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ORIGINAL PAPER

Cellulase immobilized on mesoporous biochar synthesized by ionothermal carbonization of cellulose

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Abstract Cellulose-based biochar was prepared via ionothermal carbonization of cellulose in [Bmim]Cl with $H_2SO_4$and subsequent pyrolysis. The biochar was analyzed by a series of characterization methods, indicating that it was a kind of mesoporous carbon suitable for the adsorption of cellulase. Kinetic analysis showed that the immobilized cellulase exhibited higher affinity to carboxymethyl cellulose than free cellulase. The immobilized cellulase, at different pH and temperatures, was more stable than free cellulase. It was used to hydrolyze pretreated cellulose in [Bmim]Cl with a total reducing sugar (TRS) yield of 99.9%. The immobilized cellulase maintained activity of 74.8% after five cycles at an immobilized cellulase/cellulose weight ratio of 30:1. When the cellulose loading was increased by a factor of 5, the TRS yield decreased by only 27.5%.

Keywords Mesoporous biochar · Ionothermal carbonization · Immobilized cellulase · Kinetics analysis · Stability

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Introduction

As the world population grows and the economy expands, the utilization of renewable resources such as lignocellulosic biomass for the production of fuels and chemicals has garnered great interest around the world (Himmel et al. 2007). It is reported that the annual global biomass production is about 220 billion dry tons, equivalent to 8.3 times the world's energy consumption in 2014 (Fang 2015). This large cache of biomass undoubtedly constitutes an important resource for biofuel production. However, due to the stiffness of the molecule and the close packing of the chains via numerous intermolecular and intramolecular hydrogen bonds, cellulose is difficult to dissolve in conventional solvents such as water, impeding its

hydrolysis into fermentable sugars. Therefore, pretreatment is a necessary step that disrupts the tight packing arrangement of cellulose fibrils in the crystalline domains, thereby enhancing the accessibility of enzymes to cellulose for hydrolysis. Numerous methods have been applied to the pretreatment of cellulose and biomass, such as fungal pretreatment and pretreatment with organic electrolyte solutions (Tian et al. 2011; 2012).

Recently, ionic liquids (ILs) have emerged as a new class of solvents for reactions because of their high thermal stability and nearly non-volatility (Hallett and Welton 2011). It was found that some ILs can dissolve cellulose (Sun et al. 2011) rendering it more susceptible to enzymatic hydrolysis. The ionic liquid [Bmim]Cl (1-butyl-3-methylimidazolium chloride) has a greater capacity to dissolve cellulose (Xu et al. 2012) and can also be used to carbonize microcrystalline cellulose for the production of mesoporous biochar via ionothermal carbonization (Sun et al. 2015) that uses IL as solvent, potential template and structure directing agent (Morris 2009). In the presence of IL [DMFH][Tf_2N] [N,N-dimethyl-N-formylammonium bis(trifluoromethylsulfonyl)imide], mesoporous carbon was synthesized by an ionothermal carbonization of glucose or fructose at 80 °C under ambient pressure (Lee et al. 2010). Hybrid porous carbon materials were also synthesized from cellulose and sugarcane by ionothermal carbonization at 200 °C (Zhang et al. 2014).

Carbon materials, including activated carbon and biochar, can be used as carriers of enzymes. Compared with other carbons, biochar is a carbon-rich and porous solid with abundant surface functional groups (e.g., C–O, C=O, COOH and OH) produced by pyrolysis (Liu et al. 2015). For example, a series of biomass waste residues were used to synthesis biochars by using microwave or conventional heating (Mubarak et al. 2014a, b; Thangalazhy-Gopakumar et al. 2015; Thines et al. 2017). The immobilization of enzymes, including methods of chemical cross-linking and physical adsorption, has been applied to the hydrolysis of raw cellulose with necessary pretreatment (Kumakura 1997; Tu et al. 2006). During chemical cross-linking, carrier and enzymes are connected via a cross-linking agent, such as glutaraldehyde and (3-aminopropyl) triethoxysilane (Long et al. 2014). During physical adsorption, cellulase is immobilized onto a carrier through physical absorption by a π-π stacking interaction with the aid of non-covalent hydrogen bonds between the carrier and cellulase enzyme (Mubarak et al. 2014a, b). Compared with chemical cross-linking, the physical method is simpler, as it does not require further modification of the carrier and deactivation of enzymes. In addition, the carrier is recyclable. Most studies mainly focus on the adsorption of enzymes using activated carbon. For example, *Aspergillus nidulans* SU04 adsorbed on activated carbon retained an average activity for the hydrolysis of carboxymethyl cellulose of 57.12 ± 0.05 U mL^{-1} after 10 cycles (Jabasingh and Nachiyar 2012). *Aspergillus niger* adsorbed on commercial activated carbon maintained its 70% initial enzyme activity after five repeated batches of enzymatic methylcellulose hydrolysis (Daoud et al. 2010). However, only a few studies have described the immobilization of cellulase on biochar via physical adsorption, such as hardwood biochar, while maintaining the stability of some soil extracellular enzymes (e.g., β-glycosidase and β-D-cellobiase) for 4-methylumbelliferyl-β-D-glycoside and 4-methylumbelliferyl-β-D-cellobioside hydrolysis, respectively (Elzobair et al. 2016).

In this study, biochar was produced by the ionothermal carbonization of cellulose in [Bmim]Cl at 150 °C and pyrolysis at 300 °C for the adsorption of cellulase. The characteristics of immobilized cellulase were systematically tested by kinetic analysis, pH and temperature resistance. The immobilized cellulase was further used to hydrolyze cellulose pretreated by [Bmim]Cl at 50 °C. Cellulase immobilized on the biochar synthesized via the ionothermal method showed better hydrolysis activity toward cellulose and raw cellulose in corncob.

Materials and methods

Materials

Carboxymethyl cellulose (CMC) sodium (≥ 99.0%) was purchased from Tokyo Kasei Kogyo Co., Ltd. Microcrystalline cellulose (Avicel® PH-101, Cat. No. 11363), cellulase [*Aspergillus niger*, ATCC 26921, 50 mL solution, CMC activity of 981.1 U mL^{-1} measured by the CMC method (Ghose 1987)], D-(+)-glucose (≥ 99.5%), bicinchoninic acid (BCA) disodium salt (≥ 98.0%) were from Sigma (Shanghai). Bovine serum albumin (BSA) (≥ 98.0%) was

purchased from J&K scientific Ltd. (Beijing). The [Bmim]Cl (≥ 99.0%) was purchased from Shanghai Chengjie Chemical Co., Ltd. Sulfuric acid (95.0–98.0%) was obtained from Diancan Medicine Co. Ltd. (Kunming, Yunnan). Trisodium citrate dihydrate ($C_6H_9O_9Na_3$, 99.0%) was from Tianjin Fengchuan Chemical Reagent Technology Co., Ltd. Citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$, 99.5%) was purchased from Xilong Chemical Factory Co., Ltd. (Chengdu, Sichuan). NaN_3(≥ 99.0%) was purchased from Beijing Dingguo Biotechnology Co., Ltd. 3,5-Dinitrosalicylic acid (DNS; ≥ 98.0%) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai). Corncob (Yingkou, Liaoning), with component content analyzed using the NREL method (Sluiter et al. 2008), was dried at 100 °C for 48 h, crushed and sieved (40–60 mesh).

Cellulose and corncob pretreatment

Referring to previous work (Liu and Chen 2006; Lee et al. 2009), microcrystalline cellulose was mixed with [Bmim]Cl {cellulose:[Bmim]Cl = 1:10 (w/w)} under mechanical stirring for 1 h at 90 °C, and corncob was mixed with [Bmim]Cl {cellulose:[Bmim]Cl = 1:10 (w/w)} under mechanical stirring for 1 and 3 h at 90 °C. The mixture was washed with hot water sufficiently to obtain regenerated cellulose and corncob. The regenerated substrates were dried at 60 °C for 48 h in a drier (WFO-710, EYELA, Tokyo Rikakikai Co., Ltd.) for enzymatic hydrolysis.

Synthesis of biochar

Primary biochar by ionothermal carbonization

Microcrystalline cellulose (2.0 g) was mixed with [Bmim]Cl (20.0 g) in a flask (250 mL) with a magnetic bar and stirred vigorously while heating in an oil bath (DF-101S, Gongyi Yuhua Instrument Co., Ltd., Henan) at 150 °C. Concentrated H_2SO_4 (98%, 12.0 mL) was added dropwise by pipette, and the resulting mixture was reacted for 15 h. The primary biochar prepared was filtered through a Millipore filter (0.22 μm pore size) and washed with distilled water until the filtrate was neutral. The sample was freeze-dried at − 47 °C (FDU-1200, EYELA, Tokyo Rikakikai Co., Ltd.) for 72 h.

Biochar carrier by pyrolysis

Primary biochar was further stabilized by heating at a rate of 1 °C/min to 300 °C for 1 h pyrolysis under N_2 flow (purity ≥ 99.9%) in a rotary tubular furnace (SGL-1100, Shanghai Daheng Optics and Fine Mechanics Co., Ltd.) (defined as ionothermal biochar) (Van de Velden et al. 2008). In this step, the Brunauer–Emmett–Teller (BET) surface area and Barret–Joyner–Halenda (BJH) pore size are further increased (the specific surface area and pore parameters were calculated by applying the BET/BJH method). In addition, crystal cellulose was also directly pyrolyzed at 300 °C (defined as conventional biochar) as a blank for comparison.

The pyrolyzed biochar was soaked with ethanol (100 mL) and stirred for 10 h at room temperature in order to remove low-molecular-weight substances like polyphenols and polycyclic aromatic hydrocarbons generated during pyrolysis, which may be toxic for enzymes (Heredia et al. 1990; Van de Velden et al. 2008). The biochar was further washed three times with citric acid buffer (0.1 M, pH 5, 100 mL) through a Millipore filter (0.22 μm), freeze-dried at − 47 °C for 72 h, and sieved through a 200-mesh sieve to obtain biochar carrier.

Biochar characterization

The surface area and pore volume of biochar carrier and pyrolyzed cellulose were analyzed by the BET method (TriStar II 3020, Micromeritics Instrument Corp., Norcross, GA, USA) with N_2 adsorption–desorption at − 196 °C. Its chemical composition (wt%) was determined by an elemental analyzer (vario EL cube, Elementar Analysensysteme GmbH, Hanau, Germany). Structural and morphological information was obtained by scanning electron microscopy (SEM; ZEISS EVO LS 10, Cambridge, UK). The samples were placed on the conductive adhesive and magnified 500 and 5000 times to observe their morphology. Both biochar structures were analyzed by a Raman microscope equipped with a 523 nm excitation wavelength laser diode and backscattering configuration (LabRAM HR800-LS55; Horiba, Kyoto, Japan). The spectral resolution was 1.5 cm^{-1} with 50 MW laser power, and a total acquisition of 3 was considered. Raman spectra in the range of 100–3500 cm^{-1} were curve-fitted using

Renishaw's WiRE [Windows-based Raman Environment] software. X-ray diffraction (XRD; X'Pert Pro MPD, PANalytical, Almelo, Netherlands), operated at 40 kV and 40 mA with Cu Kα radiation, was performed to compare the crystallinity of the crystalline cellulose, conventional biochar, ionothermal biochar and IL-pretreated cellulose, and the data were collected in the 2θ range of 5–80°.

Immobilized cellulase

Immobilizing cellulase on biochar

Cellulase solution (2.0 mL), biochar (2.0 g), NaN_3 (2%, 0.5 mL, for keeping cellulase from the infection of germs) and citric acid buffer (0.1 M, pH 5) were mixed to 50.0 mL in a bottle (100 mL), sealed, and ultrasonicated for 10 min (AS10200BDT, Tianjin Automatic Science Instrument Co., Ltd.) to disperse the mixture. The bottle with sample was placed in a water bath at 30 °C (SHA-C, Jintan City Baita Jinchang Experimental Instrument Factory, Jiangsu) and stirred for 24 h to reach adsorption equilibrium. The mixture, in a centrifuge tube (25 mL), was centrifuged (3–30 K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 20,000 rpm (33,987 relative centrifugal force) for 20 min, and the supernatant was collected. The solid phase in the tube was collected and washed with 100 mL of citric acid buffer (0.1 M, pH 5) through a Millipore filter (0.22 μm), and the filtrate was collected. The solid with immobilized enzyme was freeze-dried at − 47 °C for 72 h. The BCA disodium salt assay method (Smith et al. 1985) was used to determine the content (mg) of protein in the cellulase solution (2 mL), the collected supernatant and filtrate that were quantified by external standard curve method with five concentrations of BSA (0.04, 0.08, 0.12, 0.16 and 0.20 mg/mL; $R^2 = 0.9994$) using UV–vis spectrometry (UV-1800, Shimadzu, Kyoto, Japan) at 562 nm. The loading efficiency (wt%) with respect to the protein immobilized on the biochar was calculated as follows:

$$\begin{aligned}&\text{Loading efficiency (wt\%)}\\&\quad=(\text{protein weight in cellulase solution}\\&\qquad-\text{protein weight in supernatant and filtrate})/\\&\qquad(\text{protein weight in cellulase solution})\times 100\%\end{aligned}\tag{1}$$

Characterization of immobilized cellulase

The biochar carrier and immobilized cellulose were characterized by Fourier-transform infrared spectroscopy (FT-IR; Nicolet iS10, Thermo Fisher Scientific, Waltham, MA, USA) via the KBr pellet method (KBr pellets with 200 mg KBr and 0.1 wt% sample were prepared for the analysis at an average of 32 scans from 400 to 4000 cm^{-1} at 4 cm^{-1} resolution) and transmission electron microscopy (TEM; JEM-2100, JEOL Ltd., Tokyo, Japan). Biomass particles were dispersed by putting in ethanol and ultrasonicated for TEM analysis.

Enzyme activity assay

The activity of cellulase was assayed according to the CMC method (Ghose 1987), where CMC was used as an indicator of cellulase activity that was calculated by the amount of glucose equivalent from the hydrolysis of CMC.

Immobilized cellulase (50 mg) was incubated for 0.5 h with 1 wt% CMC in citrate buffer solution (pH 5, 5 mL) at 50 °C. The reaction was terminated by removing the immobilized cellulase, and total reducing sugars (TRS) were measured by the DNS method (Miller 1959) and calibrated with five standard glucose points (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL; $R^2 > 0.999$). The activity unit of immobilized cellulase (U/mg) is defined as unit weight (per mg) of immobilized cellulase producing 1 μmol of glucose equivalent per minute, and it is calculated as follows:

$$\begin{aligned}&\text{Immobilized cellulase activity (U/mg)}\\&\quad=1000\,\mathrm{W}/(\mathrm{M}\times\mathrm{m}\times\mathrm{t})\end{aligned}\tag{2}$$

where W weight of released glucose equivalent (mg); M molecular weight of glucose; m weight of the measured sample (mg); t reaction time (min).

Characteristics of immobilized enzyme

Kinetic parameter (K_m) of enzyme

The kinetic parameter (Michaelis constant, K_m) of free and immobilized enzyme was determined by measuring the velocity of reaction using a series of CMC concentrations (1–3 mg/mL) in citric acid buffer (0.1 M, pH 5) at 50 °C. The velocity of reaction (v)

is defined as the activity of unit weight of cellulase (U/mg, free or immobilized cellulase) for CMC. K_m was calculated by the Michaelis–Menten equation as follows (English et al. 2006):

$$1/v = 1/v_{max} + K_m/v_{max} \times [S] \quad (3)$$

where ν reaction velocity (U/mg); v_{max} the maximum velocity for reaction (U/mg); *[S]* CMC concentration (mg/mL); K_m Michaelis constant (mg/mL).

Effect of temperature and pH on enzyme activity

The effect of temperature on enzyme activity was studied in the range of 30–70 °C in a batch operation with 1% CMC in citric acid buffer (0.1 M, pH 5). The effect of varying pH from 3.0 to 7.0 in citric acid buffer (0.1 M) on the activity of free or immobilized enzyme was investigated at 50 °C with 1% CMC.

Hydrolysis of corncob raw material, pretreated cellulose and corncob with immobilized enzyme

Regenerated cellulose (10 mg) was mixed with immobilized cellulase (300 mg) (m_{IC}/m_{RC} = 30/1; subscript IC indicates immobilized cellulase; subscript RC indicates regenerated cellulose), 2% NaN_3 (0.5 mL) in citric acid buffer (0.1 M, pH 5) to maintain a total volume of 10 mL in a vial (25 mL). Because corncob contains raw cellulose, a higher dosage of 300 mg was loaded in accordance with the experimental conditions of the hydrolysis experiment.

The mixture was then incubated in a thermostatic shaker (SPH-100B, Shanghai Shipping laboratory equipment Co., Ltd.) with a rotation of 130 rpm at 50 °C for 24 h. The reaction was terminated by the removal of immobilized cellulase via filtration (0.22 μm pore size). The immobilized cellulase was freeze-dried at – 47 °C for 72 h for the next cycle. The activity of the collected immobilized cellulase was examined by testing the retaining activity via the enzyme activity assay described above. Glucose and cellobiose, in aqueous solution, were analyzed by high-performance liquid chromatography (HPLC; CTO-20A, Shimadzu) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 60 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min, and the results were calibrated with five standard points (glucose: 0.1, 0.2, 0.4, 0.8 and 2.0 mg/mL, R^2 = 0.9998; cellobiose: 0.09, 0.18, 0.27, 0.36 and 0.90 mg/mL, R^2 = 0.9996). The absorbance of TRS in aqueous solution was measured at 540 nm using a UV–vis spectrometer and the above-referenced DNS method. The yields (wt%) of glucose, cellobiose and TRS were calculated as follows:

$$\text{Glucose yield (wt\%)} = (\text{weight of glucose} \times 0.9)/(\text{weight of regenerated cellulose}) \times 100\% \quad (4)$$

$$\text{Cellobiose yield (wt\%)} = (\text{weight of cellobiose} \times 0.9)/(\text{weight of regenerated cellulose}) \times 100\% \quad (5)$$

$$\text{TRS yield (wt\%)} = (\text{weight of TRS} \times 0.9)/(\text{weight of regenerated cellulose}) \times 100\% \quad (6)$$

All the experiments were carried out in triplicate to improve accuracy, and the standard deviation (σ) was 2.35–3.98% for glucose, 0.60–3.76% for cellobiose, and 1.78–3.08% for TRS.

Results and discussion

Biochar was synthesized for the purpose of immobilizing the enzyme for hydrolysis of pretreated cellulose. Figures 1, 2, 3 and 4 present N_2 adsorption–desorption, Raman, SEM images and XRD for biochars and cellulose, respectively. Figures 5 and 6 show FT-IR spectra and TEM images for biochar and immobilized cellulase, respectively. A Lineweaver–Burk plot of immobilized cellulase and free cellulase is presented in Fig. 7 to compare the affinity of the enzymes to CMC. The effects of pH and temperature on the activity of free cellulase and immobilized cellulase are given in Fig. 8. Figure 9 shows sugar yield vs. m_{IC}/m_{RC}. Figure 10 illustrates hydrolysis yields and cycles of the immobilized cellulase. Tables 1 and 2 show the components of raw corncob and hydrolysis results from raw corncob and regenerated corncob using the immobilized cellulase, respectively. Detailed results are presented and discussed below.

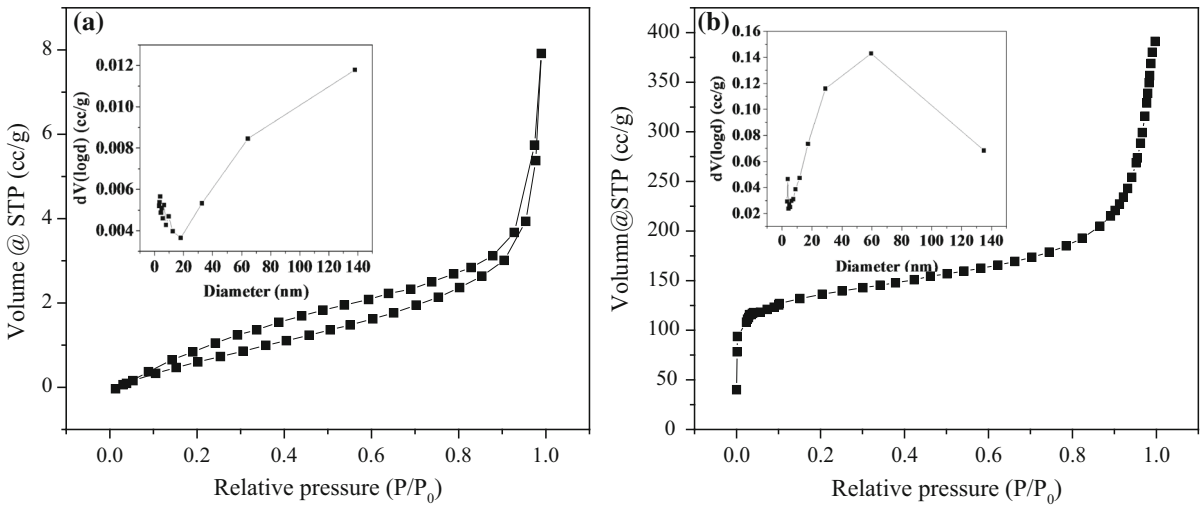


Fig. 1 N_2 adsorption–desorption curves of **a** conventional biochar and **b** ionothermal biochar (experiment conditions: – 196 °C, biochar was vacuum-activated at 150 °C for 2 h until pressure was within 0.1 MPa in the sample tube)

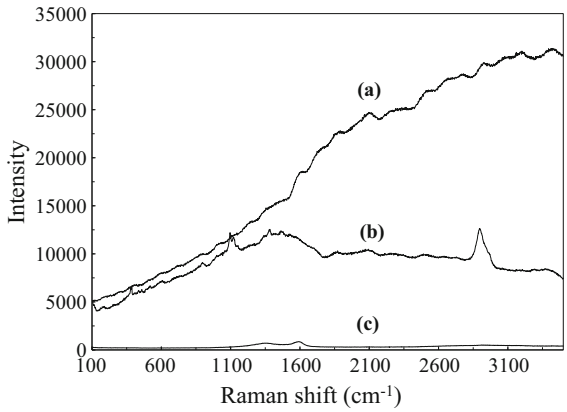


Fig. 2 Raman spectra (100–3500 cm^{-1}) for **a** conventional biochar, **b** cellulose and **c** ionothermal biochar

Biochar characterization

BET and BJH

The generation of N_2 adsorption–desorption isothermal curves is an important method used to characterize the structure of solid materials. The specific surface area, pore volume and pore size distribution of materials are obtained from an N_2 adsorption–desorption curve (Webb and Orr 1997).

Figure 1a shows the BET isotherm and BJH plot of biochar produced by conventional pyrolysis, with a specific surface area of 4.3 m^2/g, pore volume of 0.013 cm^3/g, and pore size of 114.6 Å. In Fig. 1b, the biochar carrier displays a typical type IV Langmuir adsorption–desorption isotherm at low temperature (– 196 °C) that has the type H4 hysteresis loop, indicating the synthesized ionothermal biochar has the characteristics of mesoporous materials (Barrer 1989). Biochar has a specific surface area of 413.7 m^2/g, a pore volume of 0.480 cm^3/g, and a pore size of 95 Å (9.5 nm). Because the average diameter of a cellulase protein is around 3–7 nm (Weimer and Weston 1985; Murmanis et al. 1987), BET and BJH data (specific surface area, pore volume and pore size) of the ionothermal biochar are more suitable for the adsorption of cellulase than conventional biochar.

Raman

In Fig. 2a, the Raman spectrum of conventional biochar is disorganized as a result of the large accumulation of tar oil from the pyrolysis process. In Fig. 2c, compared with conventional biochar (Fig. 2a) and cellulose (Fig. 2b), significant bands between 1200 and 1700 cm^{-1}, arising mainly from aliphatic chains and aromatic compounds (such as C–C, amorphous C structure, alkene C=C and carbonyl group C=O), are observed for ionothermal biochar (Liu et al. 2016).

SEM

In Fig. 3a, the biochar appears as an agglomeration formed by amorphous carbons (Sun and Li 2004; Xie

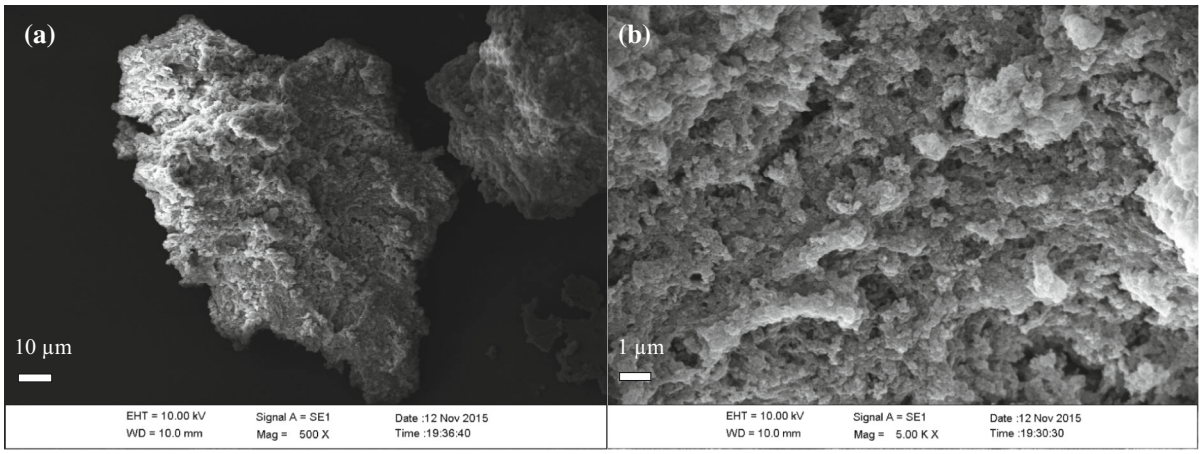


Fig. 3 SEM images of the ionothermal biochar carrier **a** with low magnification (×500) and **b** high magnification (×5000)

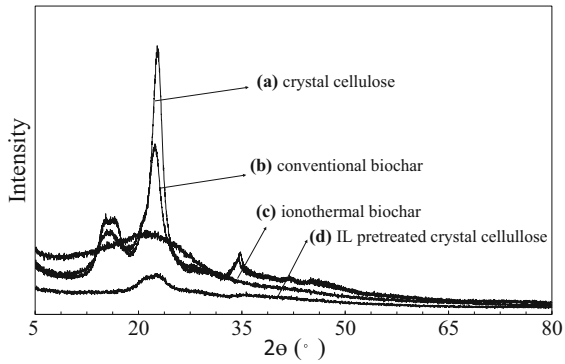


Fig. 4 XRD patterns: **a** crystal cellulose, **b** conventional biochar, **c** ionothermal biochar and **d** IL-pretreated cellulose

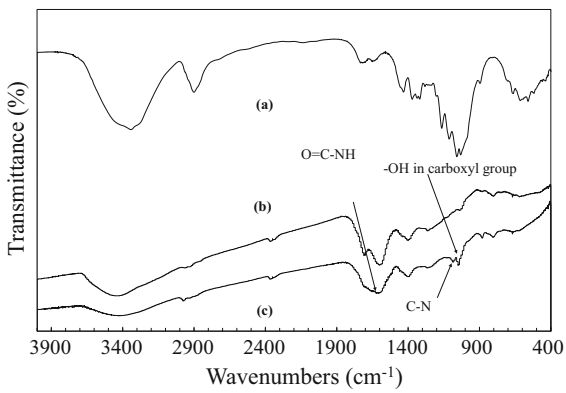


Fig. 5 FT-IR spectra: **a** conventional biochar, **b** ionothermal biochar and **c** immobilized cellulase

et al. 2011) with numerous pores and irregular protrusions at high magnification (Fig. 3b). Owing to these pores and irregular protrusions, biochar possesses a higher BET surface area and an appropriate pore size for the adsorption of cellulase protein.

XRD

XRD patterns of crystalline cellulose, conventional biochar, ionothermal biochar and IL-pretreated cellulose are shown in Fig. 4. The XRD patterns of crystalline cellulose and conventional biochar show diffraction peaks at 16°, 22° and 35°. For ionothermal biochar and IL-pretreated cellulose, the peaks at 16° and 35° almost disappear and transform into an amorphous halo peak at 22°. These results demonstrate that the crystallinity of cellulose was noticeably decreased and the porosity of the biochar carrier was enhanced. More importantly, the resulting hydrolysis reaction rate is significantly affected by these results (Zhu et al. 2016).

Elemental analysis

The elemental composition of the biochar is 56.2% C, 39.6% O, 3.3% H, 0.9% N and 0.11% S with an empirical formula of $C_{1.89}H_{1.31}O$ (N and S free basis), whereas the original cellulose contained around 44.4% C, 49.4% O and 6.2% H [formula of $(C_6H_{10}O_5)n$]. Trace amounts of N and S in the biochar likely originated from [Bmim]Cl and H_2SO_4 during ionothermal carbonization.

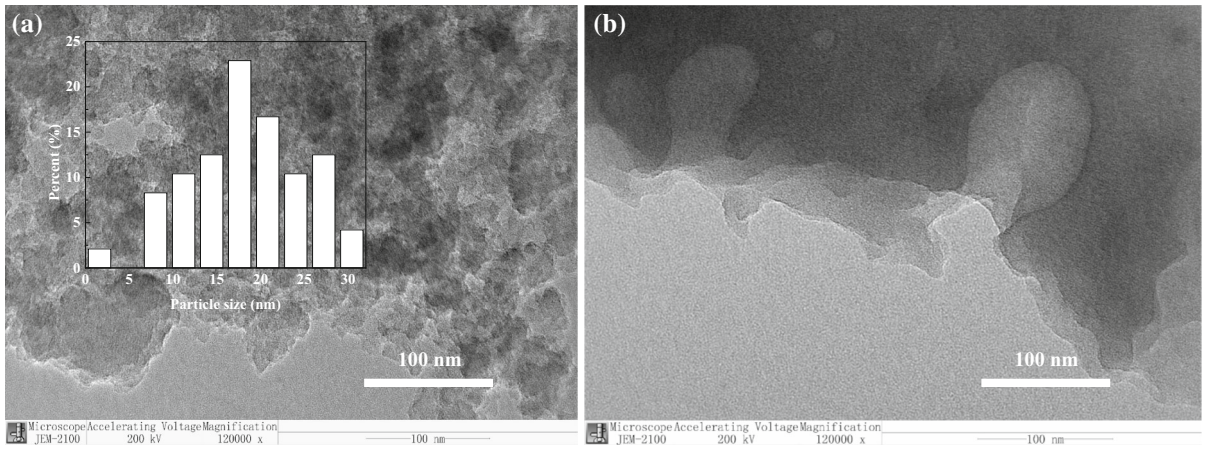


Fig. 6 TEM images: **a** ionothermal biochar carrier and **b** immobilized cellulase on ionothermal biochar carrier

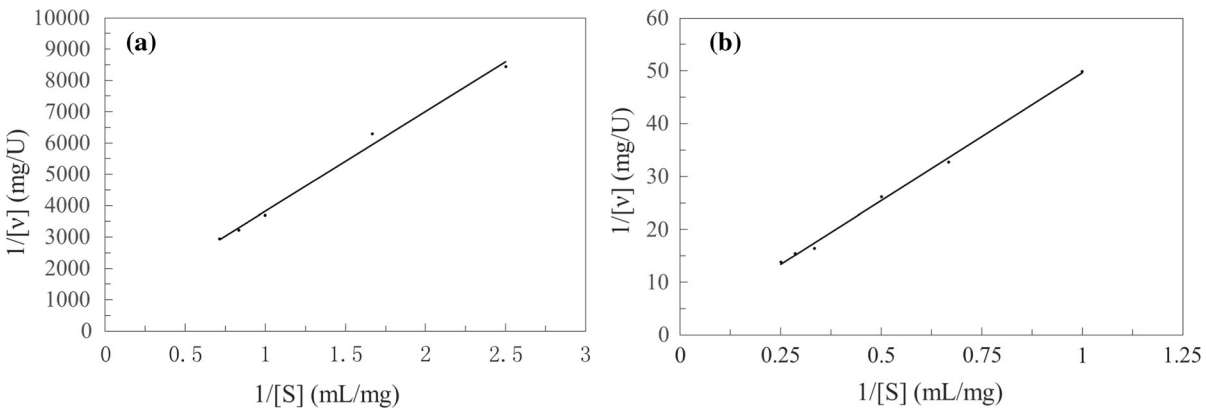


Fig. 7 Lineweaver–Burk plot for CMC hydrolysis with **a** immobilized cellulase and **b** free cellulase [reaction condition: 1% CMC in 5 mL citric acid buffer solution with pH 5 at 50 °C **a** 100 mg immobilized cellulase for 2 h, and **b** 25 μL free cellulase for 10 min]

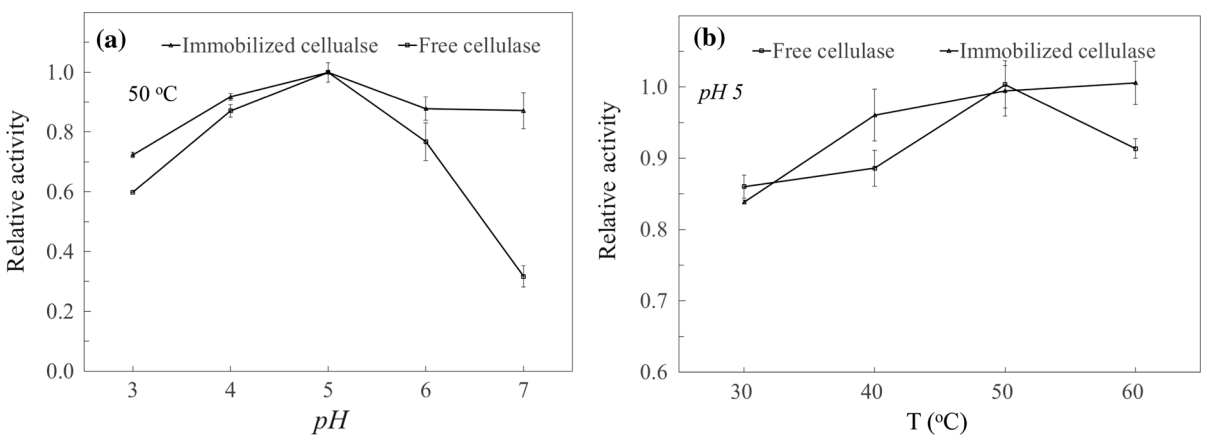


Fig. 8 Effects of **a** pH and **b** temperature on the relative activity of free cellulase and immobilized cellulase

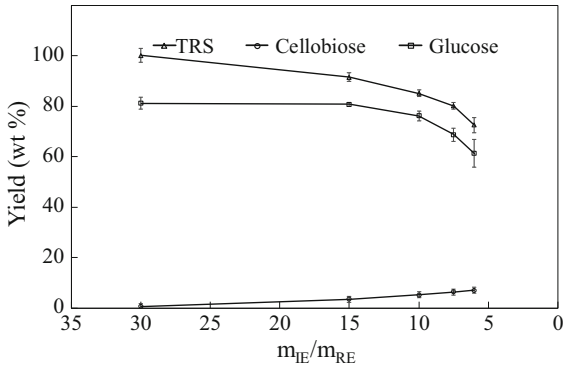


Fig. 9 Sugar yields vs. m_{IE}/m_{RC} ratio (hydrolysis conditions: m_{IC}/m_{RC} = 30/1, 15/1, 10/1, 7.5/1 and 6/1, respectively, 50 °C, 24 h, 130 rpm in 10 mL citric acid buffer solution)

Immobilized cellulase

FT-IR

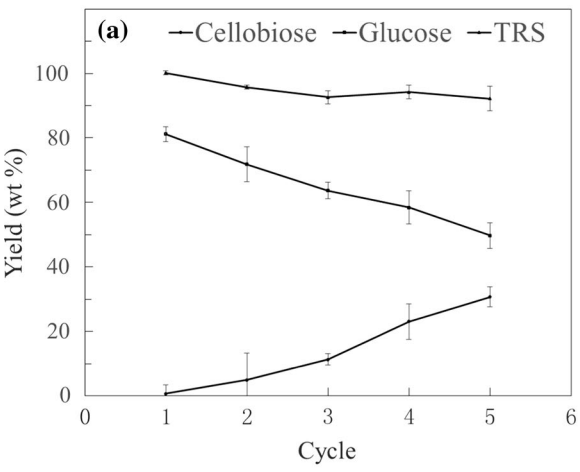
Figure 5a presents the IR spectum of the biochar produced by conventional pyrolysis. Relative to the ionothermal biochar, the conventional biochar contains more unnecessary functional groups that can damage the stability of cellulase (Fig. 5b). In Fig. 5c, IR bands at 1653 and 1067 cm^{-1} result from the amide group (O = C-NH) and aliphatic amide bond (C-N) vibrations in protein molecules (Mubarak et al. 2014a, b), respectively. The absorption at 930 cm^{-1} implies the deformation vibration of –OH of the carboxyl groups (Chandra et al. 2011) of cellulase compared with Fig. 5b. These results show that cellulase was adsorbed on biochar.

TEM

TEM images (Fig. 6) show that after immobilization, biochar particles are covered by a cellulase layer (Fig. 6b vs. a). In Fig. 6a, the mean size of the biochar particles is estimated to be 18.3 nm.

Loading efficiency and activity of immobilized enzyme

Protein weight is measured as 924 mg in cellulase solution, 180.25 mg in supernatant and 52.8 mg in



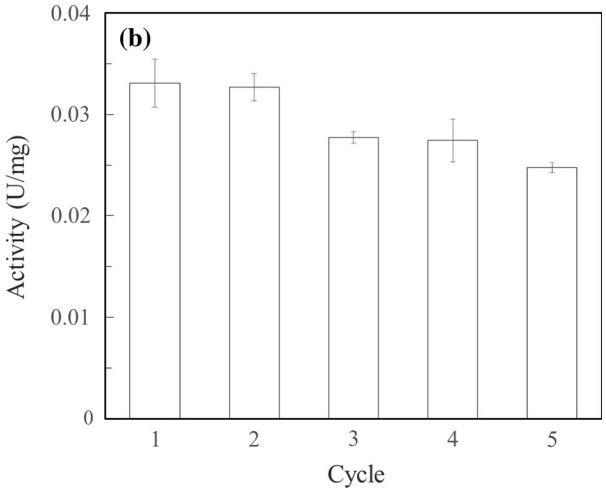


Fig. 10 Cycle of the immobilized cellulase. **a** Sugar yield vs. cycle (reaction conditions: m_{IE}/m_{RC} = 30/1, 50 °C, 24 h, 130 rpm) and **b** activity of the immobilized enzyme vs. cycles (reaction conditions: 50 °C, 2 h, 100 mg immobilized cellulase, 1% CMC in 5 mL citric acid buffer solution with pH 5.0, 130 rpm)

Table 1 The components of raw corncob

Component	Cellulose	Hemicellulose	Lignin	Ash	Pigment	Others
Content (wt%)	38.7	24.5	17.3	0.6	11.8	7.0

Determined by NREL method (Sluiter et al. 2008)

Table 2 Hydrolysis of raw and regenerated corncob with the immobilized cellulase

Entry	Substrate	Yield (wt%)		
		TRS	Glucose	Cellobiose
1[a]	Corncob-0	14.0	10.6	2.6
2[b]	Corncob-1	16.5	11.9	3.7
3[c]	Corncob-2	18.7	13.3	4.4

Reaction conditions: 300 mg raw corncob/regenerated corncob, 300 mg immobilized cellulase, 50 °C, 24 h, 130 rpm in 10 mL citric acid buffer solution

[a]Corncob-0 represents raw corncob without IL pretreatment

[b]Corncob-1 represents regenerated corncob after IL pretreatment for 1 h

[c]Corncob-2 represents regenerated corncob after IL pretreatment for 3 h

filtrate, respectively. Loading efficiency of the enzyme on biochar is calculated as 74.8% (Eq. 1). CMC (1 wt%) with immobilized cellulase (50 mg) was hydrolyzed in citrate buffer solution (pH 5, 5 mL) at 50 °C for 0.5 h, and the production of TRS was about 7.12 mg. Consequently, according to Eq. 2, the activity of immobilized enzyme is 0.033 U/mg (Fig. 10b).

Characteristics of immobilized enzyme

Kinetic parameters of enzyme

The K_m of the immobilized enzyme was calculated from Eq. 3 to be 4.89 mg/mL, while the K_m of the free enzyme was 36.31 mg/mL as calculated from Fig. 7.

Because K_m can indicate the affinity between an enzyme and its substrate, the higher the K_m value, the weaker the affinity between an enzyme and substrate, and vice versa. Therefore, the affinity of the immobilized enzyme toward the CMC substrate is higher than that of the free enzyme. After immobilization, the steric hindrance of the enzyme molecules decreased, which affects the orientation of the active center to the CMC substrate. It is concluded that the ability of an enzyme to attach to the surface of biochar particles can be enhanced by the interactions between the immobilized enzyme and the substrate to avoid the potential aggregation of the free enzyme molecules (Jia et al. 2003).

Effects of pH and temperature on enzyme activity

The effects of pH and temperature on the free cellulase and immobilized cellulase were studied. Figure 8a provides the relative activity (%, relative to the maximum value) of immobilized and free cellulases under different pH values at 50 °C. At a pH of 5, both cellulases reach the maximum activity of 100%. However, the relative activity decreases to 68.3% for the free enzyme and 87.2% for the immobilized enzyme at a pH of 7. When pH < 5 or pH > 5, the activity for both cellulases decreases, while the immobilized enzyme has a higher activity than that of free enzyme, indicating that immobilized cellulase can more effectively resist pH fluctuation. This is because the negative charge on the surface of biochar can attract cations in solution (including $[H^+]$), resulting in higher $[H^+]$ concentration in the diffusion layer of the immobilized enzyme than the external solution around it. Thus, pH in the external solution should be shifted to balance pH fluctuation and counteract the effect of the microenvironment. Briefly, it is caused by the charge distribution on the surface and microenvironment of an enzyme changed by the immobilization (Chen and Dong 2003; Zhang et al. 2004). Therefore, a pH of 5 is selected as optimal for further studies.

The relative activity of the immobilized and free cellulases under temperatures of 30–60 °C at a pH of 5 is presented in Fig. 8b. The activity for free cellulase reaches the maximum of 100% at 50 °C, but decreases to 91.4% as temperature increases to 60 °C. However, for the immobilized cellulase, the activity does not decrease at 60 °C. Thus, the immobilized cellulase is more resistant to higher temperatures (e.g., 60 °C).

Thermal inactivation of the free enzyme is mainly caused by the change of interactions between groups in enzyme molecules. The original balanced state is destroyed, and the native conformation of enzyme molecules tends toward an increase in thermodynamic entropy. This opens the folding structure, eventually leading to the loss of enzymatic function. After the enzyme is immobilized, the rigid structure of the carrier can effectively relieve the fluctuation of the enzyme conformation caused by a temperature increase, and the degrees of freedom of an immobilized cellulase structure are fewer. This may be caused by electrostatic interactions, hydrogen bonds and Van der Waals forces, etc., generated between the cellulase

molecules and biochar carrier that limit the freedom of the protein structure (Zhao et al. 2006; Singh et al. 2013).

In summary, the optimal conditions for higher immobilized enzyme activity for the hydrolysis of regenerated cellulose include a pH of 5 and a temperature of 50 °C.

Hydrolysis of pretreated cellulose, raw and pretreated corncob with the immobilized enzyme

Sugar production at different cellulose loading

Enzymatic hydrolysis of cellulose involves the combined action of exoglucanases or cellobiohydrolases (CBHs), endoglucanases (EGs), and β-glucosidases (β-Gs). CBHs cleave cellulose to release cellobiose from the ends of the cellulose chain. Generally, in enzymatic hydrolysis, cellulase primarily acts on cellulose, and the substrate is sheared into small pieces and hydrolyzed to cello-oligosaccharides (by CBHs and EGs) until glucose is formed (by β-Gs) (Dutta and Wu 2014). To further study the action of immobilized cellulase on substrate hydrolysis, different loadings of regenerated cellulose (10, 20, 30, 40, and 50 mg) were mixed with immobilized cellulase (300 mg, m_{IC}/m_{RC} = 30/1, 15/1, 10/1, 7.5/1 and 6/1) in 10 mL citric acid solution for hydrolysis at pH 5 and a temperature of 50 °C.

When the m_{IC}/m_{RC} ratio changes from 30/1 to 6/1, TRS yield decreases from 99.9 to 72.5%, glucose yield decreases from 81.2 to 61.4%, while cellobiose yield increases from 0.7 to 7.1% (Fig. 9). As cellulose weight increases by a factor of 5 to 50 mg (m_{IC}/m_{RC} of 6/1), the yield of TRS only decreases to 72.5%.

The decrease in TRS and the increase in cellobiose highlight the activity loss for immobilized cellulase in the heterogeneous enzymatic system. Upon increasing substrate dosage, the enzymatic hydrolysis products of cellulose, such as cellobiosyls, are strong competitive inhibitors of the immobilized cellulase (Rui and Dias 2004). In addition, the solid phase of the biochar carrier also can block the diffusion of cellulase toward cellulose molecules.

The experiments to test the stability of the recycled immobilized enzyme during hydrolysis were conducted at an m_{IC}/m_{RC} ratio of 30:1, a pH of 5 and a temperature of 50 °C.

Recycle experiments of immobilized enzyme

The immobilized enzyme is cycled for sugar production at an m_{IC}/m_{RC} ratio of 30/1 and a reaction time of 24 h to test its stability. After reaction, it is separated by filtration, washed with citrate buffer three times, and dried at – 47 °C for 72 h for subsequent cycles. In Fig. 10a, yields of TRS decrease from 99.9 to 92.2%, and yields of glucose decrease from 81.2 to 49.7%, while cellobiose increases from 0.7 to 30.7% from the first to fifth cycle under identical conditions. In Fig. 10b, the immobilized cellulase retains 74.8% of its initial activity after five cycles under the same conditions, indicating the immobilized cellulase has better usability after five cycles than cellulase immobilized on calcium alginate beads recently described by Sankarraj and Nallathambi (2015).

The loss of activity of the immobilized enzyme is explained as follows: (1) cellulase leaching from biochar carrier; (2) after hydrolysis, cellulase is surrounded by the hydrolysis products that prevent polysaccharides from accessing the enzyme; (3) the residues ($[Bmim]^+$, Cl^-) on biochar may deactivate cellulase [for example, cellulase can be unfolded by [Bmim]Cl (Zhao et al. 2009)]; and (4) the electrostatic force of cellulase molecules in the biochar carrier may slightly affect the conformation of the cellulase active center.

Corncob hydrolysis with immobilized cellulase

In Table 1, at 38.7% (wt%), cellulose comprises the largest portion of corncob. The immobilized cellulase can only hydrolyze part of the corncob, leaving most of the residue. In Table 1, the immobilized cellulase exhibits lower enzymatic activity on raw corncob, which is caused not only by the lower cellulose content but also by the inhibition effect of other components (hemicellulose and lignin, etc.) in corncob. However, after pretreatment with [Bmim]Cl over prolonged time, higher sugar yield can be achieved. Thus, pretreatment of corncob to remove lignin and hemicellulose in conjunction with the use of immobilized cellulase can improve sugar production.

In this work, the preparation of biochar by ionothermal carbonization requires a large amount of concentrated H_2SO_4, which is hazardous to the environment. It is necessary to develop new methods that do not rely on concentrated H_2SO_4. For example,

acidic ILs such as $[Bmim]^+[H_2PO_4]^-$ and $[Hmim]^+[Tfa]^-$ (1-methylimidazolium trifluoroacetic acid) or $[Bmim][FeCl_4]$ and hydrothermal carbonization to synthesize biochar can be used.

Conclusions

Cellulose-based biochar was synthesized by ionothermal carbonization under mild conditions via pyrolysis and was used as an enzyme carrier. FT-IR and TEM results showed that cellulase was adsorbed on the mesoporous biochar. The immobilized cellulase had better stability against pH and temperature than free cellulase. The hydrolysis of ionic liquid-pretreated cellulose was catalyzed by the immobilized enzyme, with a TRS yield of 99.9%, and cellulase activity remained at 74.8%, with a TRS yield of 92.2%, after five cycles. Lastly, the immobilized cellulase showed a certain degree of hydrolysis activity toward corncob, which warrants further investigation.

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