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# Efficient cellulase production from low-cost substrates by *Trichoderma reesei* and its application on the enzymatic hydrolysis of corncob

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**In this work, cellulase production by *Trichoderma reesei* CH11 using different low-cost raw materials as carbon sources and its application on the enzymatic hydrolysis of corncob were systematically investigated. Among all the studied carbon sources, rice straw was the most suitable one for *T. reesei* to produce cellulolytic enzymes. In addition, adding wheat bran and ammonium sulfate improved the cellulase activity and reduced the production cost. *T. reesei* was cultivated at 28°C and 160 rpm for five days to generate the liquid enzyme for hydrolysis. The enzyme activity of FPase reached 2.40 IU/mL, which was improved about 21% compared with that obtained from original culture medium without wheat bran or ammonium sulfate. Pretreated corncob was then hydrolyzed by this crude enzyme. After optimization of solid-to-liquid ratio and dilution rate of crude enzyme, 95.7% of hydrolysis yield was obtained. This work could offer one promising low-cost platform for bio-refinery.**

**Key words:** *Trichoderma reesei*, cellulase, low-cost substrate, corncob, enzymatic hydrolysis.

## INTRODUCTION

Recently, the concept of bio-refinery was a hot topic since this potential technology could help in solving the energy crisis or environmental problem (FitzPatrick et al., 2010). Lignocellulosic biomass, the most common substrate for bio-refinery, could be considered as the next generation of energy source (Kumar et al., 2008).

However, lignocellulosic biomass cannot be utilized by most microorganisms for fermentation directly. Thus, hydrolysis is usually necessary for the utilization of lignocellulosic biomass and thus it is the key factor that determines the possibility and profits of bio-refinery (Wyman et al., 2005). Among the two main kind of hydrolysis (chemical and enzymatic one), enzymatic hydrolysis was more efficient and "green" for environment, and thus was the focus for many works (Rubin, 2008). However, the cost of commercial cellulase was too expensive for industrial application (Lynd et al., 2008). Unfortunately, little work focused on the low-cost production of cellulase for enzymatic hydrolysis.

To reduce cost of cellulase production, using low-cost fermentation substrates could be a promising strategy. And among various microorganisms, *Trichoderma reesei* was shown to be an ideal one for cellulases production (Martinez et al., 2008). In this work, the efficient

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#Both authors have the same contribution and are co-first authors.

production of cellulases by *T. reesei* strains using ramie powder, bamboo, rice-straw, corn-straw, corncob and cotton stem as the inexpensive carbon source, respectively, and the characteristics of the produced cellulases were investigated. In addition, the medium for cellulases production by adding wheat bran and ammo-nium sulfate to reduce the cost was further evaluated. The produced cellulases were used to hydrolyze the pretreated corncob. At the same time, the effect of solid-to-liquid ratio and dilution rate of liquid enzyme on the enzymatic hydrolysis were also evaluated. This work can offer one low-cost platform for bio-refinery and is beneficial for its future industrialization.

## MATERIALS AND METHODS

### Microorganism for cellulase production

*T. reesei* CH11 (stored by the Laboratory of Energy and Biochemical Engineering, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences) was used for cellulases production. It was maintained on potato dextrose agar (PDA) and stored at 4°C until use.

### Lignocellulosic biomass and its pretreatment

In this work, bamboo and ramie were obtained from southern China while rice straw, corn straw, corncob and cotton stem were collected from Liaoning Province of China. All these lignocellulosic biomasses were air dried and crushed into particles with diameter less than 2 mm, and they were used as carbon source directly without any pretreatment.

Pretreated corncob was offered by ZHONGKE New Energy Co., LTD (Ying-Kou, China) and used as the raw material for enzymatic hydrolysis in this work. According to ZHONGKE New Energy Co., LTD, the pretreatment method was the modification of the previous work (Teramoto et al., 2008) that used organic solvents to treat the lignocellulosic biomass. The composition of the pretreated corncob was (% w/w): cellulose 57%, hemicellulose 31%, lignin 7%, and others 5%.

### Medium

The medium for seed culture contained 1.0% (w/v) glucose, 0.1% (w/v) peptone, 0.05% (w/v) citric acid, 2% (v/v) Vogel's Medium N (Vogel, 1964), and 0.015% (v/v) Tween 80. The initial pH of the medium was 5.0 to 5.5.

The medium for induction and production of enzyme contained 0.1% (w/v) glucose, 0.1% (w/v) peptone, 0.05% (w/v) citric acid, 2% (v/v) Vogel's Medium N, and 0.015% (v/v) Tween 80. Otherwise to culture *T. reesei*, 2%(w/v) ramie powder, 2% (w/v) bamboo, 2% (w/v) rice-straw, 2% (w/v) corn-straw, 2% (w/v) corncob and 2% (w/v) cotton stem as different carbon source were added into the culture media, respectively. The media was then autoclaved at 121°C for 20 min.

### Cellulase production by *T. reesei*

Spores of *T. reesei* were incubated into 500 mL flak containing 200 mL pre-cultural medium metioned above and shaken at 30°C and 200 rpm for 36 h to prepare seed cultures. Then, 10% (v/v) seed cultures were translated into flask containing 100 mL fermentation medium. Cultivation was performed in a rotary shaker at 28°C and 160 rpm for five days. After fermentation, the fermentation broth was centrifuged at 4000 rpm for 10 min and the clear supernatant stored at 4°C was used as liquid enzyme for hydrolysis later.

### Determination of enzyme activity

The enzyme activity was determined by using filter paper as reaction substrates. A solution (1 mL) of 50 mg filter paper, 800 µL of 0.1 M citric acid buffer (pH5.0) and 200 µL enzyme liquid were mixed and incubated at 50°C for 30 min (Ghose, 1987). To determine CMCase activity, 800 µL of 1% CMC-Na (in 0.1 M citric acid buffer, pH 5.0) and 200 µL liquid enzyme were mixed and then incubated at 50°C for 30 min (Ghose, 1987). To determine *β*-glucosidase activity, 800 µL of 1% salicylic acid (in 0.1 M citric acid buffer, pH 5.0) and 200 µL liquid enzyme were mixed and then incubated at 50°C for 30 min (Ghose and Bisaria, 1979).

After hydrolysis, the mixture was boiled for 5 min to terminate the reaction. The residual sugars concentration was determined by DNS methods (Miller, 1959). The FPase, CMCase or *β*-glucosidase activity was defined as the amount of cellulase required to produce one µmol reducing sugars in one minute. All the measurement was performed at least twice and data were expressed as the average.

### Enzymatic hydrolysis by the enzyme produced by *T. reesei*

Enzymatic hydrolysis of pretreated corncob was performed in 250 ml conical flasks, containing a 100 mL liquid enzyme and 10 g corncob. The initial pH of enzymatic hydrolysis was kept natural and the hydrolysis temperature was 50°C. The hydrolysis was carried out for 72 h without specification.

### Sugar concentration analysis by HPLC

Concentration of various sugars (D-glucose, D-xylose, D-cellobiose, etc.) in the corncob hydrolysate were analyzed by HPLC (Waters 2685 systems, Waters Corp., USA), with a RI detector (Waters 2414), and on Shodex Sugar SH-1011 column using 0.5 M $H_2SO_4$ solution at a flow rate of 0.5 ml/min at 50 °C.

The yield of enzymatic hydrolysis was defined according to previous work (Chen et al., 2007), that is:

Hydrolysis yield (%) = reducing sugar×0.9×100/ polysaccharide in substrate.

## RESULTS AND DISCUSSION

### Cellulase production by *T. reesei*

As mentioned above, to fulfill effective and efficient enzymatic hydrolysis for bio-refinery, suitable cellulase is important (Himmel et al., 2007). Two aspects should be considered, one is the fermentation cost for cellulase production, and undoubtedly low-cost substrate is beneficial for reducing its cost. On the other hand, the cellulase activity should not be influenced by the low-cost substrate used. Base on both points, the effect of various fermentation substrates including carbon sources and nitrogen sources on cellulase production by *T. reesei* was evaluated.

**Table 1.** Cellulase production by *T. reesei* using different carbon sources in culture medium.

| Carbon source (2%, w/v) | Enzyme activity (IU/mL) | |
|---|---|---|
| | FPase | CMCase |
| Bamboo | 0.38 | 0.87 |
| Ramie powder | 1.67 | 1.27 |
| Rice-straw | 1.97 | 1.34 |
| Corn-straw | 1.39 | 1.25 |
| Corncob | 1.78 | 1.22 |
| Cotton stem | 1.42 | 0.88 |

### *Carbon sources for cellulase production*

Carbon sources are the most important nutrient materials for fermentation and take a great part in the cost of fermentation substrate. Thus, an effective and low-cost carbon source could undoubtedly reduce the fermentation cost significantly. Compared with other low-cost raw materials, lignocellulosic biomass could be considered a promising one for its great availability in nature and renewable characteristics (Rubin, 2008). Six kinds of lignocellulosic biomass as the carbon sources for cellulase production by *T. reesei* were evaluated in this work, namely, bamboo, ramie powder, rice straw, corn-straw, corncob and cotton stem, respectively.

The enzyme activity of cellulase generated by *T. reesei* on the medium containing the above carbon sources was evaluated (Table 1). The highest cellulase activity was obtained on the medium containing rice straw; PFase and CMCase activity reached 1.97 and 1.34 IU/mL, respectively. Thus, rice straw was the most suitable one for cellulase production by *T. reesei* among these six carbon sources, which was in good accordance to previous works (Karimi et al., 2006; Kim and Dale, 2004).

Rice straw is one common agricultural residue in South China. Also, it is cultivated widely among the tropic and semi-tropic area throughout the word. More than 50 countries generate at least 100,000 tons of rice annually (Kadam et al., 2000). It is also worth noting that the price of rice straw is not expensive. For example, in China, its average price was merely about 300 RMB/ton. Thus, rice straw could be one promising feedstock for industrial production of cellulase for bio-refinery field.

### *Additive nutrient for cellulase production*

Besides carbon sources, adding some suitable additive nutrients could also help in increasing the cellulase activity after fermentation (Wen et al., 2005). In this work, to further improve the cellulase activity, wheat bran was chosen as additive nutrient for cellulase production. Bran is the main byproduct of wheat processing and it is rich in carbohydrates, protein, vitamins and minerals, which is more suitable for *T. reesei* growth (Brijwani et al., 2010; Hassan and Bullerman, 2009). Different wheat bran concentration (0 to 3.0 % w/v) was added into fermentation medium and its effect on the cellulase activity was measured. As shown in Figure 1, additional wheat bran was actually useful to improve the cellulase activity. The highest CMCase and FPase activity was got (1.09 and 2.48 IU/mL respectively) when the wheat bran concentration was 1.0% (w/v). However, further increase of the wheat bran did not contribute to further increase in cellulase activity. Thus, the optimal wheat bran concentration for cellulase production was 1.0%.

### *Nitrogen sources for cellulase production*

Initially, the organic nitrogen peptone was used as nitrogen source for cellulase production by *T. reesei* since it grows fast on the medium containing organic nitrogen source (Ryu and Mandels, 1980). In order to reduce the cost of nitrogen source, inorganic nitrogen ammonium sulfide was used instead of peptone, and the effect of its concentration was also evaluated (Figure 2). When the concentration was lower than 2 g/L, the enzyme activity of cellulase increased as the concentration of ammonium sulfide was higher. However, when its concentration continued to increase, both CMCase and FPase activity decreased instead. It is possible that higher ammonium sulfide concentration would inhibit the microbial growth of *T. reesei*. Overall, the highest cellulase activity was got when the ammonium sulfide concentration was 2 g/L. Obviously, ammonium sulfide could replace expensive peptone for cellulase production.

### *Calculation on the fermentation cost for cellulase production*

Base on the experiment above, three low-cost nutrients including carbon and nitrogen sources were used. To reduce the cost of cellulase production, ammonium sulfate was used to take the place of peptone. Besides, wheat bran and other specific components [0.1% (w/v) glucose, 0.2% (w/v) ammonium sulfate, 0.05% (w/v) citric acid, 2% (v/v) Vogel's Medium N, 0.015% (v/v), Tween 80, 1% (w/v) wheat bran, 2% (w/v) rice straw] were also added into the medium. In China, the price of ammonium sulfate, rice straw, wheat bran, is about 1000, 300, and 1200 RMB/ton, respectively. Thus, the substrate used for cellulase production in this work was low-cost and suitable for bigger application.

When the flasks containing 100 mL of enzyme production medium were inoculated (10% (v/v) inoculum) and cultivated in shaker at 28°C and 160 rpm for 5 days, interestingly, the FPase activity could reach 2.40 IU/mL, and the activity improved about 21% when compared

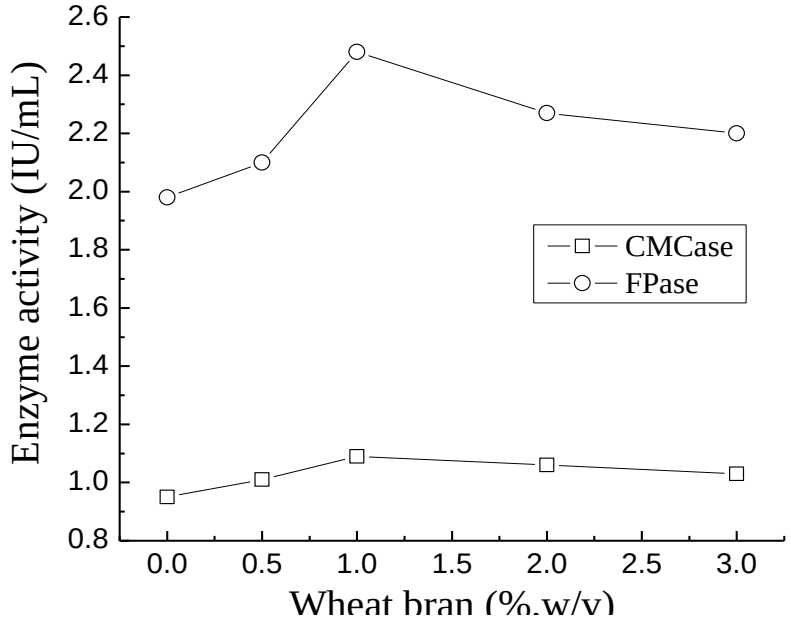


**Figure 1.** The influence of different concentrations of wheat bran. (□) CMCase; (○) FPase. Xiong et al. (2012).

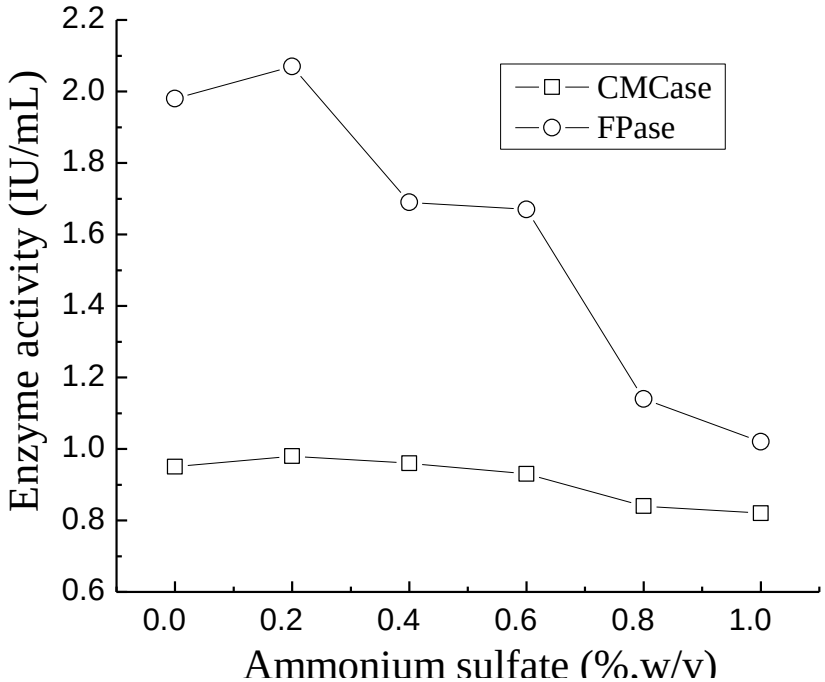


**Figure 2.** The influence of different concentrations of Ammonium sulfate. (□) CMCase; (○) FPase: Xiong et al. (2012).

with original culture medium without wheat bran or ammonium sulfate. Thus, not only the cost is low for application, using this low-cost fermentation system but was also beneficial for the hydrolysis itself.

Overall, the cost of the substrates mentioned above is low and thus could be ideal feedstock for cellulase

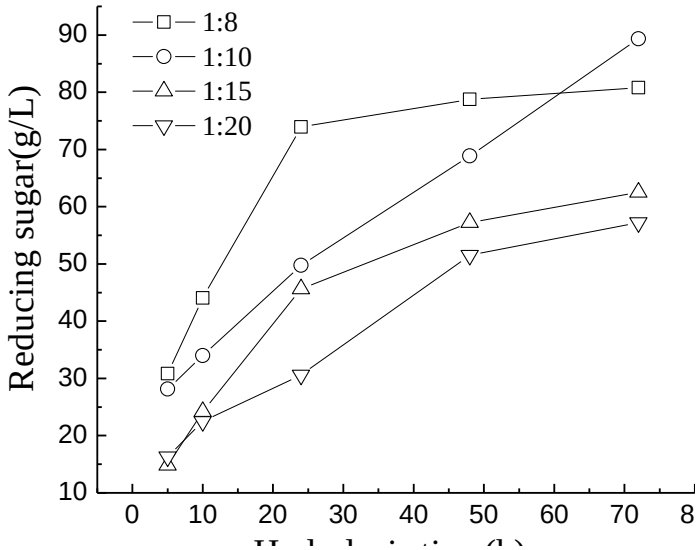


**Figure 3.** The influence of different solid-to-liquid ratio. □, 1:8; ○, 1:10; △, 1:15; ▽, 1:20: Xiong et al. (2012).

production.

## Enzymatic hydrolysis of corncob by cellulase produced by *T. reesei*

In northern and middle China, corncob is one abundant lignocellulosic material that could be used for bio-refinery industrialization (Yinbo et al., 2006). In this work, pretreated corncob kindly offered by ZHONGKE New Energy Co., LTD (Ying-kou, China) was used as the model material for enzymatic hydrolysis by cellulase produced by *T. reesei*.

### *Effect of solid-to-liquid ratio*

Traditionally, solid-to-liquid ratio was one important factor that influences the efficiency of enzymatic hydrolysis. Too low solid-to-liquid ratio might result in low sugar concentration of corncob enzymatic hydrolysate which is not suitable for fermentation and industrialization. Thus, it is critical to increase the solid-to-liquid ratio to get high hydrolysis efficiency (Roche et al., 2009). However, too high solid-to-liquid ratio would bring substrate inhibition and influence the mass transfer during hydrolysis.

To get the optimal solid-to-liquid ratio, its effect on the hydrolysis yield was evaluated. As shown in Figure 3, during the first 24 h of hydrolysis, the fastest hydrolysis rate was at the system with solid-to-liquid ratio of 1:8. At this system, the reducing sugars concentration was 73.9 g/L after 24 h of hydrolysis. However, after that, its hydrolysis rate became much slower. It is possible that too high solid-to-liquid ratio brought substrate inhibition to the enzymatic hydrolysis and made the hydrolysis rate lower. In contrast, at the system with solid-to-liquid ratio of 1:10, the hydrolysis rate was influenced little by the hydrolysis time. After 72 h of hydrolysis, the reducing sugars concentration in this system could reach 89.4 g/L. At the system with solid-to-liquid ratio of 1:15 and 1:20, the reducing sugar concentration was merely 62.5 g/L and 57.2 g/L, respectively after 72 h of hydrolysis.

### *Effect of diluted rate of liquid enzyme*

As mentioned above, the low-cost liquid enzyme produced by *T. reesei* was used for enzymatic hydrolysis. When the hydrolysis was carried out, the liquid enzyme was mixed with the hydrolysis system. For different fermentation, the reducing sugars required in the fermentation medium were different (Cheng et al., 2008; Huang et al., 2009, 2012).

In this work, we simply added the liquid enzyme into the hydrolysis system. Thus, the amount of liquid enzyme added into the system might influence the hydrolysis efficiency. The hydrolysis system was designed as follows: (1) 10 g corncob, 100 mL crude liquid enzyme; (2) 10 g corncob, 50ml enzyme liquid, 50 mL water; (3) 10g corncob, 25 mL enzyme liquid, 75mL water. Surprisingly, we found that tap water was more suitable for enzymatic hydrolysis than pure water.

Also, it is not necessary to adjust the initial pH of tap water. The enzymatic hydrolysis was carried out at 50°C and 150 rpm. As shown in Figure 4, using crude liquid enzyme without dilution for the hydrolysis of corncob, the

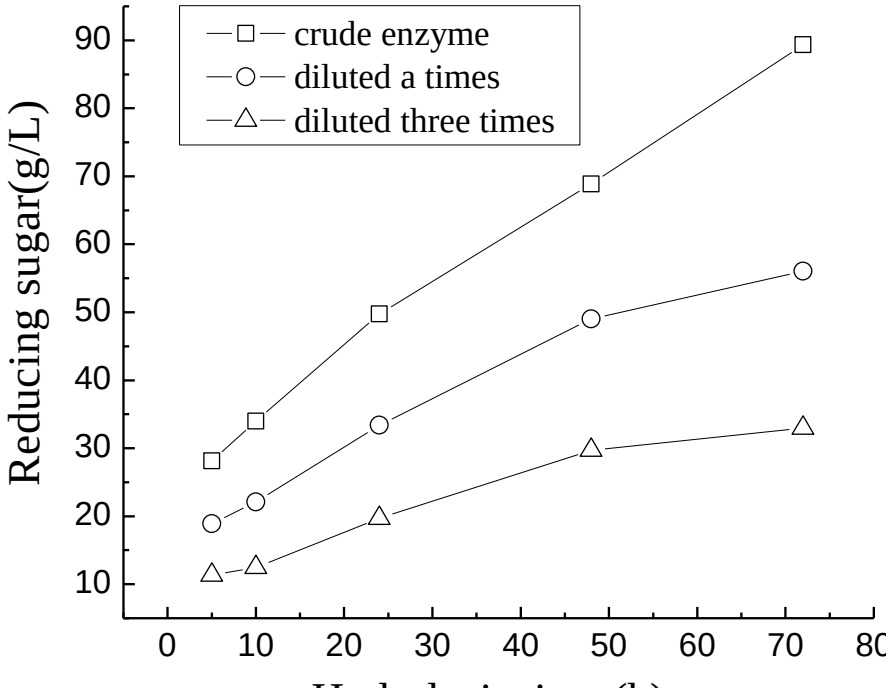


**Figure 4.** The influence of different diluted times. □, crude enzyme; ○, diluted a times; △, diluted three times: Xiong et al. (2012).

**Table 2**. Sugars in the hydrolysis system with a solid-liquid ratio of 1:10.

| Sugars in hydrolysate | Concentration (g/L) | Hydrolysis yield (%) |
|---|---|---|
| Cellbiose | 7.8 | |
| Glucose | 65.9 | 95.7 |
| Xylose | 20.0 | |

generation of reducing sugars was faster. After 72 h of hydrolysis, 56.0 and 33.0 g/L reducing sugars were obtained on the system with liquid enzyme diluted for one time and three times, respectively. Obviously, it is not necessary to use medium with high sugar concentration in many fermentation. Thus, in spite that using dilute liquid enzyme for hydrolysis might result in lower sugar concentration obtained, the enzymatic hydrolysis cost could be saved by using lower amount of cellulase.

### *Analysis of sugar composition of enzymatic hydrolysate*

Generally, the sugar composition might influence the later fermentation process. For example, many strains could not utilize xylose for growth and products accumulation (Rubin, 2008). Thus, after enzymatic hydrolysis, we further measure the sugar composition of the enzymatic hydrolysate by HPLC and the results were shown in Table 2.

As shown in Table 2, the enzymatic hydrolysate in this work mainly contained three kinds of sugar, namely, glucose, xylose, and cellobiose. The total sugar concentration of this hydrolysate was 93.7 g/L. As the substrate of enzymatic hydrolysis, composition of corncob was as follows: 56.7% cellulose, 31.5% hemicellulose, 7.1% lignin, and 4.7% others. As shown by the experimental results, the cellulose of corncob was completely degraded and most of hemicellulose was also hydrolyzed. After 72 h of hydrolysis, 65.9 g/L glucose and 20.0 g/L xylose were generated, and the hydrolysis yield could reach 95.7%.

Pretreated corncob was also hydrolyzed by other work using *Trichoderma reesei* ZU-02, and its hydrolysis yield was merely 67.5% (Chen et al., 2007). Although the hydrolysis rate could be improved to 83.9% by adding cellobiase from *Aspergillus niger* ZU-07 (Chen et al.,

2007), this would further increase the cost of enzyme and might not be suitable for the industrialization of bio-refinery. In contrast, in the present work, we merely used the liquid enzyme generated by *T. reesei* CH11, and this could save the cost in further application.

## Conclusion

In this work, one promising method for cellulase generation and its enzymatic hydrolysis system was built in order to offer a suitable platform for bio-refinery. After enzymatic hydrolysis by *T. reesei* CH11, the hydrolysis yield could reach 95.7% and the total reducing sugars obtained were close to 100 g/L. All the fermentation substrates used in this work was low-cost one and also the liquid enzyme generated mixed with low-cost tap water was used for enzymatic hydrolysis. Overall, this process was green and easy for operation. Thus, it could be further applied in industrialization. Further work should focus on the pretreatment method to make the enzymatic hydrolysis more efficiency. Also, the modification of *T. reesei* is also necessary to have a higher hydrolysis yield.

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