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Short communication

Feasibility of nanocrystalline cellulose production by endoglucanase treatment of natural bast fibers

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ABSTRACT

Whereas straw management presents a continuing challenge among farmers, tremendous opportunities may exist within the natural fibers in terms of value- added products or chemicals. This research provides the first assessment and demonstration of feasibility of an enzyme-assisted production of nanocrystalline cellulose (NCC) from hemp and flax fibers. A newly cloned endoglucanase (AoEG), derived from *Aspergillus oryzae* and characterized to be a thermostable enzyme with a half-life of 50 h at 50 °C was used for the hydrolysis. Atomic force microscopy (AFM) imaging of NCC produced from acid swollen cellulose and flax fibers indicated that they form aggregated network, showing rod-like nanofibrils of about 10 nm in height, and 200 nm in length. The yield of NCC using physical pretreatment only or combined physical–chemical pretreatment was compared. The highest yield of NCC was obtained under the conditions of 300 mg of flax fiber treated by 100 IU enzyme at 50 °C for 24 h after pretreatment of the fibers by sonication-microwave in 2% NaOH solution.

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1. Introduction

The objectives of this work are threefold: first, to provide proof-of-concept of production of nanocrystalline cellulose (NCC) from natural bast fibers such as flax and hemp; secondly, to produce NCC using enzymatic hydrolysis. These two goals are largely driven by the need to seek cleaner and less energy-intensive processes for bioproduction (OECD, 1998) and to find new biomaterials, alternative products or chemicals that can be extracted from the renewable, low-cost and abundantly available natural bast fibers to offset the economics of straw management (Mohanty et al., 2002; Vaisey-Genser and Morris, 2003; Ranalli and Venturi, 2004). The third goal is to assess the yield and characteristics of the NCC produced after physical pretreatment only or combined pretreatment processes to demonstrate the feasibility of bioproduction.

NCC, also known as cellulose nanocrystals, cellulose nanoparticles or cellulose whiskers and variations thereof, refer to those nanomaterials that are typically 2–6 nm wide and 50–500 nm in length. They have become highly desirable engineering and reinforcement materials globally due to key features that include low density (1.6 g cm^{-3}) and high mechanical strength (modulus of 110–220 GPa) that result in a specific modulus (modulus/density) greater than those of steel, concrete, glass and aluminum. Besides, its high aspect ratio (length/diameter) is important for stress transfer in a biocomposite setting (Siqueira et al., 2010; Habibi et al., 2010; Moon et al., 2011; Klemm et al., 2011 Brinchi et al., 2013). More recent applications of NCCs include enzyme immobilization (Mahmoud et al., 2009) and a delivery vehicle for drugs (Jackson et al., 2011).

Present methods of NCC production include acid treatment, typically sulfuric acid (63.5%, w/w), various physical and mechanical processes, e.g., high-pressure homogenizers, cryocrusher, microfibrillation by super-grinder, microwave (Siró and Plackett, 2010), and more recently use of a strong oxidizing agent (Leung et al., 2011), The use of enzymatic hydrolysis has been rather limited (Henriksson et al., 2007; Pääkkö et al., 2007; Filson et al., 2009; Brinchi et al., 2013). Feedstocks for these enzymatic processes so far have made use of the more readily available cellulose materials such as microcrystalline cellulose from cotton fibers (Satyamurthy et al., 2011), bacterial cellulose (George et al., 2011)

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or crystalline cellulose powder (Watanabe et al., 2011). Readers are referred to the accompanying electronic supplementary material in Appendix A for additional references. In this study, we present the first demonstration of enzyme-assisted production of NCC from flax and hemp bast fibers.

2. Materials and methods

2.1. Source of enzyme, production and characterization

For a complete description of cloning of the potential endo-1,4-β-glucanase (AoEG) encoding gene from *Aspergillus oryzae* (GenBank: BAD72778.1, NCBI, USA), the construction of recombinant clone for expression in *Pichia*, purification of the enzyme and assays, see the Supplementary data in Appendix A.

2.2. Fiber materials and pretreatment by mechanical and chemical process

Flax and hemp fibers were purchased from Biolin Research Inc. (Batch no. RCF 2008, Saskatchewan, CA). Whatman filter paper (Ashless Filter Aids #1700-025, Whatman, USA) was used as a positive control for cellulose. Acid swollen cellulose (ASC) was prepared by treating Avicel PH-101 (Fluka Chemical Group, Cat #1363) with phosphoric acid (Klyosov and Sinitsyn, 1981). The final ASC product was lyophilized.

After washing with distilled H_2O (dH_2O) and drying at 70 °C in air oven for 24 h, the fibers were put through a grinding mill followed by size exclusion using a 1 mm screen (Retsch SM2000, ATS, CA). The fiber powder was used as starting material in the following experiments:

Chemical pretreatment: 2 g of fiber powder suspended in 25 ml of 12% NaOH was processed according to Wang et al. (2007).

Ultrasound pretreatment: 0.5 g of fiber powder in 30 ml of solution [50 mM phosphate buffer (PB, pH 7.5)] or dH_2O was sonicated in an ice-water box at 60% output (10 s on/off) for 30 min (S-4000, Misonix, USA).

Microwave pretreatment: 0.5 g of fiber powder in 30 ml of solution (50 mM PB buffer, pH 7.5), dH_2O, or 2% NaOH was microwaved for 30 min at 80 °C or 120 °C (MarsXpress, CEM, USA). After microwave pretreatment (Monteil-Rivera et al., 2012), the fibers were washed with dH_2O, and for the fibers in 2% NaOH solution, they were washed with dH_2O until pH ~ 8.

2.3. Enzyme-assisted preparation of NCC

A 250 ml flask containing 100 ml of 0.3% substrate [Whatman filter paper, ASC, fiber powder (flax or hemp), or the above mechanical and chemical-treated fibers] in acetate buffer (pH 6.0) was supplemented with AoEG (58 IU) and incubated in a shaker at 50 °C, 150 rpm for 24 h. After the reaction, the enzyme was removed by centrifugation (30,000 × *g*) for 10 min. The solid particles were rinsed 3 times with dH_2O or until the supernatant turned opaque. The opaque solution kept in ice-water was sonicated at 60% outputs (10 s on/off) for 30 min. After sonication, NCC was separated from residual substrate and other impurities by centrifugation. NCC was visualized by microscopy as follows. After NCC solution was concentrated by evaporation in 70 °C oven, the final NCC product was lyophilized.

2.4. Atomic force microscopy (AFM)

AFM (MFP-3D, Asylum Research, Santa Barbara, CA, USA) with tapping imaging method was used. The AFM images were scanned in air using light tapping force to optimize height data. Silicon probes (ACT, Applied NanoStructures, USA) with radius of less than 10 nm having driving frequency of 280–300 kHz were used for the measurements. A drop of properly diluted NCC suspension was placed on a freshly cleaned mica disk coated with 2% poly-L-lysin. The drop of sample was gently washed off after the disk was air dried at room temperature. The magnification scale of all the presented images were labeled in the individual image and at least three different locations were measured for each sample.

3. Results and discussion

3.1. Characteristics and properties of AoEG

AoEG was characterized to be a new endoglucanase of ~35,000 molecular mass, glycosylated and its optimal pH and temperature for activity was determined to be between 6.0–7.0 and ~60 °C, respectively. The half-lives of AoEG at 50 °C, and 70 °C, were estimated to be 50 h and 23 h, respectively, and hence regarded as a thermostable enzyme. (see Supplementary data, Appendix A).

3.2. AoEG-assisted preparation of NCC

As a positive control, the AFM image (Supplementary Fig. 1) of Whatman filter paper, a source of pure microfibrillated cellulose (MFC), showed successful production of some NCC of dimensions around 200 nm in length and 5–7 nm width as well as large aggregates containing cellulosic material of different sizes that can be attributed to NCC or microfibrils.

3.3. Effect of pretreatment

The yield of NCC from non-pretreated hemp and flax fibers was generally low (not shown) unless various forms of pretreatment were carried out (Section 2.2). Mosier et al. (2005) reported that acid swelling treatment loosens the bonds between the cellulose chains making them more susceptible to enzyme attack. Fig. 1a shows the high yield of NCC from ASC after AoEG treatment. In the AFM image, 150–300 nm long nanocrystals are well represented. Longer nanofibrils (300 nm–over 1 μm) are present almost equally. This means the longer fibrils tend to connect cellulosic material as a network structure, which is a well recognized behavior of nanofibrillar cellulose. The thinnest of the cellulosic nanoparticles are ~5 nm thick, others are mostly 10–30 nm. Branching of the thicker nanofibrils to thinner ones through opening of the fibril bundle is evident. At the same time, thinning out of the fibril gave rise to shorter fibrils.

AFM image of flax treated by AoEG (Fig. 1b) shows shorter NCCs than those observed in the case of AoEG-treated ASC. Most of the NCCs are 100–400 nm long with thickness of 5–15 nm; others are mainly <1 μm. In general, the AoEG-treated flax produced better defined nanocrystals than those from ASC. The production of NCC with narrow size distribution from flax was supported by scanning electron microscopy (data not shown). Similar results were obtained with AoEG-treated hemp samples but not investigated further (data not shown).

3.4. Comparison of NCC yield

The yield of NCC from flax using physical pretreatment only, especially ultrasound alone, is much less than that obtained from a combined physical–chemical pretreatment. For the latter (ultrasound-microwave and ultrasound-microwave [2% NaOH]), the final NCC products are not only of better quality but also of higher yield. The decreasing order of results for the AoEG-assisted production of NCC from the bast fiber is: ultrasound-microwave (2% NaOH, 120 °C) > ultrasound-microwave

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