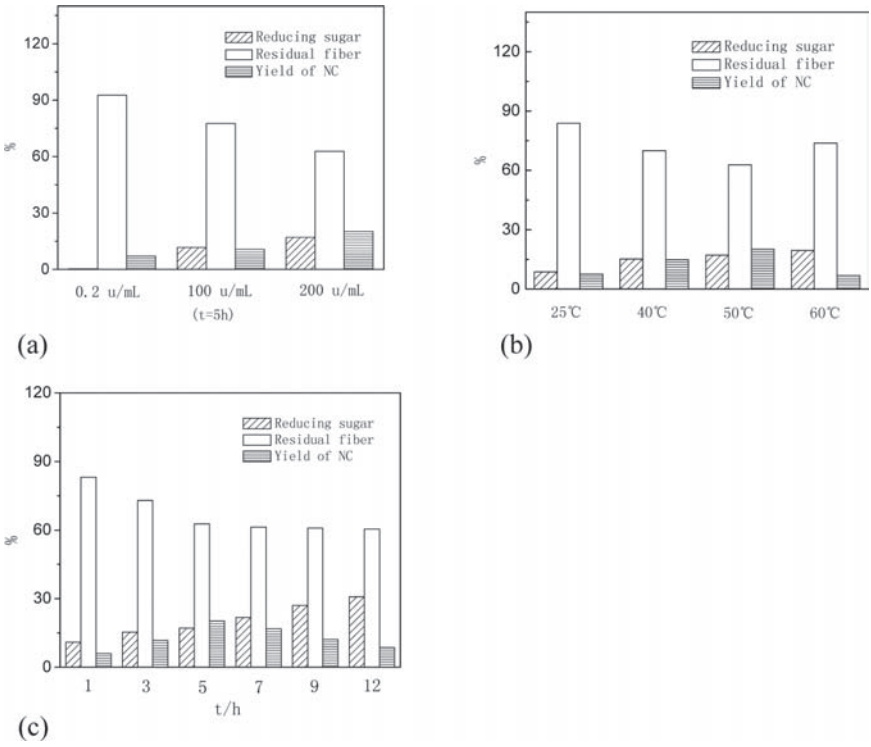

Figure 3. Effects of different experimental conditions on preparation of nanosized cellulose with hydrolysis of cellulase. (a) Enzyme concentration; (b) Reaction temperature; (c) Reaction time

3.2.2 Influence of reaction temperature

The effect of reaction temperatures on the yield was shown in Figure 3(b), and results indicated that the temperature rose from 25 °C to 60 °C, the yield increases, and the reducing sugar content also increased. At 50 °C, the yield of nanosized cellulose was the highest, when the reaction temperature went up to 60 °C, the nanosized cellulose formation was decline, but the reducing sugar content still increased. This was because that with the rise of temperature, the cellulase activity was lost, and the exonuclease in cellulase was more inactivated than the endonuclease. The formed nanosized cellulose was sequentially enzymolysized into reducing sugars. Therefore, when the reaction temperature rose to 60 °C, the yield of nanosized cellulose reduced but the amount of reducing sugar still increased. Most of the enzyme had a slow rate of enzymatic reaction at low temperature, but at relative higher temperature such as 60 °C, the enzymatic inactivation happened [20]. So, it was necessary to choose an appropriate temperature in the enzymatic hydrolysis process for the preparation of nanosized cellulose.

3.2.3 Influence of reaction time

The effect of different reaction times on the yield of nanosized cellulose was shown in Figure 3(c), the results showed the yield of nanosized cellulose firstly increased then decreased, as the reaction time was prolonged. The yield was the highest at 5 h. In the enzymatic hydrolysis of cellulose, the endonucleases cut and split the microfiber of cellulose at the amorphous region to form the nanosized cellulose. The longer the reaction time was, the more the yield of nanosized cellulose was. However, with the increase of the concentration of nanosized cellulose, the formation rate of reducing sugar was also increased, resulting in the consumption of the nanosized cellulose. Therefore, the enzymolysis preparation of nanosized cellulose has an optimal reaction time, the 5 h reaction time in this study was optimal.

3.3 Preparation of nanosized cellulose by enzymolysis of cellulase-xylanase complex

The hemicellulose content in the primary wall of the natural plant cell wall was even higher than the content of cellulose, and they tightly connected with the cellulose fiber, which increased the rigidity of the microfiber, greatly reducing the effectiveness of enzymolysis reaction [21]. Therefore, we tried to introduce xylanase in the process of enzymolysis preparation of nanosized celluloses to remove the hemicellulose in order to increase the enzymolysis reaction efficiency.

3.3.1 Influence of xylanase concentration

The cellulase concentration was fixed at 10 u/mL, changing the xylanase concentration in the experimental, the experimental results were shown in Figure 4(a). It can be concluded that the amount of reducing sugar was increasing with the increase of the complex enzyme concentration, but the yield of nanosized cellulose was unchanged basically. The excessive xylanase could not increase the nanosized cellulose, because it did not directly product from the enzymolysis of xylanase, only being used for remove of hemicellulose in favor of the enzymolysis of cellulase. Most of the reducing sugars may be contributed from the hydrolysis of hemicellulose by xylanase, so that the higher the concentration of xylanase was, the more the reducing sugar content was.

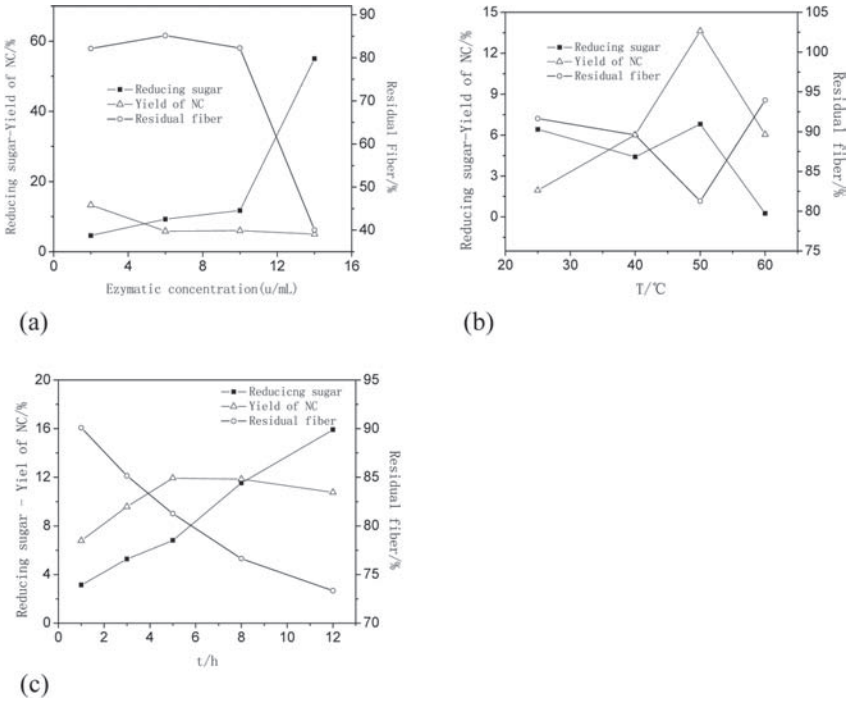

Figure 4. Effects of different experimental conditions on hydrolysis of complex enzyme: (a) Xylanase concentration in complex enzyme; (b) Reaction temperature; (c) Reaction time.

3.3.2 Influence of reaction temperature

The effect of reaction temperature on the yield of nanosized cellulose was shown in Figure 4(b). The yield of nanosized cellulose was the highest at 50 °C, when the temperature increased to 60 °C, not only the yield of nanosized cellulose decreased, but also the amount of reducing sugar decreased, and the unreacted pulp fiber increased. This indicated that the complex enzyme had been severely inactivated at this temperature, a little of cellulose was hydrolyzed.

3.3.3 Influence of reaction time

The effects of reaction times on the yield of nanosized cellulose were shown in Figure 4(c). As the time extending from 1 h to 12 h, the yield of nanosized cellulose increased then decreased, it reached at 12% at 5 h, the highest one. The cleavage of the endonuclease to the microfibrillar in the amorphous zone plays a major role in the formation of nanosized cellulose, but in the process the endoglucanase will undergo the denaturation and inactivation. On the contrary, the exoenzyme that was responsible for the formation of reducing sugars was rarely denatured and inactivated in its enzymolysis process [22], and with which the formed nanosized cellulose particles would be steadily translated into reducing sugars. Therefore, as the reaction time was overly extended, the endonuclease was gradually inactivated, not only the nanosized cellulose particles no longer produced, but also the formed ones were converted into reducing sugar by exonuclease, resulting into the decreasing of the yield of nanosized celluloses.

3.4 SEM observation of nanosized celluloses

3.4.1 As-Prepared Sample from the Enzymolysis of Single Cellulase

Figure 5(a) and Figure 5(b) were SEM figures of the sample prepared from the enzymolysis of cellulose with single cellulase, the enzyme concentration was 200 u/ml, the reaction temperature was 50 °C, and the time was 5 h. As shown in the figures, the product was spherical particles with particle size about 30 nm, being mixed with the irregular rod-like particles with several hundreds of nanometer length, which may be the intermediate state of the final spherical particles product. In the process for the preparation for the sample, the reaction conditions had been already optimized, and the enzyme concentration of 200 u/ml was very high, but there were still many irregular strip particles. It showed the incompleteness and low efficiency of the enzymolysis reaction, this was because the hemicellulose outside the microfiber restrains the cutting and splitting action of cellulase to the microfiber.

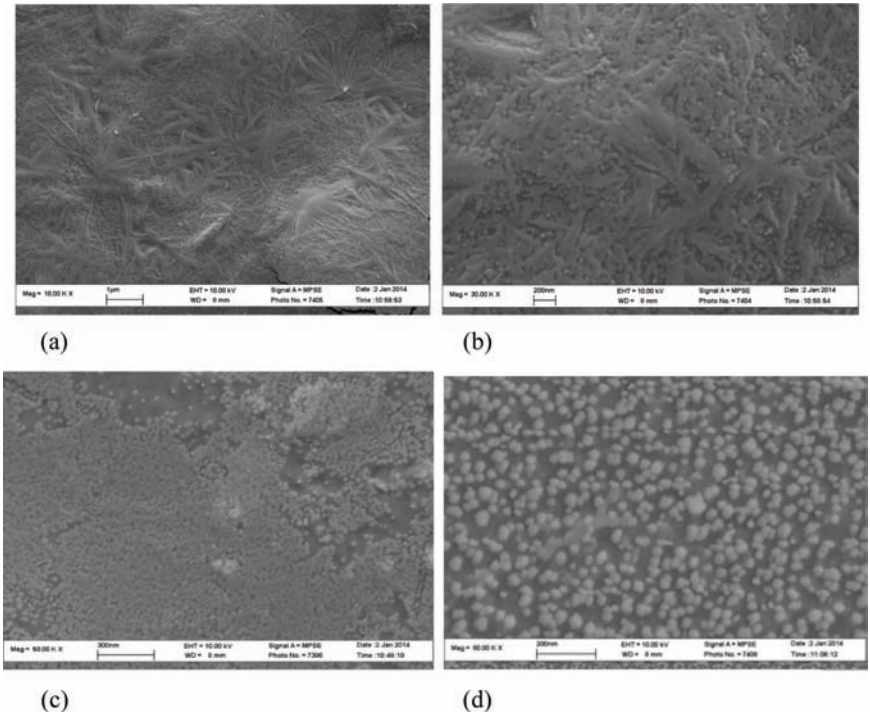

Figure 5. Electron microscopic picture of enzymatic hydrolysis products: (a)/(b) Enzymatic hydrolysis products of cellulases (30kx/50kx); (c)/(d) Enzymatic hydrolysis products of complex enzyme (50kx/80kx).

3.4.2 As-Prepared sample from enzymolysis of complex enzyme

Figure 5(c) and Figure 5(d) were SEM figures of the sample prepared from the enzymolysis of cellulose by the complex enzyme of cellulose and xylanase. The concentration of complex enzyme was 20 u/ml (cellulase : xylanase = 9 : 1), the reaction temperature was 50 °C, and the time was 5 h. As shown in the figures, the obtained spherical particles had a good monodispersity and uniform particle size with about 30 nm. Compared with the enzymolysis process of the single cellulase with higher concentration (200 u/ml), the enzymolysis of the complex enzyme had the faster reaction speed and higher efficiency at lower enzyme concentration (20 u/ml). Obviously, xylanase had hydrolyzed the hemicellulose outside microfibers to be favorable of cutting and splitting of the microfibers by the endoglucanase in cellulase, which confirms there was the synergetic action when the cellulase and xylanase was complexed to prepare the nanosized cellulose through the enzymolysis of wood cellulose.

3.5 Controlling of reducing sugar

The reducing sugar is the by-product in the preparation process of nanosized cellulose by enzymolysis, but it is just the material for the biomass energy, not waste. Zhu and colleagues [23] suggested a strategy of integrating the production of nanosized cellulose with sugar (material of biofuel ethanol) by enzymatic fractionation of pulp fibers. Of course, if the manufacture of nanosized cellulose is fundamental purpose, the yield of reducing sugar in the enzymolysis process should be lowed as far as possible. For the purpose, the activity of the exoglucanase that is mainly responsible for the formation of reducing sugar will be inhabited, meanwhile the impact on the activity of the endoglucanase should be minimized.

Metal ions are the general activity or inhibition agents of the enzyme. In the study, the used metal salts included $ZnSO_4$, $ZnCl_2$, $CaCl_2$, $CuSO_4$, KCl. The reaction conditions were set as the concentration of complex enzymes (cellulase : xylanase = 9 : 1) 20 u/mL, the reaction temperature 50 °C, and the reaction time 5 h. The results were shown in Figure 6, as shown in the figure, the used salts all can low the reducing sugar content, especially $CuSO_4$ from 6.8% to 2.53%, and the yield of nanosized cellulose did not decrease.

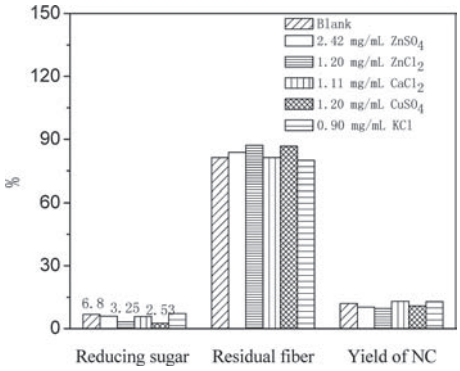

Figure 6. Influence of metal ions on the reducing sugar content in the enzymolysis of complex enzyme

4 CONCLUSIONS

The wood fiber can effectively be swelled by glycerol. The nanosized cellulose can be obtained from the enzymolysis of the swelled wood fiber by the single cellulase, but mixing the rodlike nanosized cellulose with several hundred

nanometers length, which can be regarded as the intermediate state of the enzymolysis process.

When the complex of cellulase and xylanase was used in the enzymolysis process of the wood fiber, the spherical nanosized cellulose can be obtained, which was about 30 nm particle size, and possessed the good monodispersity and the uniformity of particle size distribution. The synergetic action of cellulase and xylanase may be owed to that xylanase has hydrolyzed the hemicellulose outside microfibers to be favorable of cutting and splitting of the microfibers by the endoglucanase in cellulase.

The optimal reaction conditions are that the concentration of complex enzymes (cellulase : xylanase = 9 : 1) is 20 u/mL, the reaction temperature is 50 °C, and the reaction time is 5 h. If it is wished to the formation of the reducing sugar is decreased effectively, the additive copper sulfate may be an alternative.

ACKNOWLEDGEMENTS

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Transcription of Discussion

PREPARATION AND CHARACTERIZATION OF SPHERICAL NANOSIZED CELLULOSE BY ENZYMATIC HYDROLYSIS OF PULP FIBRES

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Most of the interesting properties in nanocellulose are derived from the fact that they are fibres with high aspect ratios as well as being small, so why did you want to make spherical nanocellulose instead of fibres?

Wen-Hao Shen South China University of Technology

The NC with different morphology have different characters. We wanted to use this NC as the raw material to conduct the researches about its application in a high level, such as the flexible screen, photo-energy conversion, and the flexible electronic device, maybe some sensors, etc.

Peter de Clerck PaperTec Solutions Pte Ltd

Thank you. That was a very interesting paper on a very difficult subject. My first question is on your technique. The cellulase you are using, does it have a CBD, a Cellulose Binding Domain, on the enzyme? Does it actually attach to the

cellulose, or is it free? So, when it attacks the fibre, does it stay in one place, or is it free to move with the solution?

Wen-Hao Shen

Do you want to know the details about the experiment? I think that the details about the experiments are explained in the proceedings.

Peter de Clerk

All right, I was asking about the characteristic of the enzymes you have used? Enzymes with the CBD, when they attack fibre, they stay in one place and work from thereon. Enzymes without a CBD are free to float around the system and to attack any free surface. In your work, because you are producing these spherical units, it seems likely that what is happening, is that the cellulase is attacking the amorphous regions of the cellulose and leaving you basically with the crystalline regions. Is this correct, is this what you find, that your microcellulose is basically microcrystalline cellulose and it's the amorphous regions that have been removed?

Wen-Hao Shen

Sorry, I can't follow you. Since Dr Chen is the corresponding author, is it possible we can discuss this later?

Peter de Clerk

Okay. Thank you.

Wolfgang Bauer Graz University of Technology

Did I understand correctly? That there was no mechanical treatment used in these experiments?

Wen-Hao Shen

Firstly, the swelling treatment and secondly the enzymatic hydrolysis reaction were used. No mechanical treatments were included in the experiments.

Zoheb Karim KTH Royal Institute of Technology

I don't have any question, but I am trying to give the answer to your question. You have correctly said that the enzyme had cut the amorphous part. Normally, the

enzyme used here is endoglucanase, so it will cut within the fibres, not from the outside of the fibres, so normally it is producing the crystalline part, as it will cut the amorphous part, that is why it is produced in the spherical, not in fibre form.

Peter de Clerck PaperTec Solutions Pte Ltd

Can I respond on that? An endoglucanase will react not from the end of the fibre, but from the end of the cellulose chain. So when you have amorphous regions in fibres, you also have ends of glucose chains in that region. It is not that the cellulose molecule extends the entire length of the fibre, you still have the free ends for endoglucanase reactions in the amorphous regions of the fibre, but thank you for the contribution.

Sara Ceccherini Aalto-University

I would like to know a couple of things. You work in very low consistencies and I wondered if you have carried out any experiment at high consistencies, such as above 15% or something similar? Have you ever tried to carry out the same experiment but at higher solid content of pulp?

Wen-Hao Shen

No, not yet.

Sara Ceccherini

And one more question. Did you utilize commercial enzymes, or did you produce your own?

Wen-Hao Shen

Commercial ones.

Sara Ceccherini

So were they cocktails, or were they pure?

Wen-Hao Shen

Pure.