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Immobilized cellulase by polyvinyl alcohol/Fe_2O_3 magnetic nanoparticle to degrade microcrystalline cellulose

Hongdong Liao[a], Ding Chen[b], Li Yuan[b], Mang Zheng[a], Yonghua Zhu[a,**], Xuanming Liu[a,*]

[a] State Key Laboratory of Chemo/Biosensing and Chemometrics, Department of Life Science and Technology, Hunan University, Changsha, 410082, PR China
[b] College of Materials Science and Engineering, Hunan University, Changsha, 410082, PR China

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ABSTRACT

In an attempt to improve the enzymatic efficiency in ball mill, a novel immobilized cellulase on polyvinyl alcohol/Fe_2O_3 magnetic nanoparticle with high activity was synthesized and characterized by transmission electron microscopy, Zetasizer, Fourier transform infrared (FTIR) spectroscopy and vibrating sample magnetometry. It was found that the immobilized cellulase was a kind of spherical complex with approximate 270 nm, 4.87 A m^2 kg^{-1}, and mainly constructed by the accumulation of 10 nm Fe_2O_3 nanoparticles with the diffused polymer. When the immobilized cellulase was applied to degrade microcrystalline cellulose by combining with wet ball milling, the yielded glucose was 1.89 mg mL^{-1}, at least three times than the sum of individual yield. The immobilized cellulose maintained 40% activity even after four cycles of reuse. From these results, it can be concluded that the immobilization of cellulase with wet ball milling is a novel method to significantly improve the efficiency of cellulose conversion.

1. Introduction

Cellulose can be specifically hydrolyzed into oligomeric or soluble sugar by cellulase and is thus expected as a renewable source of fuels and chemicals. But the susceptibility of cellulose to enzyme degradation was limited for the structural complexity, particularly for hydrogen-bonded and ordered microcrystalline cellulose (MCC) (Mansfield & Meder, 2003). As a result, the complete conversion usually required large cellulase loadings, which increase cost and accordingly limited its economic feasibility.

Several methods have been developed to improve the efficiency of enzyme degradation. Among of them, ball milling has been recognized as one of the most effective treatments for its contribution to reducing particle size and crystallinity of cellulose (Hendriks & Zeeman, 2009). The rate and extent of saccharification of cellulose could be improved by simultaneous ball milling and enzyme hydrolysis (Mais, Esteghlalian, Saddler, & Mansfield, 2002). However, our research showed that severe wet ball milling greatly destroyed the activity of cellulose (Zhou et al., 2010). Many documents have reported that the immobilization on nanoparticles can efficiently maintain enzyme activity and get some applicable performance such as low cost and high stability (Dincer & Telefoncu, 2007; Ho, Mao, Gu, & Li, 2008; Li, Yoshimoto, Fukunaga, & Nakao, 2007; Liao et al. 2008; Wu, Yuan, & Sheng, 2005), however, there was rare report about the technology of nano-immobilized cellulase combining with mechanical pretreatments for effective hydrolysis of cellulose, not to mention MCC with high-crystallinity property.

In this work, a novel magnetic nanoparticle immobilized with cellulase under microemulsion system (ICM) was synthesized and applied to improve the hydrolysis efficiency of MCC combing with ball milling.

2. Experimental

2.1. Materials

MCC and cellulase (R-10) were purchased from Dingguo Biologic Technique Company (Beijing, China). PVA with 1750 ± 50 degree of polymerization and 98% of degree of hydrolysis was purchased by Yufeng Chemical Reagent Glass Instrument Co. Ltd. (Changsha, China). All other agents used were of analytic grade.

2.2. Immobilized cellulase by polyvinyl alcohol/Fe_2O_3 magnetic nanoparticle

The magnetic Fe_2O_3 nanoparticle was prepared as described in the literature (Chastellain, Petri, & Hofmann, 2004). PVA/Fe_2O_3 nanoparticle-bound immobilized cellulase was prepared by cyclically freezing and thawing procedure under microemulsion system. PVA (20 mg) was dissolved in 4 mL sodium citric acid buffer (0.05 M,

* Corresponding author. Tel.: +86 731 88821721; fax: +86 731 88821721.
** Corresponding author. Tel.: +86 731 88821565; fax: +86 731 88822606.
E-mail addresses: zyh20@hotmail.com (Y. Zhu), xml05@126.com (X. Liu).

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pH 4.5) at 93 °C for 4 h and cellulase was added to a final concentration of 25 mg mL^{-1} at room temperature, followed by being stirred for 10 h at 4 °C. Magnetic Fe_2O_3 nanoparticle was then added with the mixing mass ratio of PVA vs Fe at 50/1. This aqueous solution was dropped into 100 mL silicone oil with span80 (4 g) by thoroughly stirring with a homogenizer at 4 °C 5000 rpm for 30 min. A water-in-oil (w/o) emulsion was formed and was further frozen at −60 °C for one night and then allowed to thaw on ice. After three freezing–thawing cycles, the PVA/Fe_2O_3 nanoparticle-bound immobilized cellulase was extracted by acetone (1:1 volume ratio of the suspension to acetone) and rinsed twice thoroughly with absolute ethanol and then twice with distilled water. Thereafter, the immobilized cellulase was vacuum dried and was kept at 4 °C for further investigations.

2.3. Characterization of ICM

The morphology of ICM was viewed by transmission electron microscopy (TEM, JEOL-1230, Japan) at 100 kV (Can, Fu, Xie, & Yao, 2010), and the diameter and size distribution of ICM after diluted with pure water was measured by a Malvern Zetasizer 3000HS (Malvern, UK) at 25 °C and pH 4.5. The structure of ICM was detected by Fourier transform infrared (FTIR) spectrometer (Rayleigh WQF-410) with the KBr pellet technique. The saturation magnetization of ICM was examined at room temperature by HH-15b vibrating sample magnetometer (VSM, I_{max} = 50 A, $P \leq 6$ kW, H_{max} = 15,000 Oe, sensibility between 6 and 5×10^{-5}) (Hong et al., 2008), made by Nanjing University Instrument Plant.

2.4. Degradation of MCC in ball mill reactor

The degradation process of MCC was completed in the planet stainless steel ball mill reactor (100 mL volume, QM-ISPO4, Nanjing university instrument plant, China) with 100 stainless steel beads having a diameter of 0.6 mm and a mass of 500 mg. The reaction solution containing cellulase (free or immobilized cellulase), 0.5 g MCC and 20 mL sodium citric acid buffer (0.05 M, pH 4.5) was sealed in the reactor and milled (300 rpm) for 6 h at room temperature. Ball milling without cellulase addition or cellulase hydrolysis without ball milling were also carried out as controls. Then the reaction solution was centrifuged at 10,000 rpm for 5 min, and the supernatant was collected for glucose measurement. To examine the reusability of enzymes, the retained mud containing ICM or free cellulase was put back into the reactor with 0.5 g MCC and 20 mL medium supplementation and the reaction was carried out at the same condition.

2.5. Assay of cellulase activity

The cellulase activity was defined as the amount of cellulase that catalyzes filter paper (1 cm × 6 cm quantitative filter paper, Xinhua, China) to generate 1 μmol glucose per minute in 1 h on the condition of 50 °C, pH 4.5. The glucose produced was measured according to the DNS assay (Ghose, 1987). The content of cellulase in ICM was determined as described by Kjeldahl in avoidance of PVA and Fe_2O_3 interference (Ng, 2004). All analytic experiments were repeated in triplicate.

2.6. The investigation of structure transformation of MCC

Native MCC, MCC milled with no cellulase, MCC milled with ICM were rinsed thoroughly by pure water and lyophilized. The morphologies of the three kinds of samples were viewed by environmental scanning electron microscopy (ESEM) using an FEI Quanta 200 FEG instrument without any coatings. The observations were performed in high-vacuum mode, using an accelerating voltage of 25 kV. The degree of crystallinity of the MCC samples was detected using a Rigaku D/max-2200 X-ray diffractometer (Rigaku Co., Tokyo, Japan). The measuring conditions were as follows: target, Cu Kα; voltage, 40 kV; current, 40 mA; scan range, 2θ = 10–70°. The apparent crystallite size (ACS) was calculated as described by Heinze and Liebert (2001).

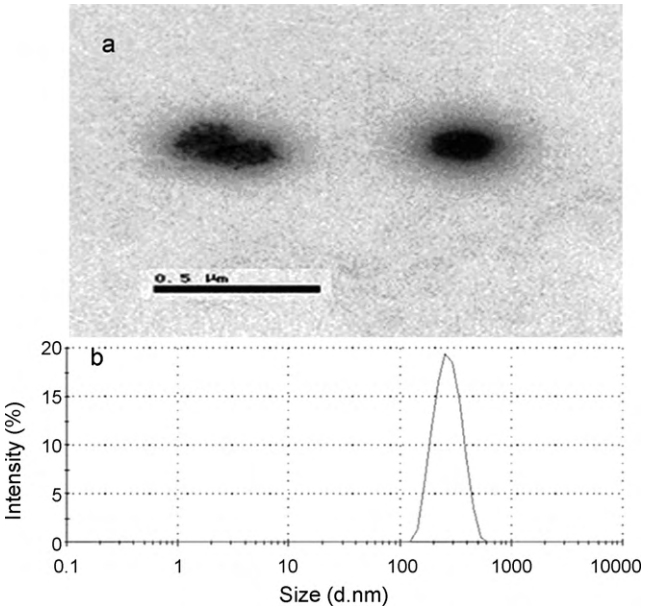

Fig. 1. Transmission electron microscopy image (TEM, ×50,000) (a) and the size distribution (b) of ICM by Zetasizer.

3. Results and discussion

3.1. Characterization of ICM

The morphology of nanoparticle immobilized with cellulase under microemulsion system was investigated by TEM and found that the spherical complex was mainly constructed by the accumulation of 10 nm Fe_2O_3 nanoparticle with the diffused polymer as indicated in Fig. 1.

The size of the spherical complex was detected by Zetasizer and the result indicated that it was approximate 270 nm, which was smaller than that of immobilized cellulase on PVA/Fe_2O_3 nanoparticle made in homogeneous aqueous solution system (IC, 1 μm) (Liao et al., 2008). The results demonstrated that the water-in-oil microemulsion system can provide an environment for the formation of spherical complex with smaller size. The saturation magnetization of ICM at 25 °C measured by vibrating sample magnetometer was 4.87 A m^2 kg^{-1}, which indicated that ICM can be easily separated from the reaction mixture for reuse due to its magnetism. Finally, FTIR spectra of ICM and PVA/Fe_2O_3 nanoparticle were depicted (Fig. 2). After immobilized with cellulase, the absorption peaks of ICM at 3400 cm^{-1} was stronger than that of PVA/Fe_2O_3 nanoparticle due to the superposition of stretching vibration of O-H and N-H (Tawansi, El-Khodary, & Abdelnaby, 2005; Wu et al., 2005). From the stronger peaks at 1658 cm^{-1} (O=C-stretching vibration) and lower peaks at 1260 cm^{-1} (–OH bending vibration) in Fig. 2a, it can be concluded that carbonyl group on a protein chain of cellulase can interact with hydroxyl group on the PVA chain in ICM.

Table 1
Property of free cellulase, immobilized cellulase on PVA/Fe_2O_3 magnetic nanoparticle under aqueous solution system (IC) and microemulsion system (ICM).

	Total activity (U)	Enzyme amounts (mg)	Specific activity (U mg^{-1})	The retained specific activity (%)
Free enzyme	57.79 ± 2.34[a]	6.54 ± 0.30	8.87 ± 1.07	100
IC	24.27 ± 1.64	4.31 ± 0.13	5.63 ± 1.26	63
ICM	29.10 ± 1.23	3.58 ± 0.23	8.13 ± 1.72	91

[a] All values are means of duplicates ± standard deviation.

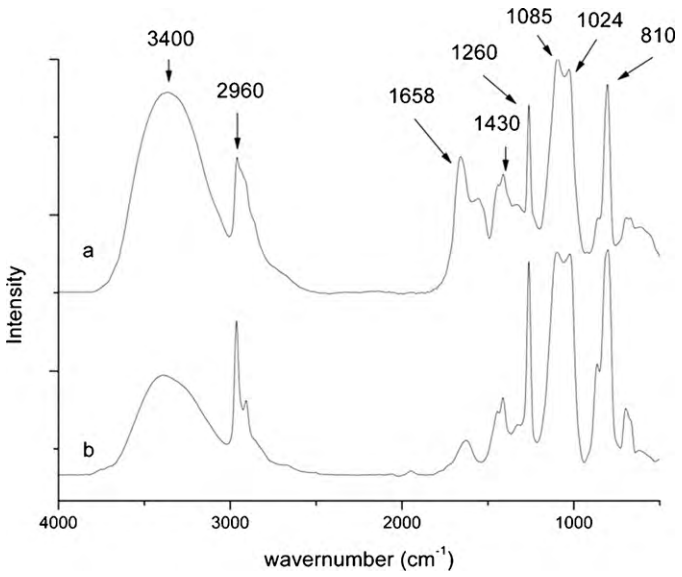

Fig. 2. Infrared transmission spectra of ICM (a) and PVA/Fe_2O_3 magnetic nanoparticle (b).

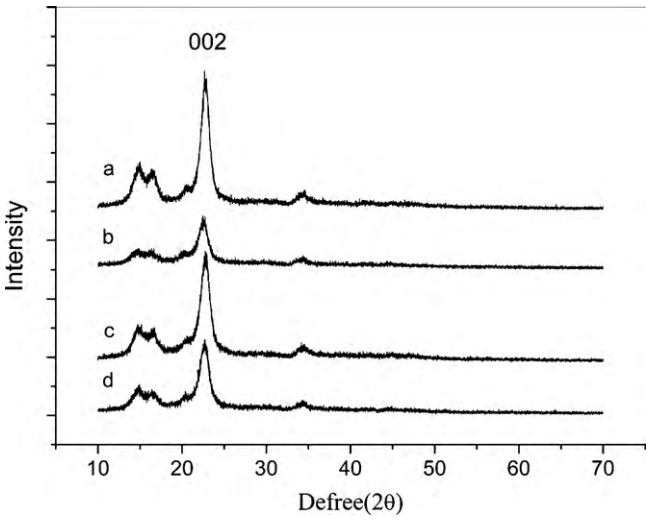

Fig. 3. X-ray diffraction intensity profiles of MCC under different treatments: native MCC (a), MCC milled without cellulase (b), MCC milled with ICM (c) and MCC milled with free cellulase (d).

3.2. Catalytic property of ICM

Theoretically, ICM with smaller size can improve enzymatic efficiency by increasing contact area between cellulase and substrate because of higher surface area. We found that the retained specific activity (91%) of ICM was not only higher than that of IC (63%) (Table 1) but also higher than that of reported data (Dincer & Telefoncu, 2007; Wu et al., 2005;). The result, which is consistent with our expectation, suggested that the novel procedure for immobilized cellulase preparation was effective.

3.3. The degradation efficiency of MCC by ICM with wet ball milling

The degradation efficiency of MCC by wet ball milling and ICM was examined by measuring glucose yield (Table 2). It can be found that the method of merely ball milling of MCC could produce about 0.07 mg mL^{-1}, while that of ball milling with enzyme (free cellulose or ICM) could obviously increase glucose yield (2.10 and 1.89 mg mL^{-1}, respectively). The similar degradation efficiency caused by free cellulase and ICM combining with ball milling further suggested the ICM maintain a high specific activity of cellulase. It is important to point out that combing ball milling and ICM hydrolysis can produce more glucose than the sum of that produced by individual treatment for the existence of synergistic effect (Table 2).

The structure transformation of MCC was investigated so as to explore the possible mechanism of the above synergistic effect. From the X-ray diffraction detection results (Fig. 3), it was found that the diffraction peak around $2\theta = 22.5^\circ$ corresponding to the maximum peak of cellulose (002) crystal plane, as well as two overlapped weaker peaks around $2\theta = 14.7^\circ$ and 16.2° corresponding to the peak of cellulose (1 0 1) and ($1\,0\,\bar{1}$) lattice planes, became blunter after milling, with ICM or not (Fig. 3a versus b and c). Clearly, long-range crystalline order was decreased by mechanical milling and more amorphous characteristic might be obtained.

Correspondingly, ESEM observation showed a lubricous fibrous morphology of initial MCC (Fig. 4a and b), while milled MCC was ground to form irregular rough surface (Fig. 4c and d). However, the milling process cannot hydrolyze MCC efficiently as it still keeps consecutive structure of MCC at the surface. It was reported that the more amorphous cellulose powder is more active in chemical modification (Zhang, Liang, & Lu, 2007). The point

Table 2
Glucose yield and retained specific activity of cellulase by different treatment methods.

Treatment method			Glucose produced (mg mL^{-1})	Cellulase retained specific activity[a] (%)
Ball milling	Free cellulase	ICM		
+	–	–	0.07 ± 0.02[b]	–
+	+	–	2.10 ± 0.15	24.10 ± 2.94
+	–	+	1.89 ± 0.04	83.80 ± 3.40
–	+	–	0.90 ± 0.13	93.90 ± 1.61
–	–	+	0.46 ± 0.08	94.52 ± 2.50

[a] The retained specific activity (%) is defined as the ratio of the specific activity of cellulase after and before treatment.
[b] All values were showed as means of duplicates ± standard deviation.

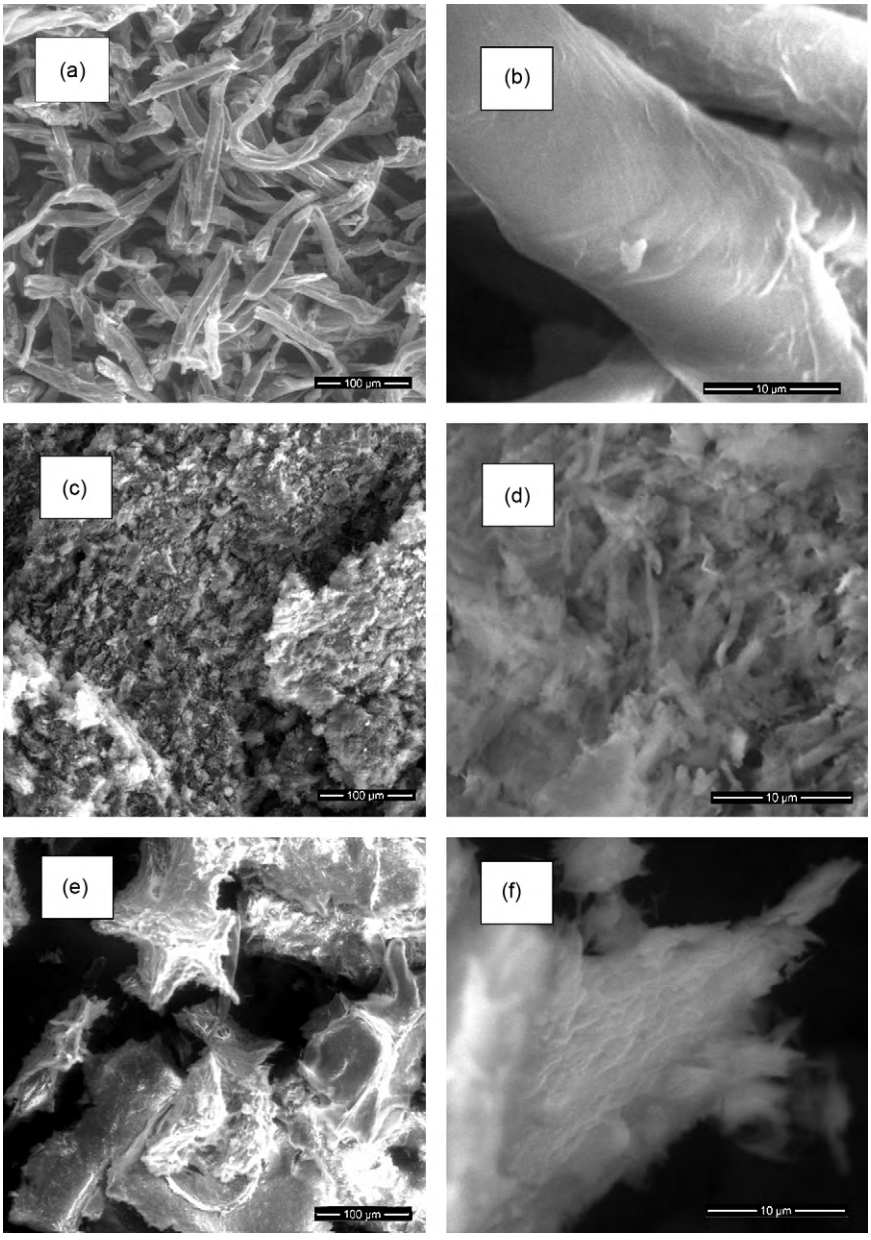

Fig. 4. The environmental scanning electron micrographs of MCC with different treatment: native MCC (a and b), MCC milled without cellulase (c and d) and MCC milled with ICM (e and f). The micrographs of a, c and e are of lower magnification (×300) and the micrographs of b, d and f are of higher magnification (×4500).

was supported by ESEM image of MCC: The fibrous structure of MCC was destroyed after treatment with milling and ICM at the same time (Fig. 4e and f). This was also confirmed by the different ACS of MCC, which showed that milled MCC simultaneously treated with ICM (1.86 nm) was smaller than that of merely milling one (2.04 nm). So, ball milling loosed the crystalline surface and made amorphous structure, which was more accessible by ICM. With the enzyme hydrolysis of MCC, the fibrous structure of cellulose was broken, which is considered to be adoptable for better mechanic and chemical modification. These two reactions proceeded repeatedly and synergistically until the MCC was degraded efficiently.

3.4. The reusability of ICM in ball mill reactor

We noticed that the retained specific activity of ICM was 3.5 times than that of free cellulase as MCC were enzymatically degraded by combining with wet ball milling (Table 2), which suggested that the immobilization method can efficiently maintain enzyme activity and bring ICM reusability characteristics in a ball mill reactor. The result of ICM reusability and the glucose productivity in Fig. 5 further confirmed it. It was found that the retained enzyme activity of ICM retained over 40% original activity even after four cycles of reuse, while that of free cellulase decreased so sharply that it could not be detected after only three cycles (Fig. 5a). In consistent with the result of retained enzyme activity in Fig. 4a, the conversed glucose by free cellulase had fallen to 0.1 mg mL^{-1} after four cycle treatments, which is the same level produced by merely ball milling treatment for its low conversion activity (Fig. 5b and Table 2). Whereas, 1.1 mg mL^{-1} glucose was produced in the case of ICM, which is approximately 10 times as that produced by free cellulase (Fig. 4b). The results demonstrate that ICM has the stability and reusability characteristics in a ball mill reactor, which was important for the potential industry application.

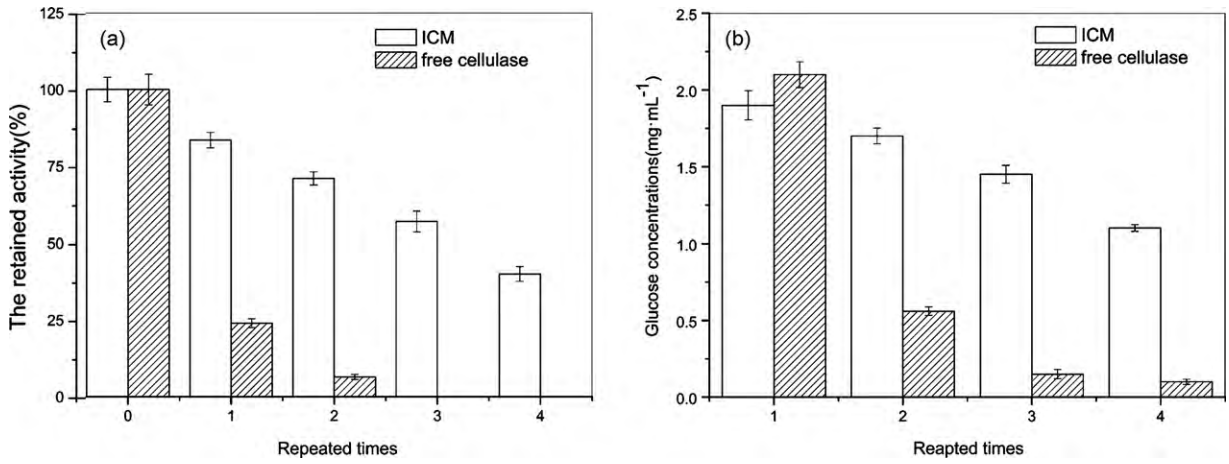

Fig. 5. The retained activity (a) and the glucose productivity (b) of ICM and free cellulase after four reused cycles in ball mill reactor.

4. Conclusion

In this paper, cellulase was immobilized on PVA/Fe_2O_3 magnetic nanoparticle by frozen and thawed method so as to form ICM with small size and excellent specific activity. ICM with good stability and enough reusability can significantly improve the efficiency of MCC degradation by combining with milling treatment for the synergy effect. These results demonstrate that the combined strategy of simultaneous ICM hydrolysis and ball milling can be recommended for efficient conversion of cellulose.

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