

# Cloning and characterization of an exocellulase and synergistic effect with recombinant endocellulase and beta-glucosidase from *Bacillus subtilis* 1AJ3

**Lingling Ma**

Northwest Agriculture and Forestry University

**Jiaxin Chen**

Northwest Agriculture and Forestry University

**Xin Wang**

Northwest Agriculture and Forestry University

**Yanglei Yi**

Northwest Agriculture and Forestry University

**Yuanyuan Shan**

Northwest Agriculture and Forestry University

**Bianfang Liu**

Northwest Agriculture and Forestry University

**Yuan Zhou**

Northwest Agriculture and Forestry University

**Xin Lü** (✉ [xinlu@nwsuaf.edu.cn](mailto:xinlu@nwsuaf.edu.cn))

Northwest Agriculture and Forestry University

---

## Research

**Keywords:** Exocellulase, *B. subtilis*, Synergy effect, Biomass lignocellulose

**Posted Date:** December 20th, 2019

**DOI:** <https://doi.org/10.21203/rs.2.19483/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

---

# Abstract

**Background:** Biomass lignocellulose provide abandon cellulosic material for bioenergy. The synergy effect of the cellulase system still needs more attention to make it more efficient. Exocellulase was less of studies in former studies, especially recombinant exocellulase from bacteria.

**Results:** An exocellulase Cbh -A from strain *B. subtilis* 1AJ3 was cloned and expressed in *E. coli* BL21. By sequence analysis, Cbh -A contains two domains: FlgJ and SH3\_8. Enzymatic properties of Cbh -A were characterized, and the maximum enzyme activity was gotten under the condition of pH 6.4 and 50°C. Moreover, the enzyme reached the highest enzyme activity and kept stable under 50°C for 4 h. Ion metal Mn<sup>2+</sup> had an activating effect on Cbh -A, while Cu<sup>2+</sup> as well as chemical EDTA had inhibition effect. Cbh -A had widely substrates specification that it could hydrolyze  $\beta$ -1,3-1,4/ $\beta$ -1,3/ $\beta$ -1,4 glucan linkage,  $\alpha$ -1,4 glucan linkage . When it took cooperation with recombinant endocellulase and  $\beta$ -glucosidase also from the strain *B. subtilis* 1AJ3, synergism effect was observed through DS (degree of synergism) value by hydrolyzing CMC-Na, Avicel, and filter paper. At the same time, the synergism effect was also examined in biomass lignocellulose by comparing the saccharification rate and DS value of each material.

**Conclusions:** Cbh -A contained a FlgJ domain and first studied as an exocellulase cloned from *B. subtilis* 1AJ3. The biochemical, enzymatic properties, ion metal, and substrate specificity of Cbh -A were analyzed, and a special character of Cbh -A could keep maximum activity after pre-warm at 50°C for 4h. Cbh-A had good synergy effect with endocellulase and  $\beta$ -glucosidase from the same strain in variety kind of cellulosic substrates, including simple and complex biomass cellulose. It laid a foundation of application by mixed cellulases via synergy effect, and supply new thought for the degradation of biomass lignocellulose.

## Background

Lignocellulosic biomass represents the main sustainable utilize substrates of biofuels since it is a wild source and high content of cellulose of them. Cellulose in different biomass always consists of long chains of  $\beta$ -1,4-linked glucose units which make lignocellulose kept stable and partly dense crystalline structure [1]. The structure of cellulose is added lignin and hemicellulose crossed into it, making lignocellulose hard to hydrolyze. Thus, pretreatment is necessary for lignocellulose biomass to hydrolyze cellulose into glucose, and further can be utilized into bioethanol, chemicals, and other value-added products [2]. For better hydrolyzation of cellulose into reducing sugars, it needs the synergism of cellulase system, which usually consists of three types of cellulase: endocellulase, exocellulase, and  $\beta$ -glucosidase. Both in bacteria and fungi cellulase systems, the three types of cellulases response for different work in different periods. Endocellulase (EC 3.2.1.4) firstly cuts long chains of cellulose into short chains and to exhibit more terminals of cellulose, and it mainly works on amorphous regions of cellulose. Exocellulase (EC 3.2.1.91) mainly targets at crystallization region of cellulose, utilizes these terminals of cellulose and hydrolyzes them into cellobiose.  $\beta$ -glucosidase (EC 3.2.1.21) responses for hydrolyzing the remaining glycosidic bonds into glucose include cellobiose, which can be converted into further fermentation [3, 4].

Cellulases have a synergistic effect, and their combined ability to degrade cellulose has a higher degradation effect than separate enzymes. Meanwhile, the ratio among different cellulases strongly determines the synergistic effect, which means that the mixing ratio of different enzymes plays an important role in the final

cellulose hydrolysis efficiency [5–7]. Additionally, different cellulose substrates require a different ratio of enzymes, even different types of enzymes. Regarding the synergistic effect of cellulase, there are few studies on the three enzymes, and a large part of the research focuses on the synergy between endonuclease and  $\beta$ -glucosidase [8], while the importance of exocellulase is neglected. There should be two reasons for this phenomenon, one is efficiency of exocellulase hydrolyzing cellulose is not as obvious as endocellulase and  $\beta$ -glucosidase. Besides, the action mode of exocellulase results in lower product yield of cellulose hydrolyzation, which will not attractive in applications of industrial production. Another important reason is that there are few studies on the existing exonuclease, which provides a less material basis for the synergistic study of the three enzymes. In present studies, exocellulase mainly focuses on fungi and minor attention has been dedicated to exocellulase cloned from bacteria [9–11]. Thus, more exocellulase needs to be studied and characterize in different ways and cloning is a convenient method to obtain it.

Besides, in the research of synergistic action of different microbial cellulases, most of these cellulases were from different microbial sources, including fungi, bacteria, and lactic acid bacteria cellulases [12–14]. Although this would be advantageous for industrial applications, the synergistic effect of cellulase produced in wild strain could not be well explained. So, if the cellulases for synergy all come from the same strain and belong to different types, do they have a stronger effect? So, we tried to use the recombinant exocellulase cloned in this study to work together with another two cellulases, endocellulase and  $\beta$ -glucosidase, which also cloned from the same strain, to explore their synergy effect.

In this study, a novel exocellulase Cbh-A was successfully obtained from the strain *Bacillus substiles* 1AJ3 by cloning and expression, and its properties were studied. Herein, an endonuclease and a  $\beta$ -glucosidase which were also cloned and expressed from the same strain *B. substiles* 1AJ3 in our former work. The synergistic action between/among them were studied by enzyme activity test via a comparison of a variety of cellulose substrates such as CMC-Na, Avicel, and filter paper. Moreover, different lignocellulose biomass was used as substrates to verify the synergistic action of three types cellulases, and reducing sugar content was used as an indicator to study its synergistic effect in lignocellulose. These results showed the novel exocellulase had potential application ability. A synergy of three types of cellulases from one strain also gave us a new insight into the cellulase system. The broadness of substrates by three cellulase together shows good potential in developing cellulase cocktail due to its exquisite cooperation with various cellulase.

## Results

### Cloning, expression, and bioinformation analyze of Cbh-A

The Cbh-A gene was successfully cloned and expressed as an active soluble recombinant protein in *E. coli* BL21(DE3). The recombinant exocellulase was verified by SDS-PAGE (Fig. 1)

A total of the whole ORF sequence including His-tag of Cbh-A was 897 bp, encoding 298 acids amino, whose theoretical molecular weight was 33.90 kDa. By blast in National Center for Biotechnology Information (NCBI) and analyzed domains on the website, Cbh-A contained two domains, FlgJ (211-212L) and SH3\_8 (213K-285E). It had 99.29% percent identification with exo-glucosaminidase LytG of *Bacillus subtilis* (WP\_032726765.1). Theoretical pI of Cbh-A was 7.0 by prediction, while the the instability index was 26.58, and the grand average

of hydropathicity (GRAVY) was  $-0.549$ . It showed that recombinant enzyme Cbh-A was hydrophilic with good stability protein.

## Homology modeling, molecular docking and active sites analysis

The homology modeling of Cbh-A was performed on the raptorX website. Similarity searches for the sequence were conducted using BLASTP searches of the Protein Data Bank (PDB) database at the NCBI server. Among the results of similarity proteins, two were selected as models for homology modeling, 5T1Q\_A and 5JQC\_A, which covered two domains separately with 40.24% and 31.17% of identity. The homology model with a p-value of  $3.36E^{-8}$  showed that the structure of Cbh-A was composed of 298 amino acid residues, which consist of 38%  $\alpha$ -helices and 21%  $\beta$ -sheet (Fig. 2a).

Based on the prediction of 3D structure, domain FlgJ was the catalytic domain contained a barrel formed by 6  $\alpha$ -helices and 2  $\beta$ -sheets which probably were active sites. The pocket sites consist of Glu88, Phe104, Gly105, Val-106, Lys107, Tyr110, Thr119, Asp120, Glu121, Leu150, Tyr183, Ala184, Thr185. Domain SH3\_8 had function of cellulose binding and with binding sites Lys232, Pro233, Trp278 (Fig. 2b). All the activity sites formed a tunnel of the loops and the antiparallel strands, which seems only allow one cellulose chain into it [15]. The domain SH3\_8 possessed cellulose binding and enhanced the connection of enzyme and cellulose substrates.

## Characterization of the recombinant Cbh-A

The maximum activity of recombinant Cbh-A was observed at pH 6.4 (Fig. 3a) by measurement in different pH buffers. At pH6.0 and 7.0, the relative enzyme activity also could reach 90.3% and 85.4%. After 30 min of maintaining in different pH buffers, the highest relativity enzyme activity showed that pH 6.0 (Fig. 3b) could make enzyme stale. Followed by it was pH 6.4, 7.0 and 7.4 could also reach 89.7%, 78.1%, and 71.6%, respectively. The results suggested that the neutral pH was much more suitable for enzyme activity and keep stable. For an optimum temperature of enzyme activity, Cbh-A had maximum enzyme activity at 50°C (Fig. 3c), and it could reach 98% relative activity at 40°C. The results suggested that action temperature between 40–50°C could keep enzyme high enzyme activity. Cbh-A was found to be highly stable under 50°C (Fig. 3d).

When detecting thermostability of Cbh-A, we found an interesting thing that recombinant enzyme Cbh-A presented highly enzyme activity when it was maintained under 50°C for 30 min. It brought us a new thought out of our mind, that how long it can stay under 50°C to keep enzyme activity. So, a time-continued experiment was done, and the change of enzyme activity with time incubated at 50 °C was investigated. It showed that in one hour, the enzyme activity could Within one hour, the enzyme activity increased rapidly with the increase in temperature. Within 1 to 4 h, the enzyme activity continued to increase with the increase of temperature, but the rate became slower. After 4 h, the enzyme activity remained stable (Fig. 3e) which could reach  $0.225 \pm 0.002$  U/mL under experiment conditions.

The influence of effectors on enzyme activity was determined by using various metal salts and chemicals. Table 1 showed that relative affection on enzyme activity of different metal ions and chemicals at a concentration of 1 mM, 5 mM, and 10 mM. The presence of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $[\text{NH}_4]^+$ , and chemical SDS

were all had little effect on enzyme relative activity under different densities. Metal ion  $Mn^{2+}$  could increase relative enzyme activity greatly in 10 mM, 5 mM and 1 mM, followed by  $Zn^{2+}$  and  $Ca^{2+}$  could increase Cbh-A relative activity to some extent under 10 mM and 5 mM.  $Cu^{2+}$  could inhibit enzyme activity under 10 mM and 5 mM, but had no effect under 1 mM. Chemical EDTA inhibited enzyme activity under all density.

Substrate specificity of Cbh-A also was measured to identify which glucan linkage could be acted on (Table 2). Relative activity of different substrates showed that Cbh-A had a high efficiency of maltose of 277.63% relative activity, and it presented better in barley glucan, laminarin and xylan from beechwood that their relative activity was 168.44%, 161.52%, and 185.09%, respectively. For CMC-Na and filter paper, it showed equally hydrolyzation ability to Avicel. Results above could identify that Cbh-A could hydrolyze  $\beta$ -1,3 - 1,4 glucan linkage,  $\beta$ -1,3 glucan linkage,  $\beta$ -1,4 glucan linkage, and  $\alpha$ -1,4 glucan linkage efficiency, but had no effect on  $\alpha$ -1,6 glucan linkage.

## Synergy effect of three types of cellulase

For cellulose degradation, three types of cellulases forms a system which could hydrolyze cellulose by cooperation efficiency. Three types of recombinant cellulase cloned from *B. subtilis* 1AJ3, endocellulase Cel-5A, exocellulase Cbh-A (this study), and  $\beta$ -glucosidase Bgl-16A, were combined according to a single enzyme, two together, and three mixed formed seven groups (Table 3). Then seven groups of enzymes were measured, namely enzyme activity of Avicelase, CMCcase, and FPase based on the substrate of Avicel, CMC-Na, and filter paper, respectively. The DS value of two by two and three mixed groups was calculated to observed the synergism among three cellulases (Table 3).

Different enzymes have their characteristics in various substrates, and not to mention the interaction between enzymes and enzymes present diversely. It showed that all the three types of cellulase could form a synergism effect in different cellulose substrates, no matter two of them or three of them. They can improve hydrolyze efficiency and cooperate when degradation cellulose. But they had different synergy ability between themselves, and also within various cellulose substrates. For CMC-Na, standing for an amorphous region of cellulose, endocellulase Cel-5A presents optimum hydrolyzation among single cellulase. Exocellulase and  $\beta$ -glucosidase could synergy best which had the highest DS value of 1.736, and even higher than the three enzymes together (DS value of 1.326). But when hydrolyzing Avicel, which mainly consist of crystalline cellulose, three single cellulase present relative equally in hydrolyzation by results of enzyme activity. But when they worked to cooperate, three enzymes together presented stronger synergy effect ability (DS value of 1.501). When using filter paper as a substrate, which containing both amorphous region and crystalline cellulose, endocellulase displayed strong hydrolyzation ability. Three enzymes cooperation (DS value of 1.525) was equal to that in Avicel, but endocellulase and exocellulase cooperation seemed like more efficiency for its higher DS value of 1.703.

## Application in varieties of biomass based on synergism

Three types of cellulase into lignocellulose biomass were applied to observe their synergism and also examine hydrolyze ability in more complex cellulose substrates. Adding buffer without enzymes as blank, then single enzyme and three mixture enzymes were added into 5% (m/V) various biomass. Figure 4 showed raw biomass materials and hydrolyzation samples. There were five samples in each group in the test tube and from left to

right were blank, endocellulase solo, exocellulase solo,  $\beta$ -glucosidase solo, and three cellulases mixture. The saccharification rate of different kinds of biomass was shown in Table 4. After the hydrolyzation by enzymes, all the biomass lignocellulose showed similar characteristics. On the whole, the particles of lignocellulose after hydrolyzation changed from large to small, crashed to thin, and some even appeared sandy. Because of the lignocellulose structure becoming loosen, most of the lignocellulose color changed into lighter. However, from the results of saccharification rate of different biomass substrates by a single or mixture enzymes, cellulases and enzyme mixture had special hydrolyzation characters in different lignocellulose substrates (Table 4). For example, it displayed the highest DS value (184.0) of three enzymes in corn stalks while single enzyme nearly had no hydrolyzation effect on corn stalks. Then followed by wheat straw and rice husk, coffee ground, and sugarcane stalk, three enzymes could present cooperation with each other than single ones. But in switchgrass, corn cob, pea straw, and ginger stems and leaves, enzymes synergism was not obvious. Besides, in all these ten kinds of biomass substrates, enzymes had less effect on peanut shell, no matter single cellulase or mixture together while saccharification was low. For saccharification rate, switchgrass and corn cob would be the benefit lignocellulose for obtaining more reducing sugar content, and then followed by corn stalks and wheat straw.

## Discussion

### A novel exocellulase cloned from *B. subtilis*

In nature, three types of cellulases play a major role in the synergetic degradation of cellulose: that resulting from the cooperation of endocellulase and exocellulase to hydrolyze amorphous region and crystalline region of cellulose, with a final of beta-glucosidase hydrolyzing the low molecular cellulose into oligosaccharides. Because of crystalline cellulose, with dense structure and it is hard to hydrolyze in lignocellulose. Exocellulase as a cellulase aims at crystalline cellulose can hydrolyze cellulose chains from one end to another by hydrolyzing cellobiose once, but it was always ignored of its low hydrolyze efficiency. It had also been found that most of the recombinant exocellulase studied were derived from fungal microorganisms, such as *Thermobifida fusca* [11, 16], *Thermomonospora fusca* [17], *Orpinomyces* sp. [18], *Irpex lacteus* [19], but exocellulase from bacteria was rarely reported, such as *Clostridium thermocellum* [20]. In this study, we cloned an exocellulase Cbh-A from *Bacillus subtilis* 1AJ3 to found out its cellulose hydrolyzation ability. Cbh-A is a 34 kDa recombinant enzyme which identified conclude two domains: catalytic domain FlgJ and binding domain SH3\_8. However, FlgJ domain was always regarded as glucosaminidase [21, 22] and peptidoglycan hydrolase [23] in previous studies, some with an exo acting function [24]. SH3 domain as a binding protein, but no studies so far had been reported that weather it had cellulose-binding function in cellulose hydrolyzation. It's the first time that FlgJ domain was shown to be an exocellulase and characterized its enzyme activity using the substrate of Avicel.

Cbh-A could not tolerate acidic or alkaline environment which led to enzyme denaturation and inactivation, while pH 6.0-7.4 would maintain its enzyme activity over 70%. Similar pH conditions for exocellulase was observed in Cel48S [20]. Cbh-A had the highest enzyme activity under 40–50 °C, that it can combine with other enzymes with same temperatures. Additionally, when considering Cbh-A temperature stability, we found out a special character with it. Prewarm under 50°C for 4 h will make Cbh-A present remain at its maximum activity. It sighed that Cbh-A had a more changeable structure that longer time needed to stretch itself. A FlgJ belonging to GH73 family also has been observed that it flexible owing to could not be completely modeled into the electron

density [25].  $Mn^{2+}$  could increase Cbh-A activity to a large extent, and EDTA and high density of  $Cu^{2+}$  (5 mM and 10 mM) could inhibit its activity. Thus, Cbh-A has widely metal ion suitable ability that could provide suggestions in industrial application. Moreover, Cbh-A had widely suitability of substrate, that it could hydrolyze  $\beta$ -1,3 - 1,4 glucan linkage and  $\alpha$ -1,4 glucan linkage in cellulosic material. These characters suggested that Cbh-A had promising potential application ability in cellulosic material hydrolyzation, no matter purify cellulose or natural complex cellulose such as biomass lignocellulose.

## Synergy effect of Cbh-A with Cel-5A and Bgl-16A

In previous studies, the synergy effect among cellulases was from different microbes, even different species, in which fungal cellulases were the majority or some consist of commercial cellulases [26], some were mixed with bacterial cellulase [27]. And in this study, for the first time, the synergy effect of three different types of cellulases derived from the same bacterium was studied. Three cellulose substrates were chosen to evaluate the hydrolyzation synergic ability of cellulase, in which CMC-Na stands for amorphous cellulose, Avicel present crystalline cellulose, while filter paper was complex cellulose consist of amorphous and crystalline cellulose. When Cel-5A, Cbh-A, and Bgl-16A hydrolyzed the cellulose by itself, the results of the enzyme activity (Table 3) indicated that Cel-5A had high enzyme activity in CMC-Na and less in Avicel. Oppositely, Bgl-16A had higher enzyme activity in Avicel than that in CMC-Na. Cbh-A showed its equal hydrolyzation ability in different substrates. Another interesting thing is that three enzymes had similarity enzyme activity when hydrolyzing Avicel. It mostly probably because of the structure of CMC-Na was more loosen that suitable for endocellulase hydrolyze beta-1,4 glucan linkage randomly. But Avicel with compact structure prevented endocellulase attachment and action.  $\beta$ -glucosidase was aimed at cellobiose and cellotriose further degraded into glucose [18], so when it singly hydrolyzes cellulose substrate, no substrates were provided for it. However,  $\beta$ -glucosidase had higher enzyme activity than that in CMC-Na, it should be attributed to Avicel was insoluble, easy to gathered than CMC-Na for  $\beta$ -glucosidase to connected, while enzyme proximity is the most important factor for activity enhancement [28], and  $\beta$ -glucosidase don't have cellulose binding domain. While cellulase mixed with another, things got much more complex. Endocellulase and exocellulase cooperated well, especially in filter paper, DS value reached 1.703. Similarly, endocellulase have been observed predominates in the initial stage of hydrolysis when hydrolyze filter paper with exocellulase [29]. And it was observed that when considered a mixture ratio and adjust the combination rations of endocellulase and exocellulase into 5, the maximum DS reached 1.8 [30] also indicated that Cel-5A and Cbh-A in this study had efficiency synergism. It also suggests the exocellulase could help to released crystalline cellulose chains and make it structure getting loosen, which benefit for endocellulase to have a chance acting on glucan linkage. Furthermore, endocellulase could produce more shorter cellulose chains for exocellulase. Exocellulase and  $\beta$ -glucosidase had stabilized cooperation effect in hydrolyzing, while exocellulase can produce more available substrate for  $\beta$ -glucosidase and  $\beta$ -glucosidase can remove substrate inhibition for exocellulase [31]. Exocellulase was the speed limit factor for  $\beta$ -glucosidase. When three enzymes mixed, they all could get better synergism and the DS of more difficult hydrolyze cellulose substrate, filter paper and Avicel, over 1.5. Additionally, we have to consider of the synergy effect among different types of cellulase is complicated, depending on such factors as their ratio, enzyme concentration, reaction time, and also the substrate characteristics [32, 33], so the maximum of three types in this study still need further study. All the results suggesting the cellulases from *B. subtilis* 1AJ3 has the ability of good synergism for biotechnological applications.

Due to these three recombinant cellulases belong to different types, and they have absolutely synergy effect with each other and all together, we tried to enlarge the substrates ranges to natural biomass lignocellulose, evaluate their synergism in various cellulosic materials. Therefore, solo cellulase and three cellulases mixture were applied to the ten different common biomass materials, with the same ratio of that in the CMC-Na, Avicel, and filter paper above. The statement of the raw materials and the state after hydrolysis were shown in Fig. 4. Not all the biomass substrates can be well hydrolyzed. The degree of hydrolysis is not only related to the single or mixed action of the enzyme, but also the raw material [34]. Switchgrass was much easier to hydrolyze [35], also with the maximum saccharification rate of 9.62% of total solid. Studies had also proved that it is considered the most promising cellulosic material as an energy crop due to its high yield, high nutrient use efficiency and its wide growth region [36, 37]. Then followed corn cob, which had an 8.72% saccharification ratio hydrolyzed by three enzymes mixture. All of the biomass substrates, peanut shell is the most difficult to hydrolyze, no matter by single enzymes or mixture enzymes, it kept stubborn. It mainly owing to peanut shell is suborned, dense, and hard structure, no pretreatment made it much harder to hydrolyze. Crude enzymes from *Aspergillus aculeatus* lizuka FR60 has been reported that could hydrolyze peanut shell effectively, but the peanut shell still had been pretreatment by removing lignin [38]. Secondly, size of cellulose fiber also could impact cellulase synergy [39]. Besides, cellulase has substrate specificity during the hydrolysis process, even if it is synergistic [40]. Corn stalks hydrolyzation perfectly exhibits the synergism of three cellulases, with the DS value of it reached 184, and the meantime with a saccharification rate of 5.74%. The same phenomenon was observed in wheat straw, rice husk, sugarcane stalk, and coffee ground, they all present the cooperation effect of three enzymes than a single one. A difference of synergism effect ability suggesting that enzymes in this study have substrate specificity. However, considering maximizing of the effects of each enzyme to achieve the larger degradation and utilization of biomass, the method of synergistic of three enzymes should be optimized. In this study, three enzymes were mixed in 1 × PBS buffer and added into a biomass substrate for synergistic degradation test. Different adding order or batch hydrolyzation should be considered in further studies because of enzyme reaction was under different suitable pH. Besides, other enzymes should be attempt to add into the enzymatic hydrolysis reaction system, such as xylanase, lignin-degrading enzymes while the presence of hemicellulose and lignin could prevent cellulose hydrolyzation.

## Conclusion

For a better understanding of exocellulase from bacteria, a novel exocellulase Cbh-A was successfully cloned and expressed from *B. subtilis* 1AJ3. It consisted of two domains and had high enzyme activity when pre-warm under 50°C for 4 h. Maximum enzyme activity was observed at pH 6.4, 50°C.  $Mn^{2+}$  could increase enzyme activities, while  $Cu^{2+}$  and EDTA had inhibited affection. It could hydrolyze  $\beta$ -1,3 - 1,4/ $\beta$ -1,3/ $\beta$ -1,4 glucan linkage,  $\alpha$ -1,4 glucan linkage efficiently, but had no affection on  $\alpha$ -1,6 glucan linkage. Cbh-A had synergism effect with endocellulase and  $\beta$ -glucosidase cloned from the same strain in different types of cellulose. Not only endocellulase produces more shorter cellulose chains for exocellulase, but also exocellulase hydrolyzes crystalline cellulose loosen for endocellulase.  $\beta$ -glucosidase enzyme activity was limited by exocellulase, and take a stable degree of synergy effect with exocellulase. Three cellulase synergism efficient in simple cellulose as well as complex biomass lignocellulose without pretreatment by DS value: corn stalks, wheat straw, rice husk, sugarcane stalk, and coffee ground. Besides, the mixture cellulase performed well in switchgrass, corn cob, and corn stalks, which presented good saccharification rates. All results suggest that the recombinant

exocellulase is promising in industrial applications. Synergism effect of three types of cellulases from the same bacterium could cooperate well, and they have substrates specification. It provides the basis for the universal application of enzymes, and also provides new ideas for the comprehensive development and utilization of biomass energy.

## Methods

### Strains, medium, and chemicals

*Bacillus subtilis* 1AJ3 (GenBank No. MG062801), a cellulolytic bacterium isolated from Qinling Mountains (China) in our previous work was growing in LB medium at 37 °C, and its' genomic DNA was used as cellulase cloning templet. The plasmid of pET-28a(+) was used for recombinant vector construction. The *E.coli* BL21(DE3) was used for recombinant enzyme expression grow in LB medium with 100 µg/mL Kanamycin (Kan).

Whatman No.1 filter paper was purchased from GE Healthcare (Stockholm, Sweden), and 6 mm in diameter holes were obtained with a puncher in the same size. Avicel (PH-101), CMC-Na, barley glucan, laminarin, pullulan, and xylan from beechwood purchased from Sigma-Aldrich (St. Louis, USA). Other chemicals in this study were all of the analytical grade.

Endocellulase Cel-5A and  $\beta$ -glucosidase Bgl-16A were recombinant enzyme cloned from *B. subtilis* 1AJ3 with a pET-28a(+) plasmid and expressed in *E.coli* BL21(DE3).

Lignocellulose biomass, conclude switchgrass, wheat straw, corn stalks, corn cob, rice husk, sugarcane stalk, pea straw, ginger stems and leaves, peanut shell, and coffee ground were crashed into 10 mesh and dried out under 60 °C.

### Enzyme activity and reducing sugar content assay

Reducing sugar content was determined by DNS (3,5-dinitrosalicylic acid) assay by absorbance measurement at 540 nm [41].

Exocellulase activity (Avicelase), endocellulase activity (CMCase), and filter paper activity (FPase) were carried out by using 1% Avicel, CMC-Na and filter paper as a substrate separately, and reacted with the enzyme at 50 °C for 30 to 60 min, and then reducing sugar content of reaction solution was measured by DNS method. All reaction system consists of 100 µL substrate and 100 µL enzyme solution of suitable concentration, a volume of 300 µL DNS solution was added into it after the reaction was finished. One unit (U) of FPase/CMCase/Avicelase was defined as the amount of enzyme that produced 1 µmol of glucose per minute under standard conditions.

The activity of recombinant  $\beta$ -glucosidase was determined by p-NPG (p-4-nitrophenyl  $\beta$ -D-glucopyranoside) as a substrate at 50 °C for 10 min [42]. The amount of p-NP (p-nitrophenol) was as standard and detected photometrically at 405 nm in a plate reader. One unit (U) of the  $\beta$ -glucosidase enzyme was defined as the amount of enzyme required to release 1 µmol of p-NP within 1 minute.

### Recombinant plasmid construction

The genomic DNA of *B. subtilis* 1AJ3 was extracted via bacterial DNA Kit (Omega Bio-tek, Inc.) and used as a template of Cbh-A. The gene of Cbh-A was amplified using a forward primer 5'-CATGCCATGGGATGGCCCGTAAAAAACTTAAAAAAT-3' (NcoI site underlined) and a reverse primer 5'-CCGCTCGAGGGCACCACCACCACCACCTTGCCTCCTTTATTT-3' (XhoI site underlined). The polymerase chain reaction (PCR) products were linked into the pET-28a(+) vector and transferred into *E. coli* BL21(DE3). Universal primer T7/T7er (5'-TAATACGACTCACTATAGGG-3' and 5'-GCTAGTTATTGCTCAGCGG-3') was used to verify the right clone via sequencing to obtain the whole open reading frames (ORF) sequence of Cbh-A. Cbh-A was finally obtained, and the sequence was submitted to GenBank database to obtain accession number.

## Exocellulase expression

*E. coli* BL21-pET-28a-Cbh-A transformant was cultured in LB medium with 100 µg/mL Kan at 37 °C until value of OD<sub>600</sub> reached 0.8-1.0. Recombinant enzyme expression was induced with 0.4 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), and the transformant was further cultured at 25°C overnight. The cells were harvest by centrifugation at 10,000 rpm for 10 min and resuspended in 1 × PBS buffer. After ultrasonication for 2 h by a SCIENTZ-IID ultrasonic homogenizer (Ningbo Scientz Biotechnology Polytron Technologies Inc., Zhejiang, China), the cell debris was removed by centrifugation at 12,000 rpm for 10 min at 4°C to obtain crude enzyme solution. Recombinant enzyme successfully expression was verified by SDS-PAGE via 15% (w/v) separation gel and enzyme activity was measured.

## Biochemical and homology modeling

According to the whole ORF sequence of Cbh-A obtained by sequencing, biochemical and theoretical characters were gotten by various software and online website. The molecular weight, theoretical pI, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) were predicted using the website ExPASy website (<https://web.expasy.org/protparam/>). The signal peptide was identified using the SignalP 5.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) Domains of Cbh-A were analyzed by Conserved Domains Database (<https://www.ncbi.nlm.nih.gov/cdd>). 3D structure of homology modeling and active sites of Cbh-A were obtained by RaptorX online server (<http://raptorx.uchicago.edu/>).

## Characterization of Cbh-A

The optimum pH condition of Cbh-A was determined at 50 °C for 60 min using the various buffers ranging from pH 2.4 to 11.0. For the pH stability assay, the enzyme was incubated in ice-water for 30 min without substrate and then measured the enzyme activity at 50 °C for 60 min.

The optimum temperature of Cbh-A was measured at optimum pH under different temperatures from 30 to 80 °C (10°C intervals) for 60 min. For the thermal stability of Cbh-A, the enzyme was incubated at different temperatures for 30 min and measured the enzyme activity under optimum temperature and pH for 60 min.

The effect of metal ions and chemicals on enzyme activity was assayed in buffer with various metal salts, including 1, 5, and 10 mM of metal ions and chemicals.

Substrate specificity was measured by using different substrates at 1% concentration: Avicel, pullulan, maltose, CMC-Na, filter paper, barley glucan, laminarin, xylan from beech wood. The enzyme reaction was performed at

50 °C for 60 min in a buffer of pH 6.0. Measuring the enzyme activity and calculating relative activity in different substrate based on the Avicel activity was set to 100%.

## Special characteristic of Cbh-A–pre-incubation at 50°C can increase enzyme activity

Surprisingly, Cbh-A had higher enzyme activity when it was incubated under 50 °C for 30 min than in ice-water before measurement. To observe whether pre-warm up at 50 °C can help to increase the enzyme activities, we measured the enzyme activity of pre-incubation at 50 °C over time. Enzyme activity of Cbh-A was measured every ten minutes within one hour, and every hour after one hour. Set the enzyme activity at 1 h as 100% and calculate the relative enzyme activity at other times.

### Assessment of synergy effect

Cel-5A (GenBank: MN795058) is an endocellulase containing a GH5 catalytic domain (CD) and a CBM3 family Cellulose binding domain (CBD). Its optimum pH is 4.5 and has a wide region of pH from 4.5 to 9.0, the relative enzyme activity all over 80%. It has an optimum temperature of 50 °C. Bgl-16A (GenBank: MN795059) is a  $\beta$ -glucosidase belongs to GH16 family. Its maximum enzyme activity condition is at pH 8.6 and 50 °C. From pH 7.0 to 9.0, it can retain over 80% relative enzyme activity. Cbh-A (GenBank: MN795060) in this study, has an optimum enzyme reaction condition of pH 6.0 and 50°C. It had a wide pH work region from 6.0 to 7.0. Based on the three enzymes suitable enzyme reaction conditions, we chose 1 × PBS (pH 7.2–7.4) buffer as an enzyme reaction system buffer, and all enzyme reaction was measured at 50 °C in further study (Fig. 5).

To measure the synergy between cellulases in the reaction process, we defined the “Degree of synergism” (DS) as the evaluation index.

$$DS = U_n / (U_1 + U_2 + \dots + U_n)$$

In which,  $U_n$  stands for enzyme activity of mixture enzymes.  $U_1$  to  $U_n$  stands for enzyme activity by a single enzyme. When the DS value is greater than 1, it indicates that there is a synergy effect between/among the cellulases, and the larger the value, the stronger the synergy. When the DS value is less than 1, it indicates that the enzymes do not have a synergistic effect, and may even inhibit each other or produce an antagonistic effect. Three cellulases of crude enzyme solution were obtained by expression in advance. For better understanding and evaluating the synergy effect between the three enzymes and their synergistic ability in different types of cellulose substrates, three cellulose substrates were chosen for this part, CMC-Na, Avicel, and filter paper.

Total enzyme reaction system contained 100 $\mu$ L substrate (within 1 × PBS buffer) and 100 $\mu$ L enzyme (mixtures) and enzyme action process take under 50°C. Exact enzyme adding volume for single or mixture was according to Table 3.

### Synergy effect of cellulase in biomass

A same volume ratio of each enzyme (three enzyme mixtures) was added into different lignocellulose biomass to make the final reaction system of 5% (m/V) of straw. Blank was adding 1 × PBS buffer without any enzyme.

Total reducing sugar content was measured via a DNS method of each reaction system and observed the phenomenon of each lignocellulose biomass. The saccharification rate ( $= \text{Total reducing sugar content (mg)} / \text{total biomass quality (mg)} \times 100\%$ ) was calculated to compare each process. Besides, for a better evaluation of enzyme's degradation ability and cellulose substrates adaption, ten biomass materials were selected in this part (switchgrass, wheat straw, corn stalks, corn cob, rice husk, sugarcane stalk, pea straw, ginger stems and leaves, peanut shell, and coffee ground).

## Statistical analysis

All experiments in this study were performed three times. Data were analyzed using Origin 9.2 statistical analysis software, and results were presented as mean  $\pm$  standard deviation (SD) value.

## Abbreviations

CMC-Na: carboxymethylcellulose sodium; HPLC: high performance liquid chromatography; FE-SEM: Field-emission scanning electron microscope; GC-MS: Gas Chromatography-Mass Spectrometer

## Declarations

### Authors' contributions

LM, JC, XW, and XL designed the experiments, conducted the study, performed the statistical analysis and drafted the manuscript. JC and YY helped to do the experiments of enzyme characters. XW and YS helped to do the experiments of synergy effect of three cellulase. XW and YY helped analyze these data and draft the manuscript. YS, BL and YZ participated in the design of the study and revisions of the manuscript. All authors read and approved the final manuscript.

### Author details

Lab of Bioresources, College of Food Science and Engineering, Northwest A&F University, Yangling, Shaanxi Province 712100, China

### Acknowledgements

Our sincere appreciation is expressed for the participation of Dr. Xin Lü, *Professor of Lab of Bioresources, College of Food Science and Engineering, Northwest A&F University, China*. The authors complete this work together, thank everyone's contribution to the experiments design, experimentation, and thesis writing. We thank Dr. Xing Guo, Dr. Qian Wang, Dr. Chenxian Yang, and Dr. Zhu Qiao for their kindly help in experiments and spiritual encouragement.

### Competing interests

The authors declare that they have no competing interests.

### Consent for publication

All authors approved the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Availability of data and materials

The dataset supporting the conclusions of this article are included within the article and Supplementary files.

## Funding

This work was supported by Special Fund for Agro-scientific Research in the Public Interest (Grant No. 201503135).

## References

1. Jager G, Buchs J: **Biocatalytic conversion of lignocellulose to platform chemicals.** *Biotechnol J* 2012, **7**:1122-1136.
2. Hendriks AT, Zeeman G: **Pretreatments to enhance the digestibility of lignocellulosic biomass.** *Bioresour Technol* 2009, **100**:10-18.
3. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS: **Microbial cellulose utilization: fundamentals and biotechnology.** *Microbiol Mol Biol Rev* 2002, **66**:506-577, table of contents.
4. Hamid SBA, Islam MM, Das R: **Cellulase biocatalysis: key influencing factors and mode of action.** *Cellulose* 2015, **22**:2157-2182.
5. Kim IJ, Lee HJ, Choi IG, Kim KH: **Synergistic proteins for the enhanced enzymatic hydrolysis of cellulose by cellulase.** *Appl Microbiol Biotechnol* 2014, **98**:8469-8480.
6. Gusakov AV, Salanovich TN, Antonov AI, Ustinov BB, Okunev ON, Burlingame R, Emalfarb M, Baez M, Sinityn AP: **Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose.** *Biotechnol Bioeng* 2007, **97**:1028-1038.
7. Zhou J, Wang YH, Chu J, Luo LZ, Zhuang YP, Zhang SL: **Optimization of cellulase mixture for efficient hydrolysis of steam-exploded corn stover by statistically designed experiments.** *Bioresour Technol* 2009, **100**:819-825.
8. Allardyce BJ, Linton SM: **Synergistic interaction of an endo- $\beta$ -1,4-glucanase and a  $\beta$ -glucohydrolase leads to more efficient hydrolysis of cellulose-like polymers in the gecarcinid land crab, *Gecarcoidea natalis*.** *Australian Journal of Zoology* 2013, **60**.
9. Tozakidis IE, Brossette T, Lenz F, Maas RM, Jose J: **Proof of concept for the simplified breakdown of cellulose by combining *Pseudomonas putida* strains with surface displayed thermophilic endocellulase, exocellulase and beta-glucosidase.** *Microb Cell Fact* 2016, **15**:103.
10. Daas MJA, Nijse B, van de Weijer AHP, Groenendaal B, Janssen F, van der Oost J, van Kranenburg R: **Engineering *Geobacillus thermodenitrificans* to introduce cellulolytic activity; expression of native and heterologous cellulase genes.** *BMC Biotechnol* 2018, **18**:42.
11. Irwin DC, Zhang S, Wilson DB: **Cloning, expression and characterization of a family 48 exocellulase, Cel48A, from *Thermobifida fusca*.** *Eur J Biochem* 2000, **267**:4988-4997.

12. Hoshino E, Shiroishi M, Amano Y, Nomura M, Kanda T: **Synergistic actions of exo-type cellulases in the hydrolysis of cellulose with different crystallinities.** *Journal of Fermentation and Bioengineering* 1997, **84**:300-306.
13. Jeoh T, Wilson DB, Walker LP: **Effect of cellulase mole fraction and cellulose recalcitrance on synergism in cellulose hydrolysis and binding.** *Biotechnol Prog* 2006, **22**:270-277.
14. Demain AL, Newcomb M, Wu JH: **Cellulase, clostridia, and ethanol.** *Microbiol Mol Biol Rev* 2005, **69**:124-154.
15. Henriksson H, Stahlberg J, Isaksson R, Pettersson G: **The active sites of cellulases are involved in chiral recognition: a comparison of cellobiohydrolase 1 and endoglucanase 1.** *FEBS Lett* 1996, **390**:339-344.
16. Zhang S, Irwin DC, Wilson DB: **Site-directed mutation of noncatalytic residues of *Thermobifida fusca* exocellulase Cel6B.** *European Journal of Biochemistry* 2000, **267**:3101-3115.
17. Zhang S, Lao G, Wilson DB: **Characterization of a *Thermomonospora fusca* exocellulase.** *Biochemistry* 1995, **34**:3386-3395.
18. Chen YC, Chen WT, Liu JC, Tsai LC, Cheng HL: **A highly active beta-glucanase from a new strain of rumen fungus *Orpinomyces* sp.Y102 exhibits cellobiohydrolase and cellotriohydrolase activities.** *Bioresour Technol* 2014, **170**:513-521.
19. Hamada N, Fuse N, Shimosaka M, Kodaira R, Amano Y, Kanda T, Okazaki M: **Cloning and characterization of a new exo-cellulase gene, cel3, in *Irpex lacteus*.** *Fems Microbiology Letters* 1999, **172**:231-237.
20. Liu YJ, Liu S, Dong S, Li R, Feng Y, Cui Q: **Determination of the native features of the exoglucanase Cel48S from *Clostridium thermocellum*.** *Biotechnol Biofuels* 2018, **11**:6.
21. Horsburgh GJ, Atrih A, Williamson MP, Foster SJ: **LytG of *Bacillus subtilis* is a novel peptidoglycan hydrolase: the major active glucosaminidase.** *Biochemistry* 2003, **42**:257-264.
22. Steen A, Buist G, Horsburgh GJ, Venema G, Kuipers OP, Foster SJ, Kok J: **AcmA of *Lactococcus lactis* is an N-acetylglucosaminidase with an optimal number of LysM domains for proper functioning.** *FEBS J* 2005, **272**:2854-2868.
23. Nambu T, Inagaki Y, Kutsukake K: **Plasticity of the domain structure in FlgJ, a bacterial protein involved in flagellar rod formation.** *Genes Genet Syst* 2006, **81**:381-389.
24. Nayyab S, O'Connor M, Brewster J, Gravier J, Jamieson M, Magno E, Miller RD, Phelan D, Roohani K, Williard P, et al: **Diamide Inhibitors of the *Bacillus subtilis* N-Acetylglucosaminidase LytG That Exhibit Antibacterial Activity.** *ACS Infect Dis* 2017, **3**:421-427.
25. Zaloba P, Bailey-Elkin BA, Derksen M, Mark BL: **Structural and Biochemical Insights into the Peptidoglycan Hydrolase Domain of FlgJ from *Salmonella typhimurium*.** *PLoS One* 2016, **11**:e0149204.
26. Suwannarangsee S, Bunterngsook B, Arnthong J, Paemanee A, Thamchaipenet A, Eurwilaichitr L, Laosiripojana N, Champreda V: **Optimisation of synergistic biomass-degrading enzyme systems for efficient rice straw hydrolysis using an experimental mixture design.** *Bioresour Technol* 2012, **119**:252-261.
27. Bhattacharya AS, Bhattacharya A, Pletschke BI: **Synergism of fungal and bacterial cellulases and hemicellulases: a novel perspective for enhanced bio-ethanol production.** *Biotechnol Lett* 2015, **37**:1117-1129.
28. Tsai SL, Park M, Chen W: **Size-modulated synergy of cellulase clustering for enhanced cellulose hydrolysis.** *Biotechnol J* 2013, **8**:257-261.

29. Luciano Silveira MH, Rau M, Pinto da Silva Bon E, Andreus J: **A simple and fast method for the determination of endo- and exo-cellulase activity in cellulase preparations using filter paper.** *Enzyme Microb Technol* 2012, **51**:280-285.
30. Zhang XZ, Sathitsuksanoh N, Zhang YH: **Glycoside hydrolase family 9 processive endoglucanase from *Clostridium phytofermentans*: heterologous expression, characterization, and synergy with family 48 cellobiohydrolase.** *Bioresour Technol* 2010, **101**:5534-5538.
31. Singhanian RR, Patel AK, Sukumaran RK, Larroche C, Pandey A: **Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production.** *Bioresour Technol* 2013, **127**:500-507.
32. Zhang YH, Lynd LR: **Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems.** *Biotechnol Bioeng* 2004, **88**:797-824.
33. Zhang YH, Lynd LR: **A functionally based model for hydrolysis of cellulose by fungal cellulase.** *Biotechnol Bioeng* 2006, **94**:888-898.
34. Sanchez C: **Lignocellulosic residues: biodegradation and bioconversion by fungi.** *Biotechnol Adv* 2009, **27**:185-194.
35. Yang Y, Sharma-Shivappa R, Burns JC, Cheng JJ: **Dilute Acid Pretreatment of Oven-dried Switchgrass Germplasms for Bioethanol Production†.** *Energy & Fuels* 2009, **23**:3759-3766.
36. Samuel R, Pu Y, Raman B, Ragauskas AJ: **Structural characterization and comparison of switchgrass ball-milled lignin before and after dilute acid pretreatment.** *Appl Biochem Biotechnol* 2010, **162**:62-74.
37. Liu JY, Sidhu SS, Wang ML, Tonnis B, Habteselassie M, Mao JD, Huang QG: **Evaluation of various fungal pretreatment of switchgrass for enhanced saccharification and simultaneous enzyme production.** *Journal of Cleaner Production* 2015, **104**:480-488.
38. Boonmee A: **Hydrolysis of various thai agricultural biomasses using the crude enzyme from *Aspergillus aculeatus* iizuka FR60 isolated from soil.** *Braz J Microbiol* 2012, **43**:456-466.
39. Pellegrini VOA, Bernardes A, Rezende CA, Polikarpov I: **Cellulose fiber size defines efficiency of enzymatic hydrolysis and impacts degree of synergy between endo- and exoglucanases.** *Cellulose* 2018, **25**:1865-1881.
40. Hu J, Arantes V, Pribowo A, Saddler JN: **The synergistic action of accessory enzymes enhances the hydrolytic potential of a "cellulase mixture" but is highly substrate specific.** *Biotechnol Biofuels* 2013, **6**:112.
41. Miller GL: **Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar.** *Analytical Chemistry* 1959, **31**:426-428.
42. Odoux E, Escoute J, Verdeil JL, Brillouet JM: **Localization of beta-D-glucosidase activity and glucovanillin in vanilla bean (*Vanilla planifolia* Andrews).** *Ann Bot* 2003, **92**:437-444.

## Tables

### Table 1

Effect of metal ions and chemical on the cellulase activity of *Cbh-A*

Metal ion and chemicals	Relative activity (%)		
	10mM	5mM	1mM
Na <sup>+</sup>	109.88±0.75	111.15±0.52	92.33±1.12
K <sup>+</sup>	107.67±1.34	105.82±0.60	95.55±1.19
Mg <sup>2+</sup>	106.88±0.97	102.40±2.38	105.40±1.27
Zn <sup>2+</sup>	122.37±2.16	119.68±2.83	106.30±0.30
Ca <sup>2+</sup>	124.27±0.82	123.69±0.60	105.24±0.75
Cu <sup>2+</sup>	30.72±0.75	25.82±0.37	98.60±0.74
Mn <sup>2+</sup>	164.32±4.10	152.09±0.89	131.44±1.42
Fe <sup>2+</sup>	119.74±1.57	113.25±1.12	101.08±0.37
Fe <sup>3+</sup>	115.20±0.97	111.09±0.37	99.55±0.15
[NH <sub>4</sub> ] <sup>+</sup>	103.40±0.82	105.45±0.07	97.71±0.22
SDS	101.03±0.45	97.65±0.37	95.13±0.74
EDTA	11.01±0.60	12.38±1.12	13.70±2.16

Activity without any metal ion or chemicals set as 100%.

**Table 2**

Substrate specificity analysis of recombinant enzyme and the original enzyme

Substrate (1%)	Glucan linkage	Relative activity (%)
Pullulan	α-1,6 glucan linkage	ND
Maltose	α-1,4 glucan linkage	277.63±5.65
CMC-Na		97.80±0.89
Filter paper		101.46±1.91
Barley glucan	β-1,3-1,4 glucan linkage	168.44±5.27
Laminarin	β-1,3 glucan linkage	161.52±3.39
Xylan from Beechwood	β-1,4 glucan linkage	185.09±3.20
Avicel	β-1,4 glucan linkage	100.00±1.51

Depending on the substrate, activity was determined under optimal conditions. ND, no detectable activity.

**Table 3**

Synergistic effect reaction system and their DS value in different substrates.

No.	Component of cellulase enzyme system			Enzyme activity in separate system and DS value of enzymes in different substrates <sup>e</sup>					
				CMC-Na		Filter paper		Avicel	
	<i>Cel-5A</i> <sub>a</sub>	<i>Cbh-A</i> <sub>b</sub>	<i>Bgl-16A</i> <sub>c</sub>	EA <sup>f</sup> (U/mL)	DS <sup>g</sup>	EA (U/mL)	DS	EA (U/mL)	DS
1	+			0.080±0.001	-	0.040±0.001	-	0.015±0.001	-
2		+		0.015±0.000	-	0.015±0.000	-	0.015±0.000	-
3			+	0.004±0.000	-	0.011±0.000	-	0.014±0.000	-
4	+	+		0.104±0.003	1.102	0.095±0.001	1.703	0.041±0.000	1.367
5	+		+	0.100±0.002	1.195	0.064±0.002	1.247	0.038±0.001	1.303
6		+	+	0.033±0.001	1.765	0.038±0.000	1.474	0.038±0.001	1.296
7	+	+	+	0.130±0.001	1.326	0.101±0.000	1.525	0.066±0.001	1.501

<sup>a</sup> Endocellulase; <sup>b</sup> Exocellulase; <sup>c</sup> β-glucosidase; <sup>d</sup> Contained in the cellulase system; <sup>e</sup> Substrates are 1% CMC-Na, 2 piece of 6mm-diameter circle filter paper, and 1% Avicel, respectively; <sup>f</sup> Enzyme activity of separate system; <sup>g</sup> Degree of synergism

All the enzyme used in this part were all crude enzyme and the same amount of each cellulase in all enzyme systems.

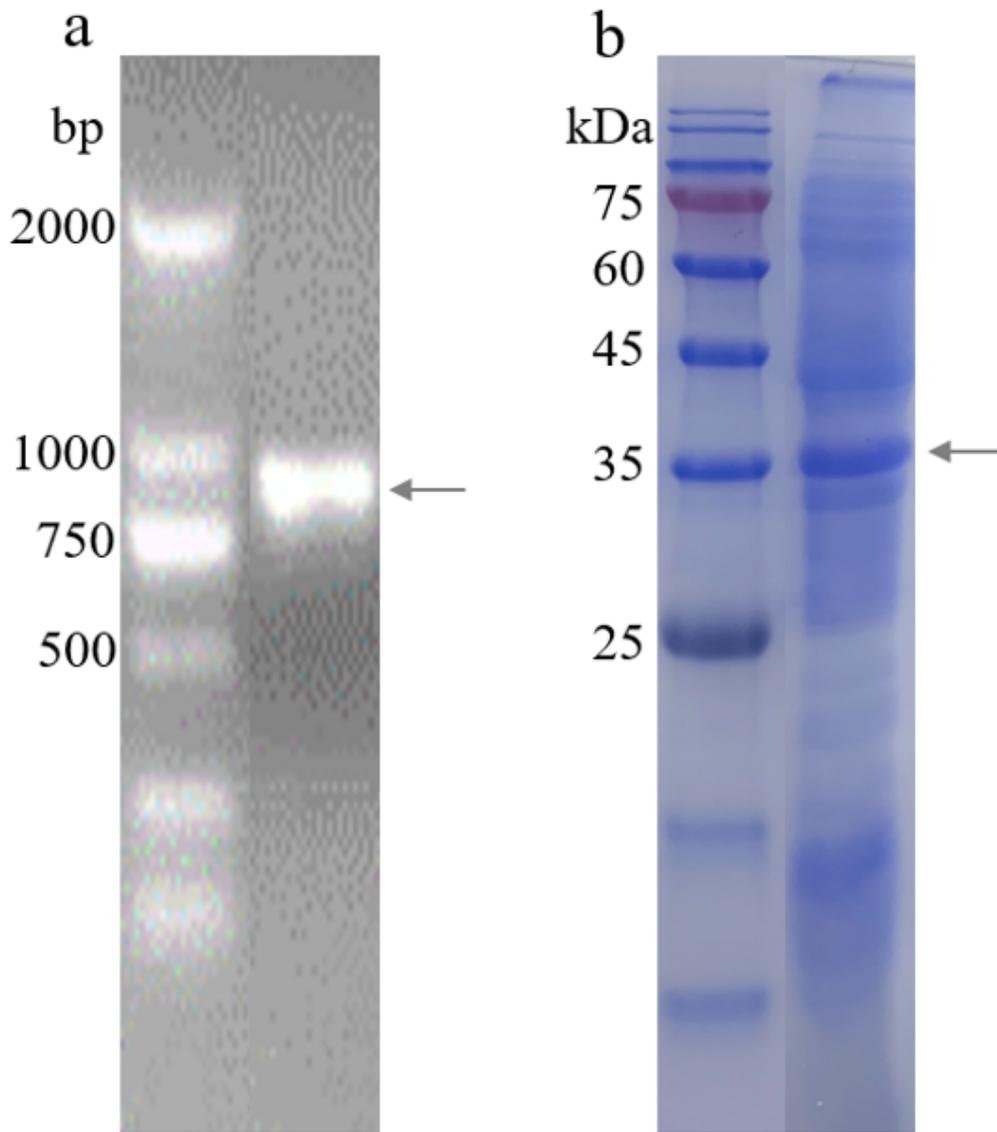
**Table 4**

Saccharification rate (%) of single enzyme and three-mixture enzymes in application of hydrolyzing different biomass lignocellulose

	Saccharification rate (%)				DS
	Endocellulase <i>Cel</i> -5A	Exocellulase <i>Cbh</i> -A	$\beta$ - glucosidase <i>Bgl</i> -16A	Three-enzymes mixture	
Switchgrass	9.020±0.026	7.318±0.018	9.338±0.092	9.615±0.022	0.37
Wheat straw	0.034±0.001	0.022±0.000	0.097±0.000	3.803±0.017	24.90
Corn stalks	0.000±0.000	0.003±0.000	0.028±0.000	5.735±0.018	184.00
Corn cob	2.649±0.015	3.475±0.019	8.718±0.093	8.755±0.140	0.59
Rice husk	0.000±0.000	0.003±0.000	0.019±0.000	0.249±0.001	11.43
Sugarcane stalk	0.024±0.000	0.009±0.000	0.000±0.000	0.217±0.001	6.73
Pea straw	0.250±0.001	0.679±0.003	0.447±0.007	1.031±0.007	0.75
Ginger stems and leaves	0.165±0.002	0.123±0.000	0.696±0.010	0.717±0.003	0.73
Peanut shell	0.003±0.000	0.026±0.000	0.038±0.002	0.047±0.000	0.70
Coffee ground	0.009±0.000	0.112±0.000	0.041±0.000	1.105±0.004	6.84

DS: Degree of synergism.

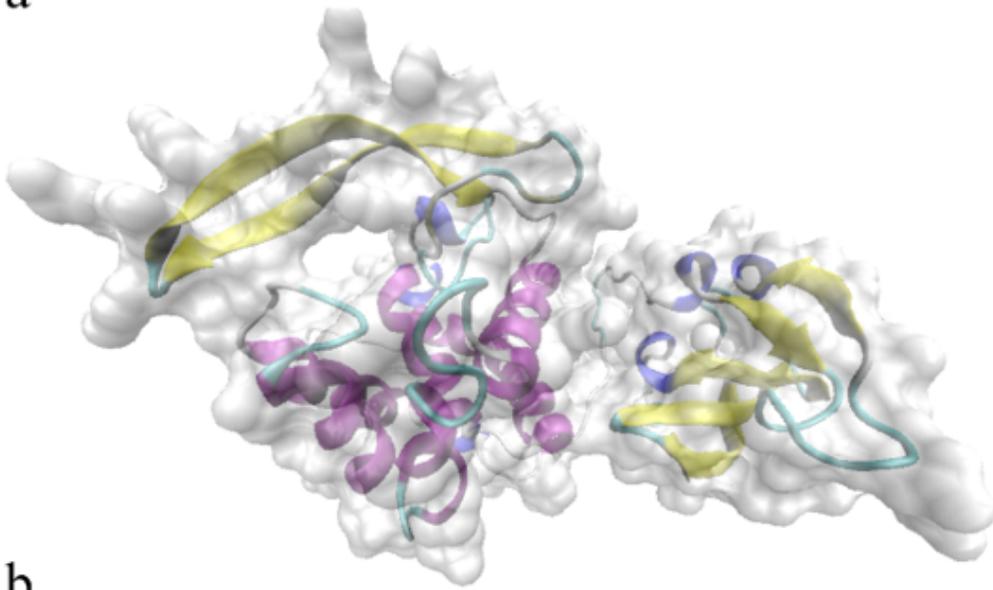
## Figures



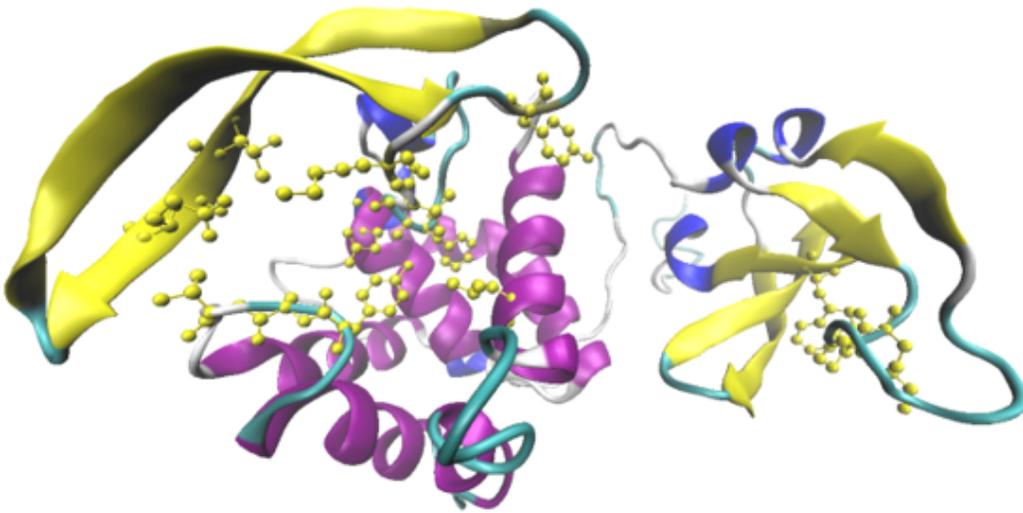
**Figure 1**

1% Agarose gel electrophoresis of PCR products (a) and 15% SDS-PAGE (b) of Cbh-A recombinant protein.

a

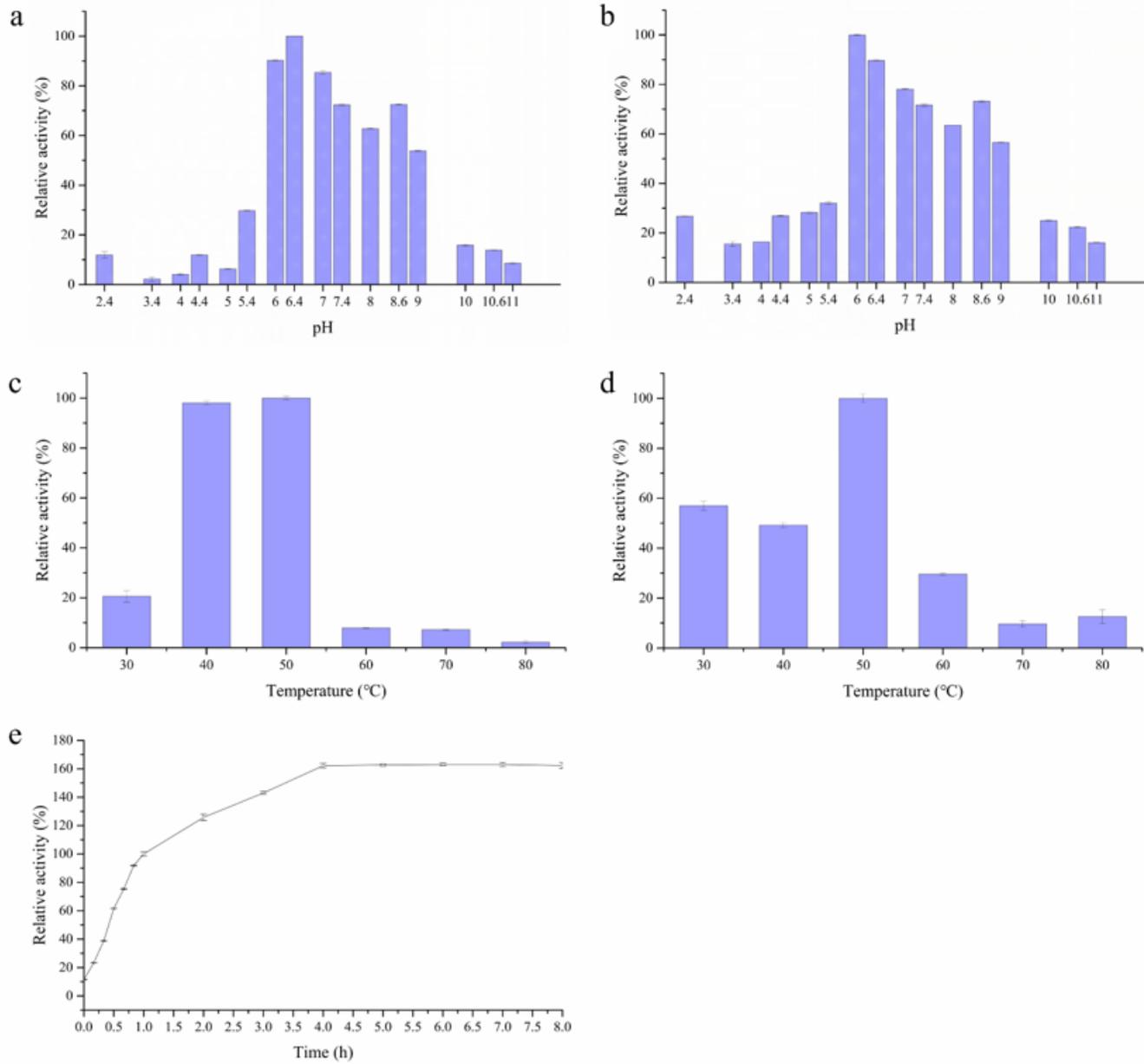


b



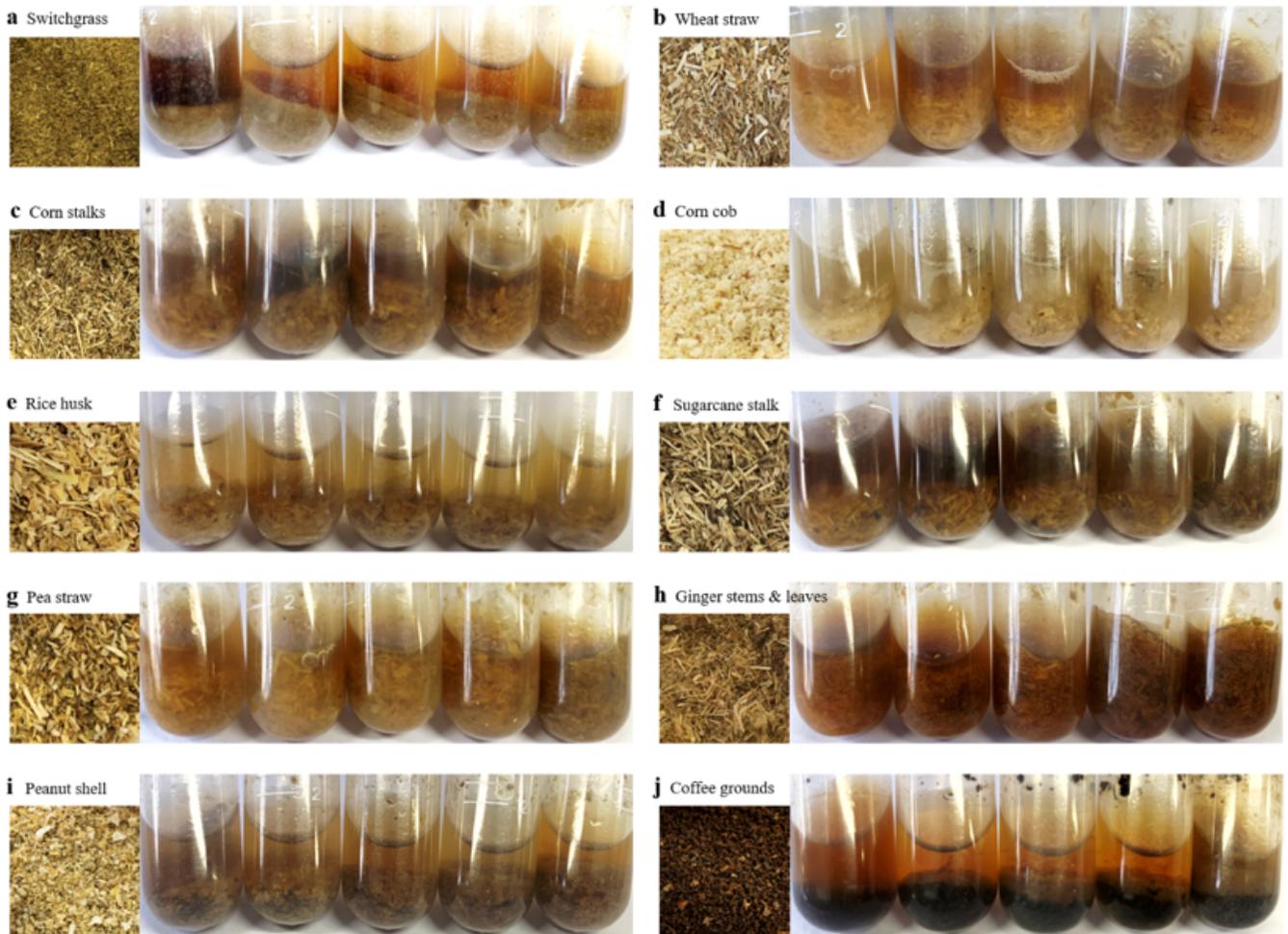
**Figure 2**

3D structure (a) and docking prediction (b) of Chb-A.



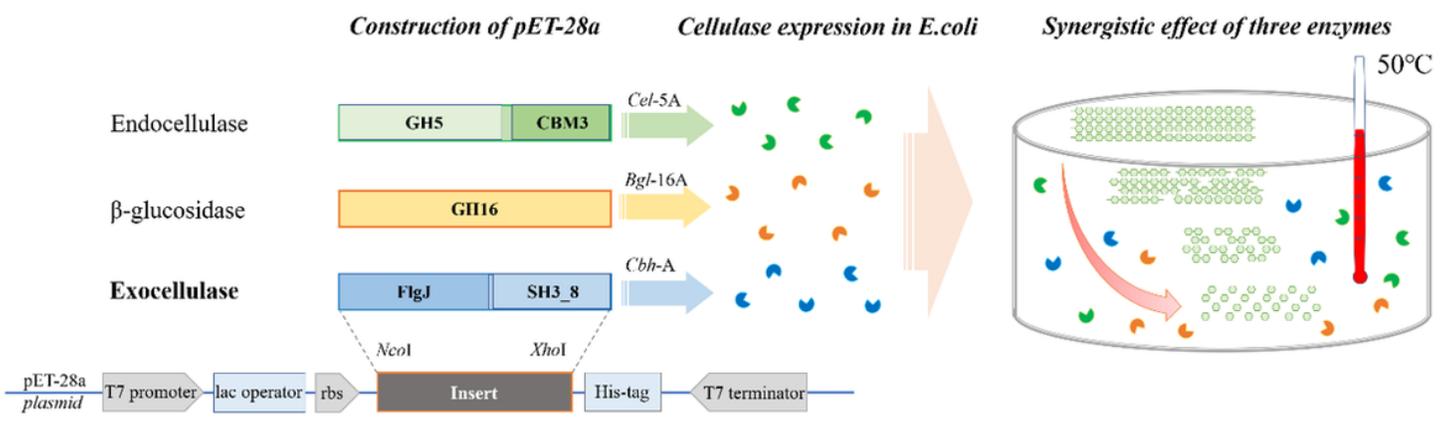
**Figure 3**

Influence pH and temperature on the Avicelase activity of the recombinant enzyme. a optimum of pH; b stability of pH; c optimum of temperature; d stability of temperature; e stability of Cbh-A at 50°C.



**Figure 4**

Pictures of raw biomass lignocellulose and samples of enzymes hydrolyzations. Every group have five tubes, and lignocellulose were hydrolyzed by different enzyme(s) from left to right: blank, Cel-5A, Cbh-A, Bgl-16A, and three enzymes mixture. a-j presents different biomass which was marked on the picture followed the letters.



**Figure 5**

Sketch map of cellulases synergy effect.