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High efficiently preparation of cellulose nanocrystals by mechano-enzymatic hydrolysis: the mechanism study

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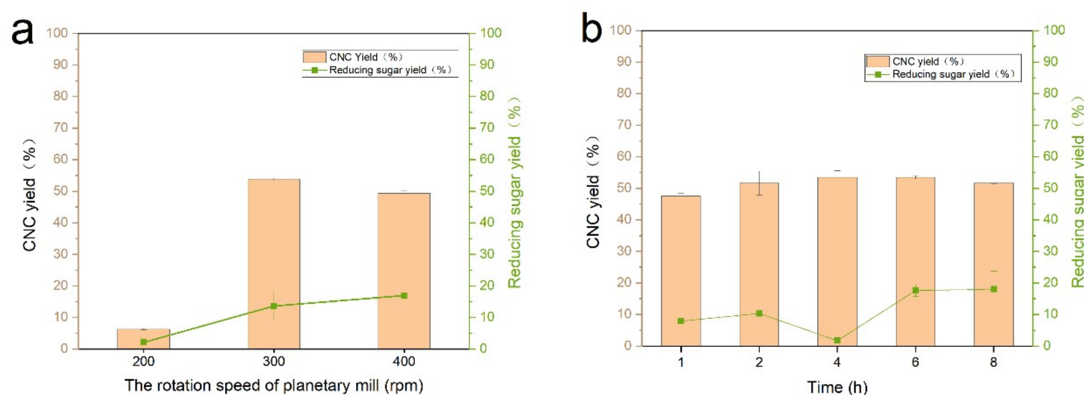

Fig. S1. The effect of rotation speed (a) and milling time (b) on the yield of CNCs and reducing sugars.

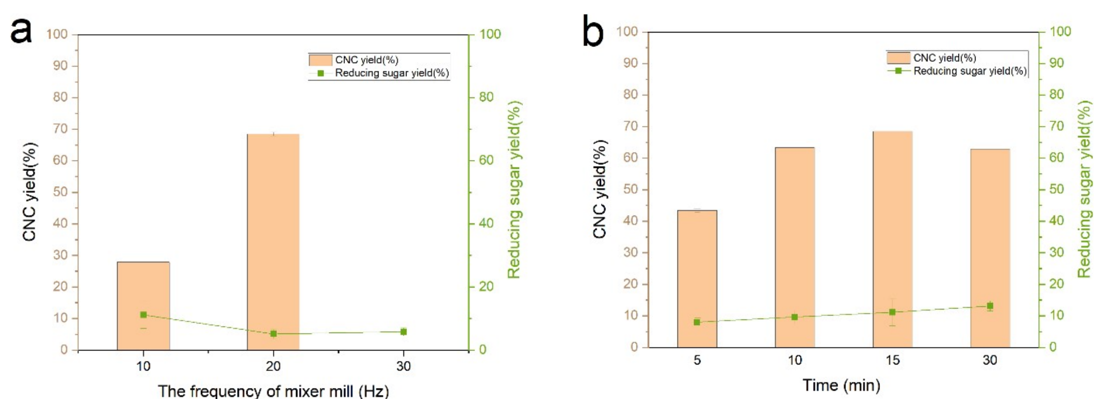

Fig. S2. The effect of mechanical strength (a) and milling time (b) on the yield of

CNCs and reducing sugars.

Experimental Section

Materials: Microcrystalline cellulose (MCC) was purchased from Sinopharm Chemical Reagent Co., Ltd. (purity: 97%). Cellulase derived from *Trichoderma longibrachiatum* (*T. l*) was purchased from Ningxia Heshibi Biotechnology Co., Ltd. (Ningxia, China). Endo-1,4-*β*-D-glucanase was purchased from Megazyme International Ireland (Wicklow, Ireland). Exo-1,4-*β*-D-glucanase was purchased from Sigma Aldrich (Saint Louis, USA). And *β*-glucosidase was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). All other chemicals used were of analytical grade.

The effect of liquid addition and mechanical force on the yields of CNCs and reducing sugar: The influence of liquid addition was systematically investigated during MEH. The experiment was carried out on roller mill according to the previous work [1]. Cellulase was added to MCC (enzyme loading was controlled to be 25 U/g), and the powders were mixed and transferred to the Teflon jars. Then 0.25~1.5μL/mg (relative to the substrate) acetate buffer (0.1 M, pH 4.8) and 840 g stainless steel balls (15 mm diameter) were added and the mixture was milled at 100 rpm for 48 h. Finally, the resulting complex was collected and suspended in water to obtain suspension. The samples were stirred at 120 °C in an oil bath for 20 min to inactivate the enzymes, and the resulting suspension was washed with distilled water several times until the final supernatant becoming neutral. Then the supernatant fluid was collected for 3,5-dinitrosalicylic acid (DNS) detection to determine the concentration of reducing sugars. Distilled water was added to the resulted precipitate and decentralized in an ultrasonic cleaner (KQ-600DE, Kunshan Ultrasonic Instruments Co., Ltd, Kunshan, China) for 10 min. The resulted suspension was centrifuged at 640 *g* for 5 min to remove large particles, and the suspension was lyophilized to obtain CNCs.

For comparison, traditional enzymatic hydrolysis of cellulose was carried out in dilute solution (5%, w/v) at 50 °C for 72 h.

According to previous work, mechanical strength has an important effect on

enzymatic hydrolysis efficiency. To evaluate the effect of mechanical force on the yields of CNCs and reducing sugar, mixer mill (Retsch MM400) with frequency of 10−30 Hz, and planetary mill (YXQM-2L, Changsha Mitrcn Instrument Equipment Co., Ltd., Changsha, China) with rotation speed of 200−400 rpm was applied in the MEH. A certain amount of the sample was withdrawn at definite intervals during hydrolysis, and then followed the method described in roller mill.

Determination the hydrolysis degree of MCC: CNCs and reducing sugars were the final products of hydrolysis of MCC. 35 mL water was added to dissolve 0.3 g mixture after ball milling, and the suspension was centrifuged at 12400 *g* for 5 min, supernatant (reducing sugars) was collected. The precipitation was further dissolved in 35 mL water and centrifuged at 640 *g* for 5 min. The cloudy supernatant was collected and dried at 85 °C. The reducing sugars were introduced in a 15 mL plug test tube and mixed with DNS reagent. The mixture was incubated for 1 min at 100 °C. After cooling down to room temperature, water was added to the scale level. The samples were measured at 550 nm using a UV-visible spectrophotometer (UV-2204, Shanghai Spectroscopic Instrument Co., Ltd, Shanghai, China).

Study on enzymatic hydrolysis kinetics: The kinetic experiments are performed as follows: 25 g MCC, 25 U/g enzyme loading and 0.5 μL/mg acetic acid buffer solution (0.1M, pH 4.8) were added in 500 mL Teflon jars in YXQM-2L. Products containing soluble reducing sugar and CNCs are withdrawn from the reaction mixture at different time intervals, and then followed the method below.

The first order kinetic equation:

$$\frac{d[C]}{dt} = k[C_{max} - C] \quad (1\text{-}1)$$

$$\frac{d[m]}{dt} = k[m_{max} - m] \quad (1\text{-}2)$$

[m] and [C] are weight of CNCs and concentration of reducing sugar, t is the time of enzymatic hydrolysis, k is the rate constant, m_{max} and C_{max} are the maximum CNCs weight and reducing sugar concentration.

$$\ln\left(C_{max}/[C_{max}-C]\right)=kt \qquad (2\text{-}1)$$

$$\ln\left(m_{max}/[m_{max}-m]\right)=kt \qquad (2\text{-}2)$$

A plot of '$\ln\left(C_{max}/[C_{max}-C]\right)$' vs 't' is used to obtain the *k* value, and the same is a plot of '$\ln\left(m_{max}/[m_{max}-m]\right)$' vs 't'.

The second order reaction kinetics equation:

$$\frac{\mathrm{d[C]}}{\mathrm{dt}}=k[C_{max}-C]^2 \qquad (3\text{-}1)$$

$$\frac{\mathrm{d[m]}}{\mathrm{dt}}=k[m_{max}-m]^2 \qquad (3\text{-}2)$$

$$\frac{1}{[C_{max}-C]}-\frac{1}{C_{max}}=kt \qquad (4\text{-}1)$$

$$\frac{1}{[m_{max}-m]}-\frac{1}{m_{max}}=kt \qquad (4\text{-}2)$$

A plot of '$\frac{1}{[C_{max}-C]}-\frac{1}{C_{max}}$' vs 't' is for getting the *k* value, and the same is a plot of '$\frac{1}{[m_{max}-m]}-\frac{1}{m_{max}}$' vs 't'.

The quasi-first order kinetic equation:

$$C=\frac{C_0}{0.9}[1-\exp\left(-\frac{0.9K}{1-h}t^{1-h}\right)] \qquad (5)$$

C_0 is the initial MCC concentrations, 0.9 is the correlation coefficient between substrate and products, *K* is the empirical rate constant, and *h* is the fractal dimension.

A plot of '$\ln(t)$' vs '$\ln\left[-\ln\left(1-\frac{0.9P}{C_0}\right)\right]$' is used to calculate the *K* and *h*.

$$\ln\left[-\ln\left(1-\frac{0.9C}{C_0}\right)\right]=ln\frac{0.9K}{(1-h)}+(1-h)ln(t) \qquad (6)$$

Effect of mechanical force on enzymatic hydrolysis: In order to study the influence of mechanical force, four groups of experiments were set up. Group MM+E,

enzymatic hydrolysis of milled MCC with un-milled enzymes under static conditions; group M+ME, enzymatic hydrolysis of un-milled MCC with milled enzymes under static conditions; group M+E, enzymatic hydrolysis of MCC with cellulase under static conditions; group M(M+E), enzymatic hydrolysis of MCC and enzyme by ball milling. All the grinding conditions were carried out in the mixer mill at 20 Hz for 15 min (η = 0.5 μL/mg).

Activity of single enzyme in mechanical enzymatic hydrolysis: First, the activity of single enzyme after grinding and static hydrolysis condition was investigated. The grinding was performed at 20 Hz for 15 min. The equal amount of acetic acid buffer and enzyme loading is added to the reaction system. The mixed system after ball milling and static reaction was centrifuged at 124000 *g* for 5 min. The supernatant was diluted to the same certain volume, and then 2 mL of EG, CBH, BG were obtained from the solution. CMC-Na, CNCs, and cellobiose were used as the substrate of EG, CBH, and BG, respectively. The enzymatic hydrolysis was carried out at 50 °C for 1 h, and the products were analyzed by DNS method and HPLC. The following formula was used to calculate the enzyme activity.

$$Enzyme\ activity = \frac{Ci\ \times V \times 1000}{2 \times 60} \quad (10)$$

C_i is the concentration of reducing sugar, cellobiose and glucose. V is the constant volume of supernatant. This formula comes from FPA measurement. We define one unit of enzyme activity as 1 mL enzyme to produce 1 μg product per min.

Secondly, the hydrolysis degree of the specific substrate by single enzyme was investigated under grinding and static conditions. MCC was added as a grinding auxiliary to simulate the conditions of ball grinding. At the same time, quantitative CBH, BG, and their specific substrate of CNCs and cellobiose were added (EG is an exception because its specific substrate is MCC). All the experiments were carried out in mixer mill at 20 Hz for 15 min with liquid addition of 0.5 μL/mg. After grinding, HPLC and DNS analysis were performed to evaluate the enzyme activity during MEH. The activity of CBH, BG, and EG was evaluated by cellobiose, glucose, CNCs and reducing sugar, respectively.

Reference

1 Q. H. Zhang, Z. H. Lu, C. Su, Z. M. Feng, H. Wang, J. B. Yu and W. K. Su, *Bioresource Technology*, 2021, **331**.