

Cellulolytic enzymes produced from ramie stalks feedstock and their synergistic effect on saccharification of ramie stalks

Chunliang Xie

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Cha Cao

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Chao Xu

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Wenbing Gong

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Zuohua Zhu

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Yingjun Zhou

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Li Yan

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Zhenxiu Hu

Chinese Academy of Agricultural Sciences Institute of Bast Fiber Crops

Yuande Peng (✉ ibfcpyd313@126.com)

Institute of Bast Fiber Crops

Research

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Abstract

Background: The high cost of cellulase is one of the main obstacles hindering the large-scale biorefining of lignocellulosic biomass. Screening strains with high cellulase producing capability and improving enzymatic hydrolysis technology are important methods to reduce the cost of enzymes. A powerful strategy to lignocellulose as a feedback combined with different enzymes synergism can reduce the time of designing high-efficient enzyme mixtures and significantly improve saccharification of specific substrates.

Results: After optimization, the maximum CMCase and FPA produced by *Trichoderma reesei* reached to 6.06 IU/mL and 0.085 IU/mL, respectively. The hydrolysis capability of *Trichoderma reesei* was induced at the presence of ramie stalk not wheat bran. Synergistic effect was observed as enzymes produced by *Trichoderma reesei* and *Aspergillus niger* were incubated together, and the highest reducing sugars yield was achieved when enzyme cocktail was prepared at the ratio of 1:1. In particular, reducing sugars yield reached to 417 mg/g dry substrate was achieved after hydrolyzing the substrate by prepared enzyme cocktail, which were 1.36 - 3.35 folds higher than different single enzymes.

Conclusion: This study indicated that (1) the carbon source exhibited a directional induction effect on the types of cellulolytic enzymes secreted by microorganisms; (2) cellulolytic enzymes from different sources could synergistically strengthen the enzymatic hydrolysis of lignocellulosic materials, providing a clue for the preparation and compounding of cellulolytic enzymes.

Background

With the decrease of fossil fuels reserves and the increasingly severe of global environmental problems, using the renewable and clean resource such as lignocellulosic feedstocks as alternative fuel has aroused widespread interest [1]. The global cellulosic ethanol market was predicted to reach to 27 billion gallons per year by 2022 [2]. However, the complex structure of lignocellulosic biomass makes it resistant to hydrolysis by cellulolytic enzymes, this causes the high cost of cellulosic ethanol production [3]. Therefore, exploring cheap and efficient technologies for converting lignocellulosic biomass into fermentable sugars has become the key to cellulosic ethanol processing.

The bioconversion of lignocellulosic biomass to target products mainly via three steps: (1) pretreatment; (2) enzymatic hydrolysis and (3) microbial fermentation [4]. As one of the crucial step, enzymatic hydrolysis of biomass require the synergistic effect of enzymes such as endoglucanase (EG), cellobiohydrolase (CBH) and β -glucosidase (BG) [5]. During this process, EG randomly hydrolyzes the glycosidic bonds in the cellulose chain to provide short chains; CBH releases cellobiose from the non-reducing end of the chain; and Cellobiose is further hydrolyzed into monosaccharides under the catalysis of BG [6]. The high cost of cellulase is a main obstacle hinders the biorefinery of lignocellulosic biomass. Therefore, the development of an efficient enzymatic hydrolysis process is essential to reduce the amount of enzymes and the cost of biomass refining [7].

Currently, commercial cellulases mainly produced by *Trichoderma* sp., but it can only produce limited β -glucosidase, the strains *Aspergillus* sp. was commonly used for β -glucosidase production [8, 9]. So far, using single natural microorganism to efficiently produce all kinds of enzymes that meets the requirement of enzymatic hydrolysis of lignocellulose is impossible. It is considered a feasible solution to compound the enzymes produced by different strains or to modify the enzymes producing pathways of the strains through genetic engineerings [10]. The synergistic effect of cellulase consortium was proved to eliminate feedback inhibition and boost enzymatic saccharification [11]. Enzymes extracted from *P. janthinellum* EMS-UV-8, *Trichoderma reesei* RUT-C30 and *Aspergillus tubingensis* were used to co-hydrolysis avicel-wheat bran, the obtained reducing sugars after hydrolyzing by the three enzymes mixture were two times higher than single enzyme at the same enzyme dosages [12]. Compared with single enzyme, the mixtures of *P. chrysogenum* P33 and commercial cellulase exhibited synergistic effect on boosting the enzymatic hydrolysis of delignified corn stover [13].

Additionally, specific cellulase mixture often not proper to all materials due to heterogeneity of lignocellulosic feedstocks in terms of composition and physical properties, which are derivated from differences in composition and content [14]. Thus, development of multi-enzyme blends which capable to perform highly-efficient degradation of specific lignocellulosic materials is necessary for reducing the demand of cellulase dosage [15].

Ramie (*Boehmeria nivea* L. Gaud) as a fiber crop that is widely planted in China, India, and other countries of southeast Asian and Pacific Rim, it can be grown on marginal lands with low irrigation and fertilizer requirements, and without competing lands with food crops, the above-ground biomass productivity reach to 14–20 Mg/ha dry matter [16, 17]. The phloem yield of ramie is accounted for only about 15% of its biomass, and the stalks reach to more than 60% [17]. Ramie stalks composed of 44% cellulose, 31% hemicellulose and 18.1% lignin, making it a suitable substrate for biofuel production [18].

The strategy of submerged fermentation (SmF) is commonly used for large scale enzymes production with the advantages of environment friendly and low costs [19, 20]. Both selecting effective cellulose-degrading organisms and optimizing operation conditions were proved as effective methods for improving the efficiency of SmF. In order to reduce the cost of enzymes, the production conditions and mediums of three different cellulolytic enzyme producing strains were optimized, SmF technology was applied to produce cellulase, and the enzymatic hydrolysis efficiency of the obtained crude enzymes mixtures was evaluated.

Results

Optimization of fermentation medium for cellulase production

The optimized medium components for cellulase production by *T. reesei* were: 3% wheat bran, 0.6% ammonium chloride and 0.1% tryptone, the achieved activity of CMCase and FPA were 2.61 IU/mL and 0.11 IU/mL, respectively. When the medium of *T. harzianum* was prepared as following: 3% ramie, 0.6% ammonium sulfate and 0.1% tryptone, the optimum activity of CMCase and FPA were reached to 2.14

IU/mL and 0.10 IU/mL, respectively. *A. niger* was used as cellulase producing strain for the first time, the optimum medium contains: wheat bran 8%, $(\text{NH}_4)_2\text{SO}_4$ 0.6% and yeast extract 0.4%. The maximum activity of FPA, CMCase and β -glucosidase were 0.027 IU/mL, 3.29 IU/mL and 6.15 IU/mL, respectively.

Effect of pH and temperature on cellulase production

It is well known that initial pH of the medium affects the metabolic ions exchange and the transport characteristic of cell membrane [23]. The effect of initial pH value on CMCase, FPA and β -glucosidase producing capability of *T. harzianum*, *A. niger* and *T. reesei* were illustrated in Fig. 3. Results exhibited that the optimum pH value for CMCase and FPA production by *T. harzianum* and *T. reesei* was 4.0, while a pH value of 7.0 is more suitable for CMCase and β -glucosidase production by *A. niger* (3.25 and 6.99 IU/mL).

The effect of temperature on different enzymes production by various microorganisms was investigated, and related results were shown in Fig. 4. The results in Fig. 4A showed that the CMCase and FPA activity of *T. harzianum* decrease when the temperature increased from 25 to 35°C. The highest CMCase and FPA of *T. reesei* was achieved at 35°C (shown in Fig. 4B). As it was described in Fig. 4C, the enhancement in CMCase and FPA activity of *A. niger* was observed as the culture temperature increased from 25 to 35 °C, whereas its β -glucosidase activities was decreased from 2.19 to 4.10 IU/mL.

Optimization of culture time

Based on the optimized medium and culture conditions, the changes in enzymes activity of different strains along with culture time were evaluated (Fig. 5). Both the activity of CMCase (3.2 IU/ml) and β -glucosidase (5.1 IU/mL) produced by *T. harzianum* were achieved their maximum when the culture was 36 h. After culturing *T. reesei* for 36 h, the highest activity of CMCase and β -glucosidase were achieved, specifically, reached to 3.2 IU/ml and 5.1 IU/ml. The maximum activity of CMCase and β -glucosidase were obtained when *A. niger* was cultured for 96 h.

Enzymatic saccharification of pretreated ramie stalks

The influence of different substrates on inducing microorganisms to produce cellulolytic enzymes

The enzymes producing capability of microorganisms were observed to be induced by lignocellulose substrate, the effect of wheat bran and ramie induced cellulolytic enzymes produced by *T. harzianum*, *A. niger* and *T. reesei* on enzymatic hydrolysis efficiency (48 h) of pretreated ramie stalk were tested. Results were showed in Fig. 6, compared with wheat bran, the obtained reducing sugar yield was obviously higher using ramie stalks induced enzymes to digest the substrate. In specific, the highest reducing sugar content of 307 mg/g was obtained when using the enzymes of *T. reesei* induced by ramie stalk to digest substrate, which was 1.98 times higher than the enzymes induced by wheat bran, while reverse result was

observed when using the enzymes of *A. niger* induced by these two substrates. Similar reducing sugars yield was obtained after hydrolyzing substrate using the enzymes of *T. harzianum* induced by wheat bran and ramie. In summary, the suitable substrate for *A. niger* to produce cellulolytic enzymes was wheat bran, whereas ramie stalk was the better carbon source for inducing the production of cellulolytic enzymes by *T. reesei* and *T. harzianum*.

Synergistic effect of cellulolytic enzymes consortium

Synergistic effects between enzymes help to reduce their dosage, thereby saving the costs for enzymatic hydrolysis of lignocellulosic biomass. The crude enzyme extracts extracted from *T. harzianum*, *A. niger* and *T. reesei* have exhibited excellent performance on cellulose digestion, the synergistic effects among these enzymes were evaluated, corresponding results of different enzyme cocktail combinations were shown in Figure 7. The highest reducing sugar concentration was achieved when using enzyme cocktails of No.5 (50% extracts of *T. reesei* cultured using ramie as carbon source + 50% extracts of *A. niger* cultured using wheat bran as carbon source), intermediate reducing sugar was obtained with No.8 (50% extracts of *T. harzianum* cultured using ramie as carbon source + 50% extracts of *A. niger* cultured using wheat bran as carbon source), while only 111 mg/g reducing sugar was obtained when using the enzyme cocktail of No.2 (50% extracts of *T. reesei* cultured using ramie as carbon source + 50% extracts of *T. harzianum* cultured using ramie as carbon source). In this study, the final reducing sugar yield was obviously influenced by the ration of different enzyme in mixture, suggesting some interactions possibly generated between different enzymes.

Discussions

Development of efficient cellulolytic enzyme cocktail can reduce the amounts of enzymes required for biomass hydrolysis and enhance the conversion efficiency of all carbohydrate into fermentable sugars [24]. However, Jian Du et, al. found that the optimal composition of enzyme mixture distinctly depended on the substrate pretreated by different methods [15]. Higher proportion of EG than CBH in enzyme mixture was proved to exhibit high efficiency for enzymatic hydrolysis of sulfite pretreated Norway spruce [25]. Due to the catalytic performance of same enzymes blends varying with different feedstuffs, developing consolidated proportion of cellulolytic enzymes is unpractical [24]. The single surface-displayed enzyme blending concept was applied to prepare compound enzymes for digestion of pretreated empty fruit bunch, the obtained sugars reached to 446 mg/mL [26]; the cheap wheat straw (WS) raw material was used as the substrate for producing cellulolytic enzymes by microorganisms, then using these crude enzymes to hydrolysis the pretreated WS, the yield of reducing sugar varied with 300–400 mg/g [27]. In this study, *A. niger* and *T. reesei* were cultured respectively using cheap wheat bran and ramie stalk as carbon source for producing cellulolytic enzymes, and then the crude enzymes were compounded to hydrolyze bio-pretreated ramie stalk, the conversion rate of reducing sugars high to 417mg/g was achieved.

Enzyme production of *T. reesei*, *T. harzianum* and *A. niger* under different carbon sources (wheat bran, lactose, avicel and avicel) were estimated. The effect of using wheat bran, lactose, avicel and other lignocellulose materials as substrates on cellulase producing were evaluated in previous studies [28–30]. Bansal et al. [31] have compared the enzymes production capability of *A. niger* NS-2 using different lignocellulose materials including leaves, orange peelings, pineapple peelings, sugarcane bagasse and wheat bran as substrates, the wheat bran was observed as the most suitable substrate for the producing of CMCase, FPA and β -glucosidase, in specific, the yields of these enzymes were 310, 17 and 33 U/g dry substrate, respectively. Avicel was used as carbon resource for cellulase production by *A. niger*, but only limited yield of endoglucanase and exoglucanase were achieved [32]. Wheat bran was used as carbon sources of *T. reesei* to produce cellulase, after cultivation for 4 days, the highest filter paper activities reached to 0.82 ± 0.08 IU gds⁻¹ [33]. It is difficult to cultivate the fungus using untreated biomass as only carbon source, due to compared with long-chain cellulose, microorganisms use soluble sugars more efficiently, so as to achieve rapid accumulation of biomass and improve the efficiency of enzymes production [24]. Bhawna Sharma et al. [27] studied the enzymes producing capability of *Penicillium janthinellum* EMS-UV-8 using untreated wheat straw (WS), WS pretreated by acid, alkali, steam exploded and organo-solve, and pure cellulosic (including avicel, cellulose-II and carboxymethyl cellulose) as substrate for cellulolytic enzymes production, results showed that severe pretreated WS and cellulose-II were contributed to high yield of enzymes. Similarly, in this study, the activity of cellulase produced by *T. reesei* and *A. niger* using wheat bran as substrate was higher than ramie stalk.

Previous studies mainly focused on cellulase producing capability of different microorganisms using different lignocellulose as substrate, but lack of researching their capacity on hydrolyzing lignocellulosic biomass [34–37]. Furthermore, Analysis of the enzymatic saccharification catalyzed by extracellular enzymes produced on two type of carbon sources (wheat bran and ramie stalk) revealed only in *T. reesei* and *T. harzianum*, the enzyme prepared using ramie stalk gave more hydrolysis of bio-pretreated ramie stalk than the enzyme prepared using wheat bran. Generally, large-scale production of cellulases use soluble sugars (e.g. lactose), and raw feedstock is not preferred, however, the type of crude enzyme produced is relatively single, which cannot achieve high-efficiency enzymatic hydrolysis of biomass. Thus, it advised that enzymes would be produced by using some part or whole raw feedstock (what straw, corn cob, rice straw, etc.) [27]. Similarly, the capability of a commercial cellulase preparation (Cellic®CTec2) on hydrolyzing pretreated ramie talk was significantly lower than cellulase induced by ramie stalk.

The combination of enzymes produced by *A. niger* and *T. reesei* was proved to have a positive effect on the saccharification of cellulose substrates [38]. A previous study showed that *T. reesei* was an ideal cellulase producing candidate, unfortunately, although a high total cellulase activity (1.7 FPU/mL) could be obtained using dairy manure as substrate, only very limited β -glucosidase was monitored [39]. Van den Brink, J et al. have evaluated the enzymes producing capability of *A. niger* cultured with two “second generation” substrates: wheat straw (WS) and sugarcane bagasse (SCB), high β -glucosidase activities were obtained after culturing on both non-washed and washed substrates [40]. The capability of a

commercial cellulase preparation (Celluclast 1.5L) on hydrolyzing pretreated corn stover (PCS) was significantly improved by adding three types of crude commercial enzyme preparations that in xylanase, pectinase, and β -glucosidase [41]. The results of this study showed that the cellulase produced from *T. reesei* and *A. niger* showed better synergistic effect than other enzyme consortiums, it is worth mentioning that the prepared enzyme consortiums have exhibited comparable performance with Cellic[®]CTec2 on enzymatic hydrolysis of pretreated ramie stalks.

Conclusions

The types of cellulolytic enzymes secreted by fungi were induced by the carbon source of the culture medium. Ramie stalk was observed as a better carbon source than wheat bran for producing cellulolytic enzymes by *T. reesei* and *T. harzianum*, while opposite effects of these carbon sources on the production of cellulolytic enzymes by *A. niger* was monitored. Obvious synergistic effects were observed between different cellulolytic enzymes, and the highest enzymatic efficiency was achieved when the crude enzymes produced by *A. niger* and *T. reesei* was compounded at a ratio of 1: 1. In particular, the reducing sugars produced after digestion of substrate by compound enzymes was 1.3–1.5 times that of different single enzymes. This research provides a cheap and efficient method for the preparation of cellulolytic enzymes, which is of great significance to the large-scale bio-refining of lignocellulosic biomass.

Materials And Methods

Microorganisms and inoculum

Trichoderma reesei (BNCC339931) was obtained from the BeNA Culture Collection. *Trichoderma harzianum* (CICC41290) was obtained from the China Center of Industrial Culture Collection. *Aspergillus niger* (ATCC16404) was purchased from the American Type Culture Collection. These fungi were stored on PDA plates at 4 °C and transferred to fresh PDA medium monthly and cultured at 25 °C for 5d. After growth on the plate, the spores were washed with 3 mL distilled water to obtain spore suspension. The inoculum was prepared by culturing the fungi under submerged fermentation in 300 mL Erlenmeyer flasks, the seed medium was prepared as following (g/L): glucose 10, peptone 1, Tween-80 0.002, $(\text{NH}_4)_2\text{SO}_4$ 1.4, KH_2PO_4 2, urea 0.3, MgSO_4 0.3, CaCl_2 0.4, ZnSO_4 0.0014, MnSO_4 0.0016, FeSO_4 0.005, citric acid buffer 500 mL/L [21].

Cellulase production by submerged fermentation

The single and combination carbon source (ramie, wheat bran or avicel) were studied to evaluate their effects on enzymes production from *A. niger*. The concentration of carbon sources was listed as following: ramie (80 g/L), wheat bran (80 g/L), avicel (80 g/L). The fermentation broth (75 mL) containing $(\text{NH}_4)_2\text{SO}_4$ (4.0 g/L), KH_2PO_4 (2.0 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.3 g/L), tryptone (3.0 g/L) and yeast extract (0.5 g/L).

The carbon source including ramie, wheat bran, lactose or avicel, and nitrogen source including ammonium sulphate, ammonium chloride tryptone or yeast extract for *T. reesei* and *T. harzianum* submerged fermentation (SmF) was evaluated, their concentrations were set as follows: ramie (10, 20 and 30 g/L), wheat bran (10, 20 and 30 g/L), lactose (10, 20 and 30 g/L), avicel (10, 20 and 30 g/L), ammonium sulphate (4, 6, 8 and 10 g/L), ammonium chloride (4, 6, 8 and 10 g/L), tryptone (1, 2, 3 and 4 g/L) and yeast extract (1, 2, 3 and 4 g/L). The fermentations of *T. reesei* and *T. harzianum* were carried out in 250 mL Erlenmeyer flasks with 50 mL working volume, the culture media contains (g/L): KH_2PO_4 , 2.0; MgSO_4 , 0.3 and Tween 80, 0.2, MnSO_4 0.0016, ZnSO_4 0.0014 mg and FeSO_4 0.005.

All mediums were autoclaved at 120 °C for 20 min, then 10% of inoculum inoculated after the temperature was decreased to room temperature. The mixtures were cultured at 30 °C, the extraction of enzymes was performed after fermentation of *T. reesei* and *T. harzianum* for 36 h, and fermentation of *A. niger* for 72 h. The supernatant was collected by filtering through nylon cloth followed by centrifugation at 10,000 rpm for 10 min. All experiments were performed in triplicate.

Optimization of pH, temperature and fermentation time

T. reesei, *T. harzianum* and *A. niger* fermentation were performed at different pH value (3.0, 4.0, 5.0, 6.0, 7.0), temperature (20, 30 and 35 °C) and fermentation time (24, 48, 72 and 96 h). The following assay conditions were identical to those aforementioned.

Measurement of enzymes

The carboxymethyl cellulase (CMCase), filter paper activity (FPA) and β -glucosidase were measured as the description of Liao et al [22]. All of experiments were performed in 0.05 M sodium acetate buffer (pH 4.8). 3, 5-dinitrosalicylic acid (DNS) reagent was used to measure the reducing sugar [22].

Pretreatment of ramie stalk

Air-dried ramie stalks from the Institute of Bast Fiber Crops at the Chinese Academy of Agricultural Sciences were cut into small chips (400–800 μm mesh). Three grams of ramie stalk was added into 7 mL H_2O , 0.1% Tween 80, 4 mmol/L veratryl alcohol, 0.2 mmol/L Mn^{2+} in 300-mL Erlenmeyer flasks and sterilized at 121 °C for 20 min. *P. eryngii* were maintained on PDA plates at 4 °C and sub-cultured in PDA medium every month at 28 °C for 7 d. Four different *P. eryngii* pre-cultures were inoculated into the substrates, then incubated at 28 °C for 21 days. All of the experiments were carried out in triplicate. The content of cellulose, hemicellulose and lignin in pretreated substrate were 40%, 21% and 20%, respectively.

Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated ramie stalks were performed using Cellic® CTec2 (Bagsvård, Denmark), the crude extracts of *T. reesei*, *T. harzianum* and *A. niger* (cultured at the optimal conditions) as

enzymes (enzyme dosage was 30 FPU/g dry biomass). Enzyme cocktails were prepared as described in Table 1.

Enzymatic hydrolysis was carried out in 50 mL centrifuge tube for 48 h. Supernatant was collected after centrifuging sample at 10,000 rpm for 5 min to measure the reducing sugar.

Statistical analysis

All the experiments values presented in graphs and tables are mean \pm SD, calculated using Excel 2017. Multiple comparison tests were performed with t test (significance levels = 0.05).

The conversion rate of reducing sugars (mg/g) were calculated as following:

Reducing sugars (mg/g) = reducing sugars obtained (mg) / biomass raw material (g)

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

CI X and CC defined the aim and scope of this research article. CC performed

the experiments, data analysis and charting. CI X and CC wrote the major parts of the manuscript. CX helped to revise the manuscript. All authors approved the final manuscript.

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Availability of data and material

All data generated or analysed during this study are included in this published article.

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Table

Table 1 The blends of enzyme cultured in different carbon sources and commercial cellulase

Codes	Combination mode
1	66.7%M1 + 33.3%M2
2	50.0%M1 + 50.0%M2
3	33.3%M1 + 66.7%M2
4	66.7%M1 + 33.3%Mc
5	50.0%M1 + 50.0%Mc
6	33.3%M1 + 66.7%Mc
7	66.7%M2 + 33.3%Mc
8	50.0%M2 + 50.0%Mc
9	33.3%M2 + 66.7%Mc
10	100%M1
11	100%M2
12	100%M3
13	100%Ma
14	100%Mb
15	100%Mc
16	Cellic®CTec2

Footnotes: Extracts of *T. reesei* cultured using ramie or wheat bran as carbon source, extracts of *T. harzianum* cultured using ramie or wheat bran as carbon source, and extracts of *A. niger* cultured using ramie or wheat bran as carbon source were numbered as M1 or Ma, M2 or Mb and M3 or Mc, respectively.

Supplementary Information

Fig. S1. Effect of different carbon sources on enzyme production by cultivating *T. reesei*.

Fig. S2. Effect of different carbon sources on enzyme production by cultivating *T. harzianum*.

Fig. S3. Effect of different carbon sources on enzyme production by cultivating *A. niger*.

Fig. S4. Effect of different nitrogen sources on enzyme production by cultivating *T. reesei*.

Fig. S5. Effect of different nitrogen sources on enzyme production by cultivating *T. harzianum*.

Fig. S6. Effect of different nitrogen sources on enzyme production by cultivating *A. niger*.

Figures

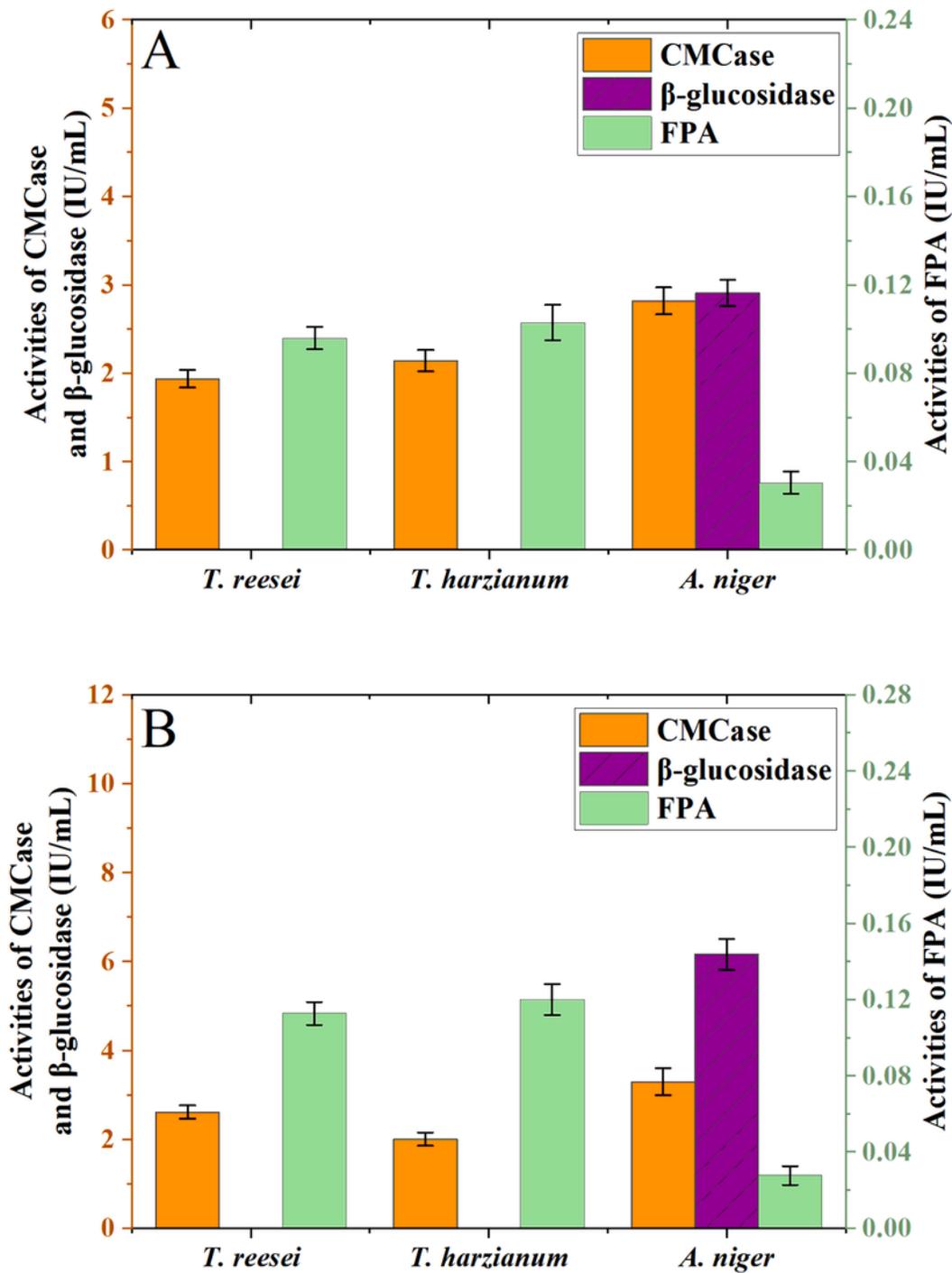


Figure 1

Effect of ramie(A) and wheat bran(B) on enzyme production by cultivating *T. reesei* in ramie 3%(A) or wheat bran 3%(B), *T. harzianum* in ramie 3%(A) or wheat bran 1%(B) and *A. niger* in ramie 8%(A) or wheat bran 8%(B).

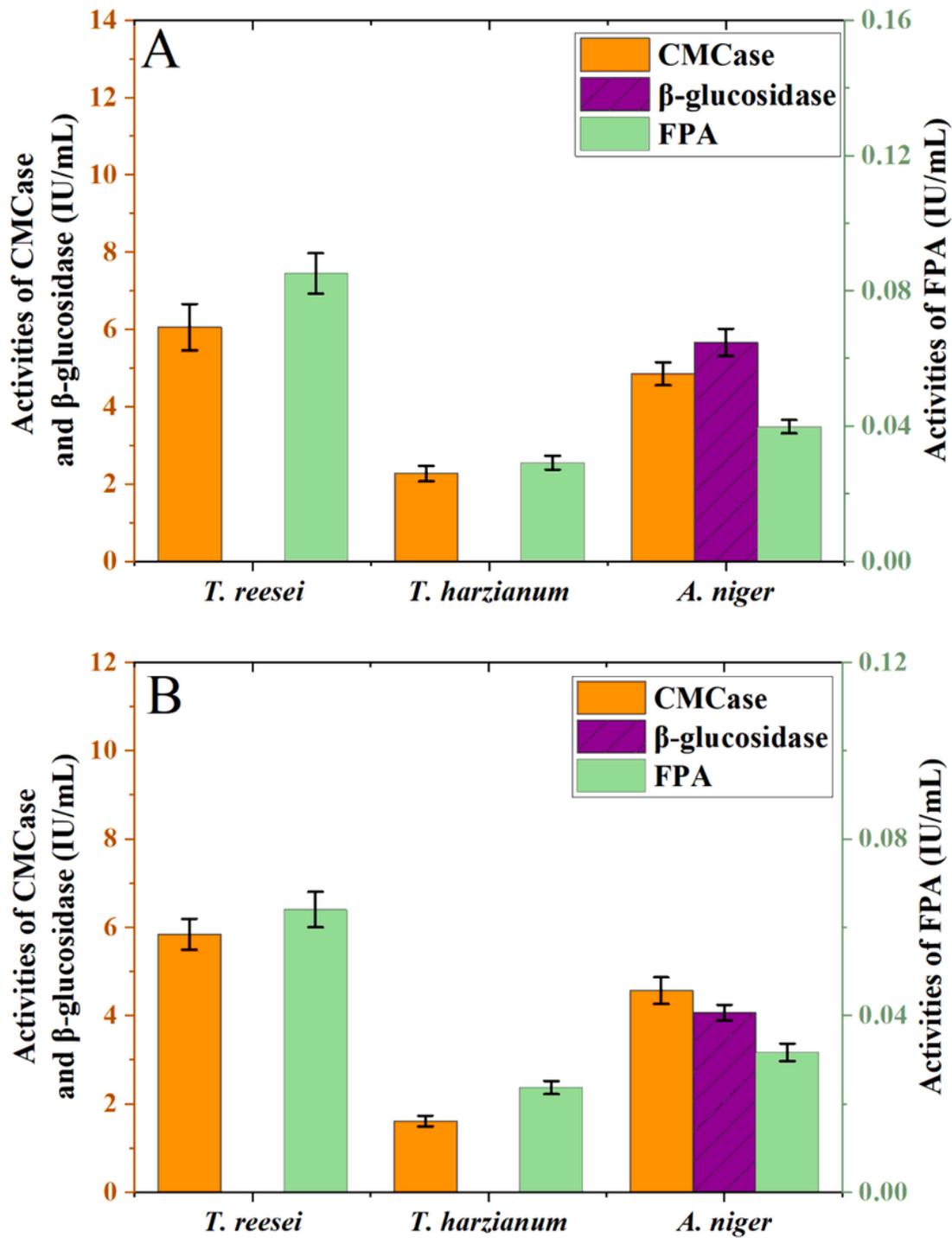


Figure 2

Effect of inorganic(A) and organic(B) nitrogen sources on enzyme production by cultivating *T. reesei* in ammonium chloride 0.6%(A) or tryptone 0.1%(B), *T. harzianum* in 0.6% ammonium sulfate(A) or 0.1% tryptone(B) and *A. niger* in ammonium sulfate 0.6%(A) or yeast extract 0.4%(B).

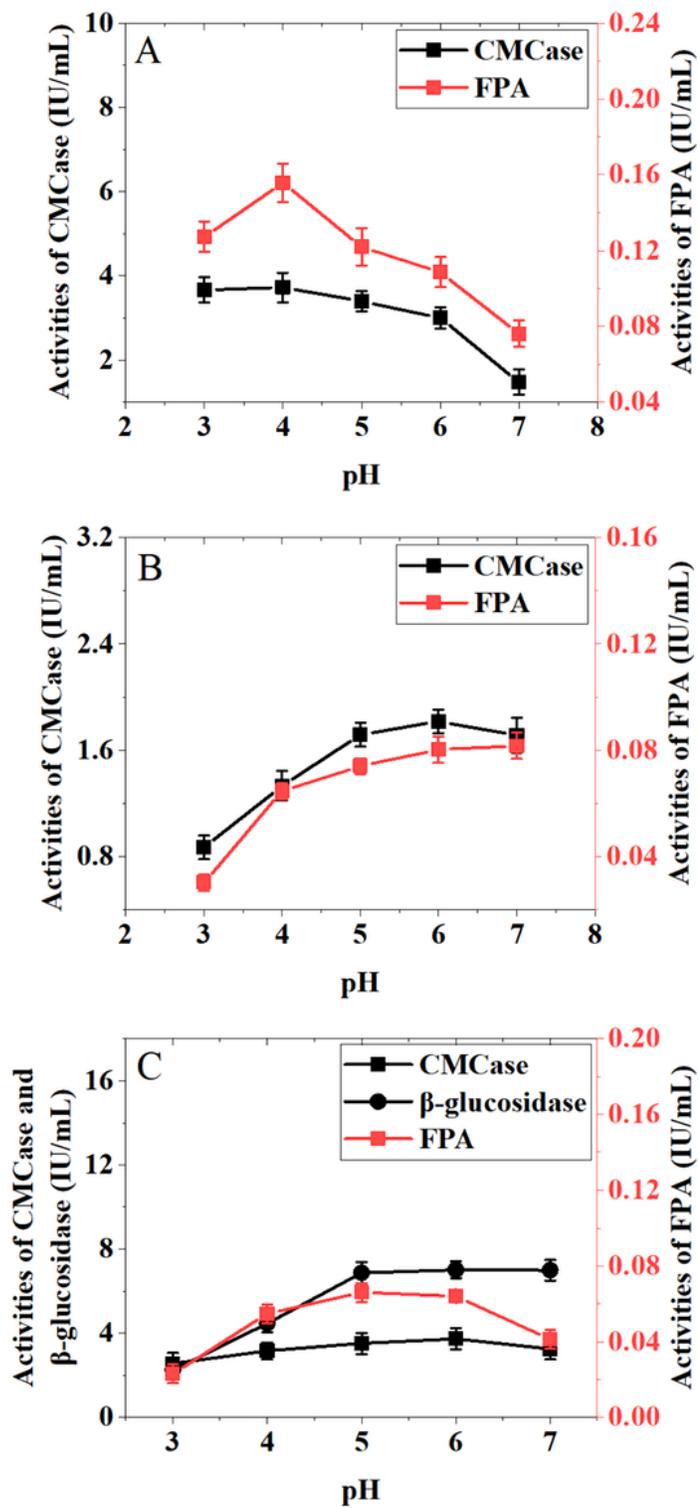


Figure 3

Effect of initial pH on enzymes production by *T. reesei*(A), *T. harzianum*(B) and *A. niger*(C).

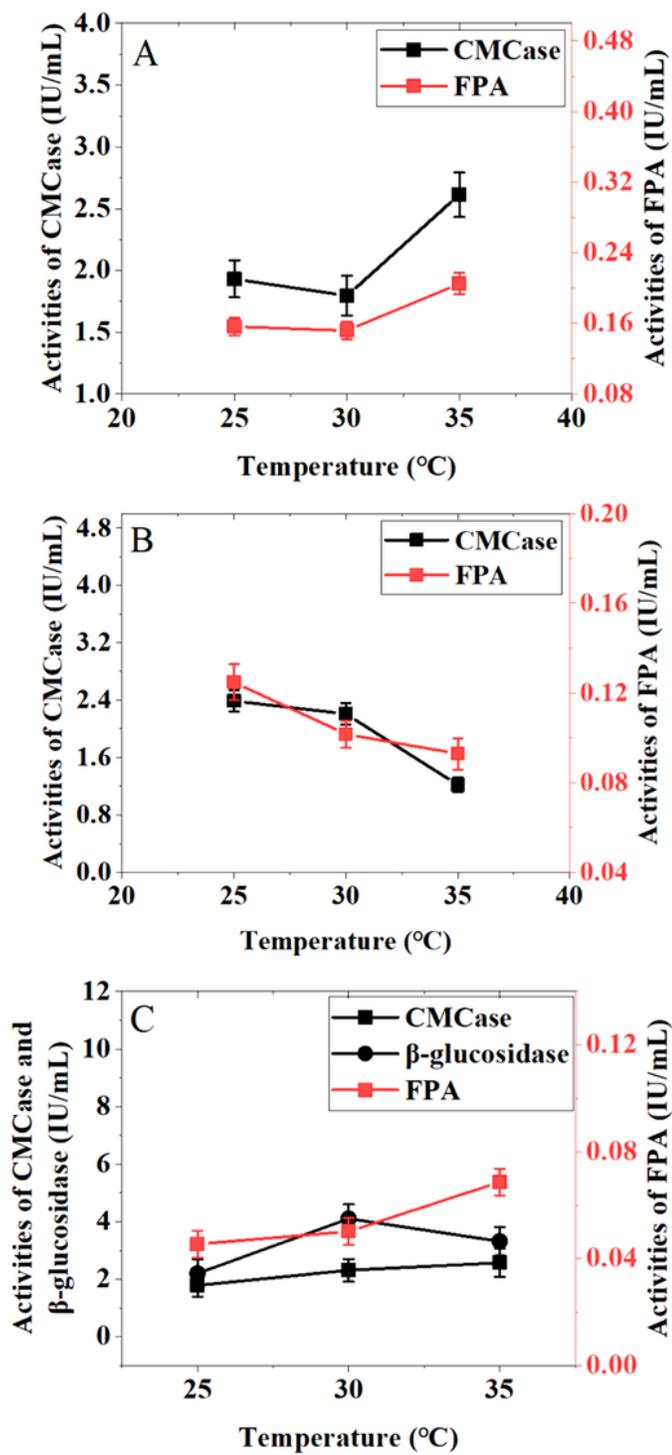


Figure 4

Effect of temperature on enzymes production by *T. reesei*(A), *T. harzianum*(B) and *A. niger*(C).

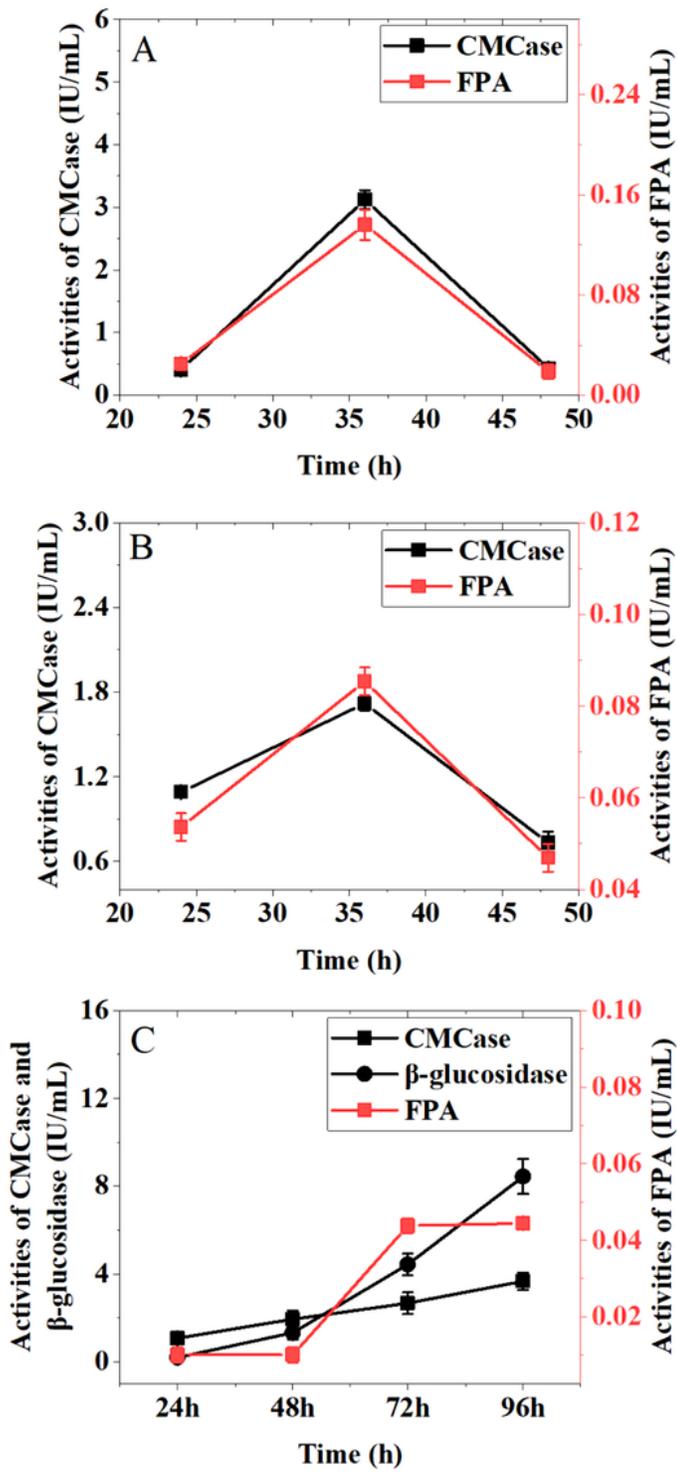


Figure 5

Effect of culture time on enzymes production by *T. reesei*(A), *T. harzianum*(B) and *A. niger*(C).

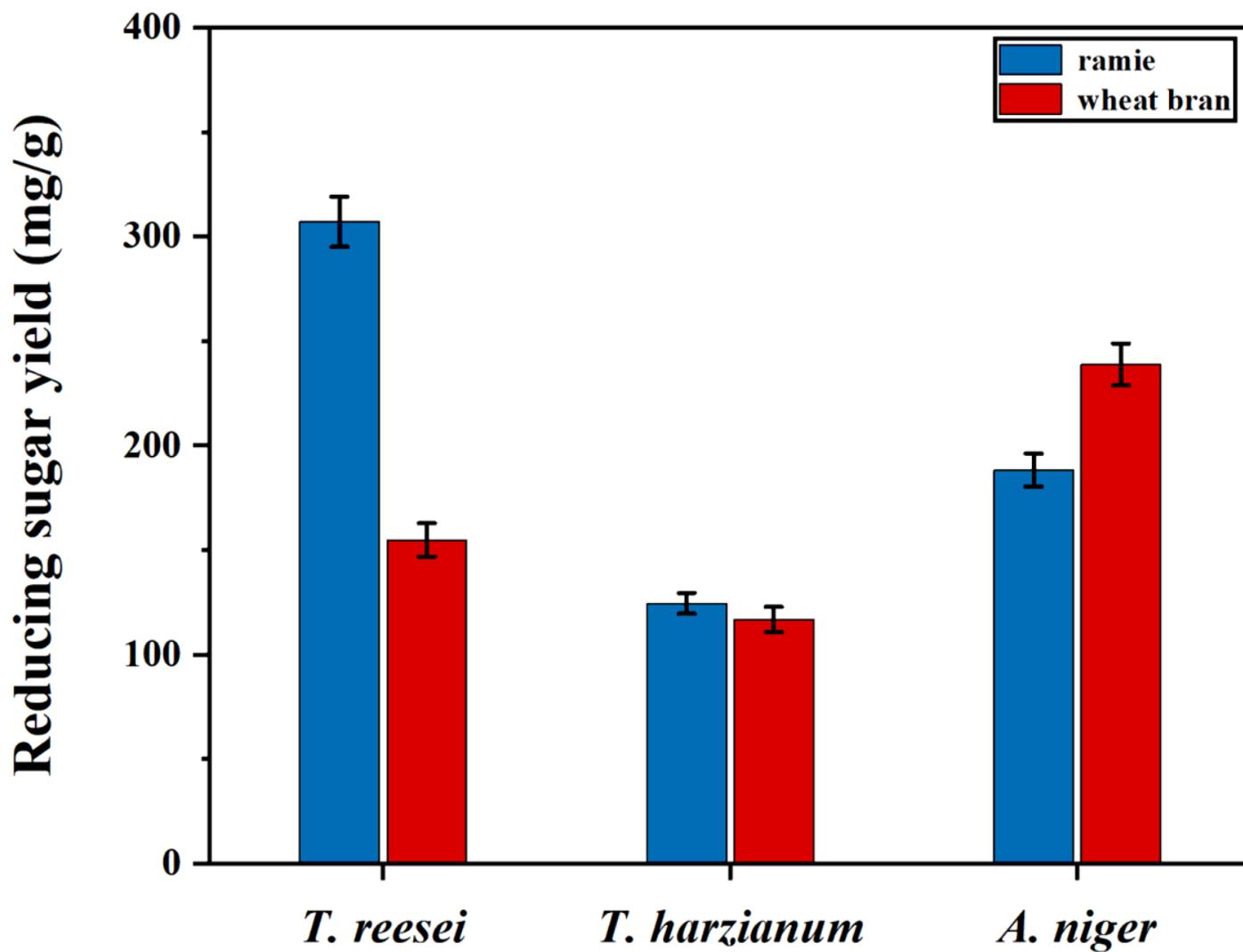


Figure 6

Effect of different carbon sources on enzymatic saccharification of pretreated ramie stalks.

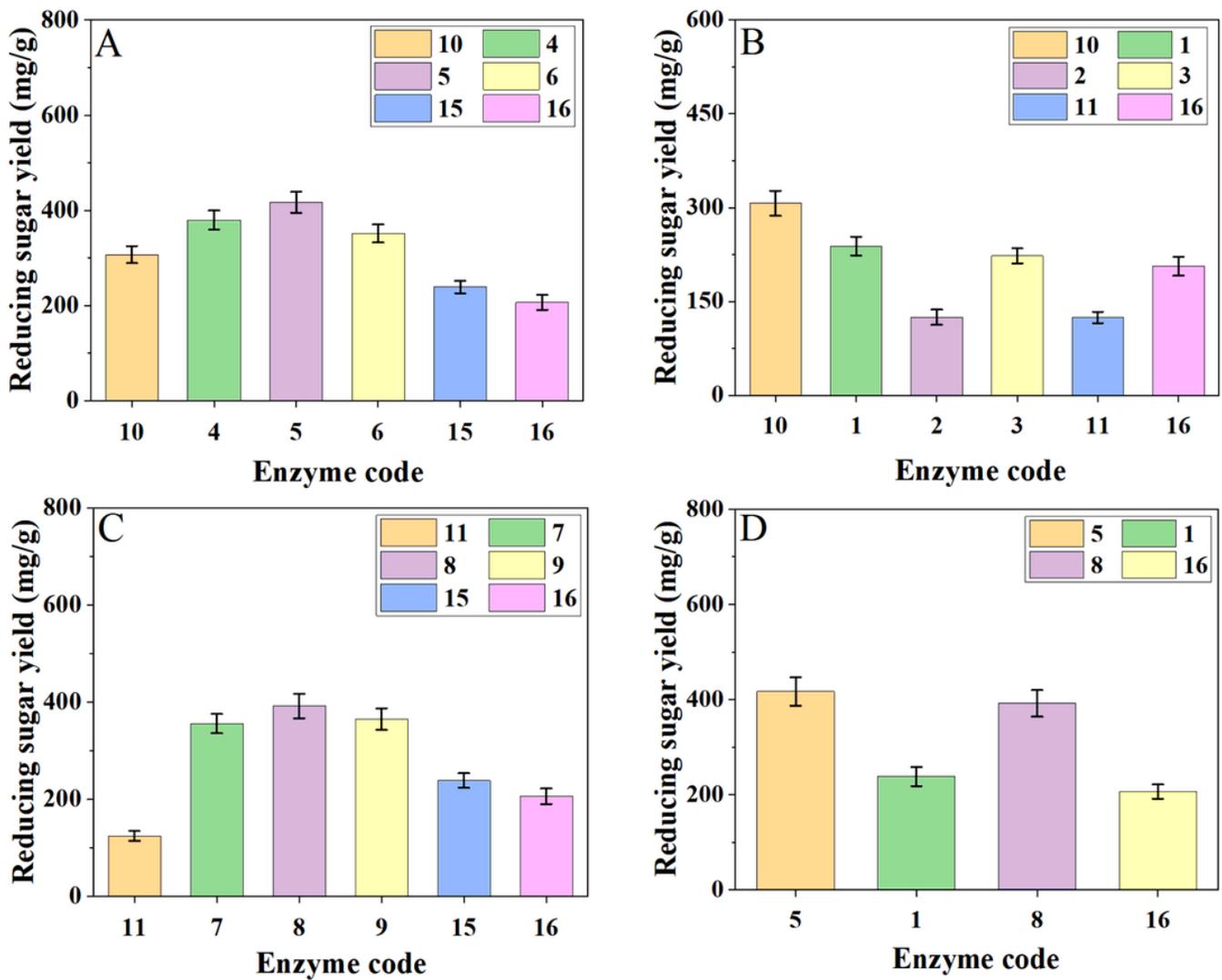


Figure 7

Enzymatic saccharification of pretreated ramie stalks by cellulolytic enzymes consortium.

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