Bamboo cellulose based single cell protein and nanocellulose by dilute sulfuric acid hydrolysis and fermentation

Ho Khac Ngoc[1], *A.P.* Rakhmatullina[1*], *Le Quang* Dien[2], *Nguyen Hoang* Chung[2], *Ha Thi* Dung[3], and *Tran Y Doan* Trang[3]

[1] Kazan National Research Technological University, 420015 Kazan, Russia
[2] Hanoi University of Science and Technology, 11615 Hanoi, Vietnam
[3] Hanoi University of Industry 11915 Hanoi, Vietnam

Abstract. The novel and facile multi-stage method was used for the conversion of bamboo into nanocellulose and single cell protein. Firstly, the bamboo chips were treated with sodium hydroxide solution followed by hydrogen peroxide bleaching for obtaining cellulose pulp with a brightness of 80% ISO. In the second stage, the obtained bleached cellulosic pulp was achieved with a dilute sulfuric acid and hydrogen peroxide followed by filtration for obtaining the sugar solution, which than was treated and used for *Candida utilis* cultivation, and nanocellulose, which than was submerged purification and refining for obtaining nanocellulose fibers. The optimal conditions of the yeast cultivation were dertermined for obtaining the single cell protein with protein content of approx. 49.5 wt.%. For isolation of nanocellulose with fiber diameter < 100 nm were proposed: concentration of hydrogen peroxide 0.25 wt.%, the concentration of sulfuric acid 0.75 wt. %, liquor to cellulose ratio 8 to 1, temperature 140°C, time 120 min. The characteristics of nanocellulose were studied by SEM, FTIR, and XRD.

1 Introduction

In recent years, cellulose from agricultural waste, industrial processing residues and energy crops has been widely used in construction, packaging, pulp production [1], textile manufacturing and wastewater treatment, etc. The fibers in natural cellulose were hydrophilic and prone to erosion in wet environments. Therefore, improving the hydrophobic properties of cellulose surface will provide more possibilities for packaging and construction materials [2]

As known the polymer produced from petroleum has a great impact on environmental pollution since it cannot be biodegraded after being discarded [3]. As renewable, biodegradable, low-density, non-toxic, green resources, biomass nanofiber materials with excellent mechanical properties have been extensively studied by scholars [4]. In recent years, researchers began to use bamboo processing residues as raw materials to prepare nano cellulose [5]. Bamboo has a short growth cycle and fast growth rate, so it is a very potential

* Corresponding author: rah-al@yandex.ru

biomass material, a kind of natural and abundant cellulose with excellent toughness, hygroscopicity, and high crystallinity [6], and it has a great potential for producing NCC [7].

In bamboo, cellulose accounts for 44% of the total bamboo, and lignin accounts for 20%. Bamboo mainly contains sclerenchyma fibers and parenchyma cells, which have different structures and compositions. Parenchyma cells make up 60% of bamboo processing residues produced in Vietnam every year, indicating that parenchyma cells are an excellent raw material for the preparation of nanocellulose [8]. Compared with wood, the parenchyma of bamboo has thinner cell walls, larger microfibril angles, lower lignification, and easy peeling of wall layers, which facilitates cell wall dispersion [9].

Different methods for producing nanocellulose have been attempted, such as acid hydrolysis, enzymolysis, and chlorine oxidation degradation (TEMPO), as well as a combination of two or several of these methods [10]. All these methods lead to different types of nanocellulose, depending on the raw material and pretreatment. Although various approaches of generating NCC have been used in recent years, the method of acid hydrolysis is well-known process used to dissolve amorphous regions effectively. The use of sulfuric acid to produce NCC imparts sulfate ester groups to the cellulose nanocrystal surfaces, resulting in electrostatically stabilized aqueous NCC suspensions. The new method for nanocellulose production using dilute sulfuric acid with adding of hydrogen peroxide was seccessfully applied for preparation of cellulose nanofibers from different biomass material, such as rice straw [11] and cassava bagasse [12]. The limitation of acid hydrolysis methods is that the cellulose hydrolysate has not been utilized yet. Therefore, the study of making use of them will improve the efficiency of nanocellulose production by limited hydrolysis.

This work aimed at developing a simple and feasible procedure to totally utilize carbohydrates of bamboo for producing valuable bioproducts, including xylose/, cellulose/nanocellulose. Lignin and silica were isolated from the black liquor via precipitation in a different procedure than the previously published works, making it more effective and cost-saving. The success of this work is believed to contribute significantly to agricultural waste management and the sustainable development of the country.

2 Experimental methodology

2.1 Materials

Bamboo was collected from the Thanh Hoa province. The material was taken randomly from 5 different positions of five chipped bamboo trunk, which were produced from approx. 10 kg of dried chips, which were collected and stored in a closed container at room temperature.

Candida utilis yeast (NBRC10707) was purchased from Biological Resource Center, NITE (NBRC), Japan. All of the chemicals used in this study were of analytical grades purchased from Sigma-Aldrich.

2.2 Methods

2.2.1 Alkaline cooking

Alkaline cooking was performed on 1L reactor with the bamboo at amount of active alkali (amount of NaOH + Na2S) of 22.5% w/w the oven dried mass of bamboo, and sulfur degree (Na2S/NaOH+Na2S ratio) is 25% weight percent, a solid-to-liquid ratio of 1-to-4. The reaction was heated from 70ºC to 150ºC within 85 min. The temperature was maintained at 150ºC for 120 min. The obtained cellulose pulp and black liquor (BL) were then discharged

from the reactor and separated by vacuum filtration. The cellulose pulp was thoroughly washed before being air dried for the determination of yield, cellulose content.

2.2.2 Cellulose and nanocellulose preparation

The unbleached cellulose pulp obtained from the alkaline cooking was bleached using a two-stage hydrogen peroxide bleaching process to reach a brightness of approx. 80% ISO.

For nanocellulose fabrication, 300 g of bleached bamboo cellulose was treated in 3.5L reactor with a mixture of hydrogen peroxide and sulfuric acid solutions at weight concentrations of 0.75% and 0.25%, respectively, a solid-to-liquid ratio of 1-to-8. The heating of reactor was conducted from 30ºC to 130oC-150ºC for 42-54 min. After hydrolysis, the product was filtered for separation of hydrolyzate and solid, which washed with water again, than treated with solution of sodium hydroxide and hydrogen peroxide before being mildly refined in a laboratory mixer for 2 min to form a nanogel. Nanocellulose was finally obtained by centrifugation at 10,000 rpm and the nanocellulose yield was determined.

2.2.3 Hydrolysate treatment and protein fermentation

The acid hydrolysate was neutralized with Ca(OH)2 and NH3 solutions, respectively, as described below. First, Ca(OH)2 (150 g/L aqueous solution) was added at a dosage of 4.0–4.1 g/L, at a temperature of 70–76ºC, to reach a final pH of 3.0–3.5. Ammonia (25 wt% aqueous solution) was subsequently added at a dosage of 2.9–3.0 mL/L to achieve a final pH of 4.2–4.3. The mixture was continuously stirred for 40 min for a complete neutralization. After this period, the solution was cooled to 35–40ºC and rested for 40 min to allow all of the precipitate settle. After separating the precipitate, the remaining liquid was aerated for 60 min with air at a flow rate of 20 L/h. Polyaluminium chloride (PAC) was then added at a dosage of 0.05 g/L and the solution was kept still for 70 min to ensure complete precipitation. Finally, xylose was filtered and stored for later fermentation.

Yeast cultivation was carried out in a 2-L Satorius Biostat® B Plus bioreactor. For 1 L of the xylose solution, 1.0 g of KCl, 8.3 g of Na2HPO4.12H2O, 6.0 g of urea, 0.75 g of yeast extract powder, and 1.8 g of MgSO4.7H2O were added. Candida utilis NBRC10707 with a biomass concentration of 3.0–3.5 g/L was used at a ratio of 40 mL of yeast per 1 L of xylose solution. The process was kept at 36–37ºC for 20 h, and the pH of the mixture was kept constant at 4.5–4.6 by using a 5% NH3 solution.

2.2.4 Products characterization

Glucose content of the hydrolysate was analyzed with an Agilent 1200 Series HPLC equipped with an RID G1328B detector and a Prevail Carbohydrate ES-GRACE 250×4.6 mm column. The stationary phase was hydrophilic polymeric gel with a particle size of 5 μm, pH 2–13. The mobile phase was a 30/70 deionized water/acetonitrile mixture that was filtered through a 0.45 μm filter and degassed. Standard sugar solutions with concentrations ranging from 0.5 to 5 g/L were prepared using deionized water. Other parameters were as follows: temperature of 30ºC, RID detector temperature of 35ºC, and flow rate of 1.5 mL/min. Data were analyzed with an Agilent 2D LC ChemStation Software (G2170BA) Version B 04.01 running on a Windows XP OS.

The morphological properties of nanocellulose samples were investigated using a JEOL JSM-7600F FESEM microscope. Structural characterization of freeze-dried cellulose and nanocellulose samples were investigated with a SHIMADZU FTIR 1S spectrometer.

The crystallographic structure of cellulosic materials was investigated using X-ray diffraction (XRD) analysis. The XRD patterns of the samples were collected using an X'pert

Pro (PANalytical) MPD diffractometer equipped with Cu Kα radiation operating at 35 mA and 40 kV. Scattered radiation was detected in the range of 5º 2θ to 70º 2θ at a scanning speed of 0.03º 2θ/0.5 s.

3 Results and discussion

3.1 Acid hydrolysis of bamboo cellulose for obtaining nanocellulose

The major chemical compositions of bamboo including cellulose, lignin, pentosan, and ash content were analyzed with TAPPI T17 wd-70, TAPPI T222, TAPPI T223, and TAPPI T211 om-93, respectively. The results showed that bamboo consists of 46.74% cellulose, 19.21% lignin, 24.03% pentosane, and 3.52% ash. After the alkaline cooking, the unbleached cellulose pulp has the yield of 46.51% (w/w) over oven dry bamboo. After bleaching, the bleached cellulose has the yield of 43.87% (w/w) over oven dry bamboo, which has the cellulose content of 85.76%, 11.24% pentosane, brightness 80.76% ISO, ash of 0.83%. Thus, up to about 46% of cellulose/hemicelulose was degradated during alkaline cooking and bleaching. This is the appropriate conditions, which have been determined by a series of experiments for cellulose, which has suitable properties for nanocellulose preparation.

Nanocellulose was produced by a limited hydrolysis method using dilute sulfuric acid and hydrogen peroxide. This is a novel method developed by the research group. It is well-known that the hydrolysis of cellulose with concentrated sulfuric acid (65–70ºC) [13] has several drawbacks in terms of the uniformity of the obtained nanofibers and acid recovery. Cellulose hydrolysis at high temperatures causes significant decomposition of cellulose and sugar, making nanocellulose purification more difficult. The concept of applying a limited hydrolysis process to cellulose using dilute sulfuric acid at a medium temperature is a suitable solution. The presence of hydrogen peroxide aids in the process of delaminating of the fibers, making purification of the resulting nanocellulose more feasible. Details on this method was reported by Dien et. al. (2019). It differs from the previously employed method (Rashid and Dutta 2020).

The appropriate cellulose hydrolysis conditions were discovered based on previous research results on untreated rice straw (Dien et. al. 2019) and conducting several experiments. The hydrolysis was performed using a mixture of 0.75% H2SO4 and 0.25% H2O2 solutions, with a solid-to-liquid of 1-to-8 at 130, 140ºC, and 150ºC for 2 h, followed by purification and refinement. Under these conditions, nanocellulose yields with average diameter of less than 100 nm as shown in Fig.1. The nanocellulose is formed in gel suspension and its viscosity is strongly influenced by the temperature of hydrolysis. The SEM of nanocelullose samples showed that at 130oC, the fibers were still clump, the number of nano-sized fibers was not much. At higher temperatures (140-150oC) all nanofibers were formed. However, the cellulose was strongly degraded at higher temperatures. The yield of nanocellulose (calculated over the dry mass of bleached cellulose) obtained from the treatments at 130, 140, and 150ºC is 71.28, 64.27, and 42.61% (w/w) over oven dry bleached cellulose, respectively. The hydrolysis of cellulose is obviously aided by high temperatures. Under comparable conditions, the decomposition of cellulose from bamboo was less stronger than that of rice straw (Dien et al. 2019). Therefore, the temperature condition of 140oC can be selected as appropriate.

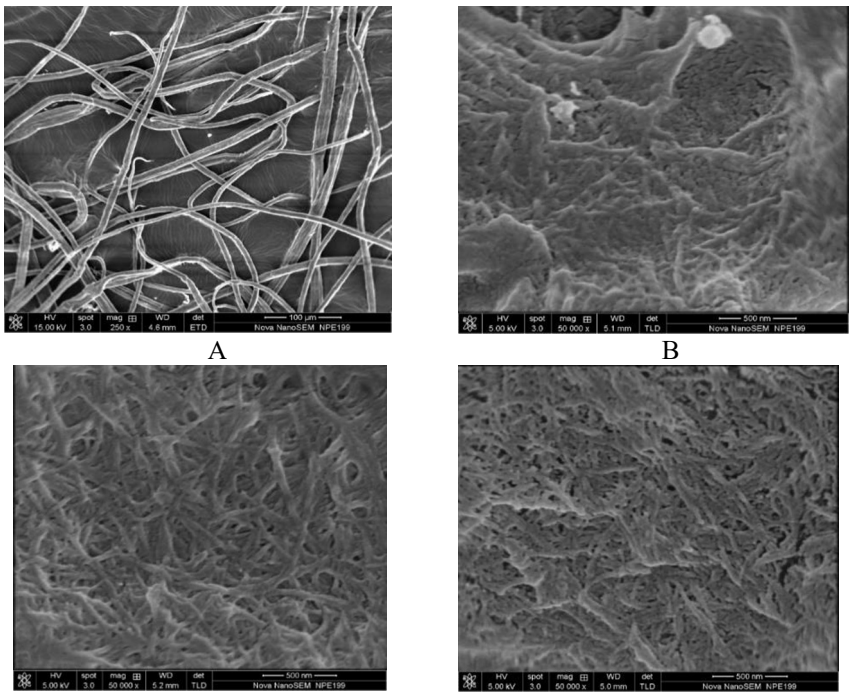
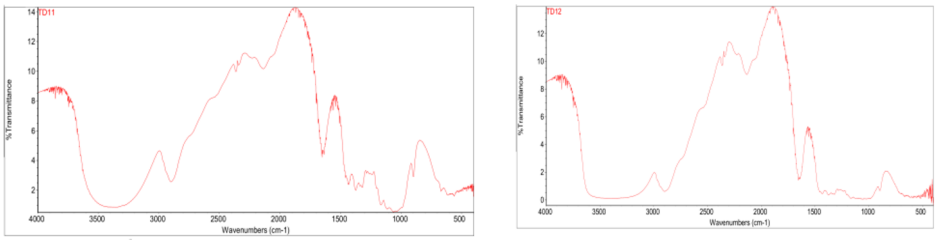


Fig.1. SEM images of bleached cellulose (A) and nanocellulose obtained at 130ºC (A), 140ºC (B), 140ºC (C) taken 50,000x magnification

The hydrolysate from hydrolysis of 300g bleached cellulose at 130, 140 and 150oC was 1620, 1687 and 1760 mL, respectively. Thus, approx. 30 % of sugar solution was unrecovered. The content of glucose in the hydrolysate at these three temperature levels, on the other hand, was 24.75, 28.4, and 34.61 g/L, respectively. Apparently a large amount of glucose was degraded into other products. The content of xylose in the hydrolysate at temperature of 130oC was 2.74 g/L. In addition, xylose was not found in the hydrolysate from hydrolysis at the 140 and 150oC. It showed that the pentosane derived xylose was fully degraded during hydrolysis at temperatures 140-150oC.

These glucose-containing hydrolysates were utilized to cultivate Candida utilis yeast according to the procedure described in Section 2.2.3, with promising results.

FTIR spectra of bleached bamboo cellulose and the obtained nanocellulose (Fig. 2) showed that the virgin bleached cellulose and derived nanocellulose had nearly identical structures. This demonstrated that the hydrolysis had no significant effect on the cellulose structure. As a result, the formation of nanocellulose fabrication is regarded as a success. The FTIR spectrum of NCC showed broadening of the OH absorption band at 3341 cm–1 shifted to 3411 cm–1 not only because of sulfuric acid hydrolysis, but also because of water adsorption. What's more, the broadening of the absorption band at 3339 cm–1 was attributed to the presence of the amorphous fraction of the cellulose [14].



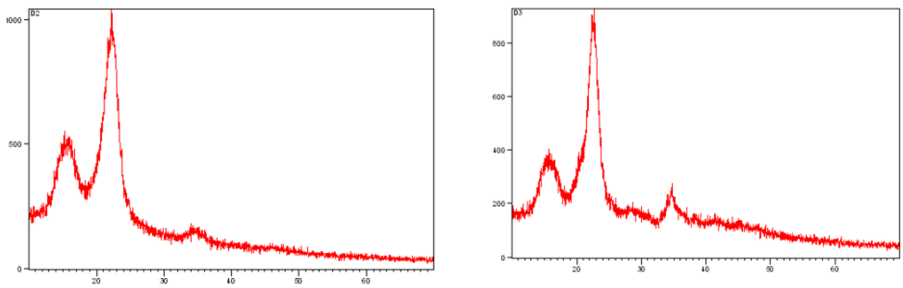
A B

Fig. 2. FTIR spectra of bleached cellulose (A) and nanocellulose obtained at 140ºC

Figure 3 depicted XRD patterns of bleached bamboo cellulose and derived nanocellulose. In both cases, the typical structure of cellulose-I is indicated by the two strong peaks observed at 2θ = 16.8º and 22.5º, which are attributed to (200) and (110) crystallographic planes, respectively. This demonstrated that the cellulose structure was unchanged after the treatments. The crystalline index (IC) of cellulose or nanocellulose was calculated according to Equation 3 based on their XRD diffractograms [15].

$$I_C(\%) = \left[\frac{I_{200}-I_{110}}{I_{200}}\right] \times 100 \quad (1)$$

where I_{200}, I_{110} are the intensity values of the peaks at 2θ = 22.5º (for 200 plane) and 16.8º (for 110 plane), respectively. It is noticed that (200) peak denotes both crystalline and amorphous regions, while (110) peak represents amorphous area.



A B

Fig. 3. XRD spectra of bleached bamboo cellulose (A) and derived nanocellulose (B)

According to Equation 1, the crystalline index increased from 62.01% for bleached bamboo cellulose to 74.31% for derived nanocellulose, which was higher than that of bamboo nanocrystalline cellulose prepared by using concentrated sulfuric acid 48% w/w at 50oC [14].

3.2 Hydrolysate treatment and protein fermentation

Acids that are normally formed during the hydrolysis process, such as formic or acetic, are not detected in the hydrolysate. Total amount of the sugars is significantly lower than the data published by Zhang et al. [16]. In their study, D-xylose was obtained with the purification yield of 71.63% after 2 h of hydrolysis with a 4% H2SO4 solution at 130ºC. In another work, when xylose was used for a similar purpose of fermentation [17], the hydrolysis was carried out at a higher temperature. Hydrolyzing with sulfuric acid at concentrations ranging from 0.6–6.0% for 12–24 h at 128–162ºC [18] resulted in a hydrolysate containing glucose, fructose, rhamnose, formic acid, and acetic acid. Apparently, the mild hydrolysis in this current work appears to produce a sugar solution that is better

suited for fermentation. As the strategy of our research, the use of sulfuric acid provides reliable efficiency and low toxicity [19].

The hydrolysate detoxification was conducted to achieve a bio-quality sugar solution suitable for the yeast development. The nutrients formula was investigated and adjusted from previous research in both laboratory and industrial scales [20]. Candida utilis yeast was cultivated in a bamboo derived glucose solution with the concentration of 28.4 g/L. The cultivation conditions and nutrients formula were described previously in Section 2.2.3. The medium had a pH in the range of 4.6–4.8, ensuring good development of the yeast and no residue sugar found after 20 h of hydrolysis (Fig. 4). Using an excess amount of nutrients allowed the fermentation to proceed steadily and effectively. This is a novel contribution to yeast cultivation on sugar-containing hydrolysate compared to the process that requires pH adjustment with NaOH or NH3 solutions Magalhães et al. [21-23] . This method can be applied to other sugar solutions and yeast species. The biomass concentration of the yeast reaches a maximum of 14.6±0.2 g/L, corresponding to an average yield of 51.4% over the sugar amount. After 18 h of cultivation, the yeast contains 49.51% crude protein, 0.29% crude fibers, and 10.26% total ash, which can be used as food additive for animals.

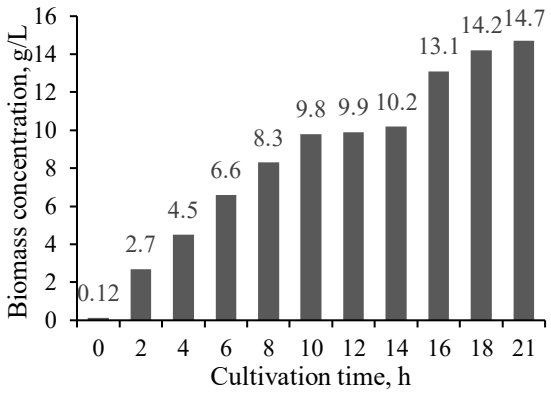


Fig. 4. Effect of Cultivation Time on Biomass Concentration

4 Conclusion

The new promising method was established for the complete conversion of bamboo cellulose using dilute acid hydrolysis of cellulose for nanocellulose production followed fermentation of cellulose derived sugars for single cell protein.

Dilute acid hydrolysis is a cheap and feasible method for nanocellulose production. nocellulose. Modified limited hydrolysis by dilute sulfuric acid with hydrogen peroxide improved the nanocellulose purification, while fermentation of cellulose derived sugars into single cell proteins makes the process more feasible on an industrial scale, as it will utilize the sugar solution compared to concentrated acid hydrolysis.

Nanocellulose from bamboo has higher crystallinity than same from hardwood, rice straw or sugarcane bagasse.

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