Functional Characterization of a Noncatalytic Protein, Athe_0181, from *Caldicellulosiruptor bescii* in Promoting Lignocellulose Hydrolysis

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Caldicellulosiruptor bescii is a cellulolytic bacterium that secretes multifunctional glycoside hydrolases for efficient hydrolysis of lignocellulose into fermentable sugars. Additionally, some abundant noncatalytic proteins accompanying multifunctional glycoside hydrolases are also secreted by *C. bescii*, but its function has not yet been demonstrated. In this study, noncatalytic protein Athe_0181 and multifunctional glycoside hydrolases CbMan5C/Cel5A were expressed and purified from *Escherichia coli* BL21(DE3). Effective binding capacity of Athe_0181 to lignocellulose was displayed, and it showed preferential affinity to rice straw. Athe_0181 was shown to be a cellulase synergistic protein. It exhibited high synergistic activity of 523% in the presence of 25 μg/mL of CbMan5C/Cel5A with microcrystalline cellulose as the substrate. The structure-modifying activity of Athe_0181 to microcrystalline cellulose was demonstrated by scanning electron microscopy and X-ray diffraction analysis. These characteristics demonstrated that Athe_0181 played a role in the synergism of glycoside hydrolases from *C. bescii* for efficient hydrolysis of lignocellulose.

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Keywords: Athe_0181; CbMan5C/Cel5A; Glycoside hydrolase; Lignocellulose hydrolysis; Noncatalytic protein; Synergism

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INTRODUCTION

Lignocellulose is the most abundant and renewable bioresource for producing biofuels and biochemicals, and microbial glycoside hydrolases (GHs) are essential in the conversion process (Liu *et al*. 2021). However, complex structure and the recalcitrance of lignocellulose play a role as barriers for enzymatic hydrolysis (Kahn *et al*. 2020). *Caldicellulosiruptor bescii*, an anaerobic and Gram-positive thermophile, is able to grow at temperatures up to 90 °C (the optimal growth temperature is 78 °C), and it can efficiently degrade crystalline cellulose, xylan, and untreated biomass (Poudel *et al*. 2018). The secretome of *C. bescii* is more efficient than *Trichoderma reesei* in hydrolyzing microcrystalline cellulose, untreated timothy grass, and rice straw (Kanafusa-Shinkai *et al*. 2013). Six multifunctional GHs, namely CelA (Athe_1867, CbCel9A/Cel48A), CelB (Athe_1859, CbMan5B/Cel44A), CelC (Athe_1857, CbXyn10C/Cel48B), CelD (Athe_1866, CbMan5C/Cel5A), CelE (Athe_1865, CbCel9B/Man5A), and CelF

(Athe_1860, CbXyl74A/Cel48C), play essential roles in the deconstruction of lignocellulose (Dam *et al*. 2011; Ye *et al*. 2012; Xue *et al*. 2015; Conway *et al*. 2018). The multi-domain architecture of these multifunctional GHs are commonly two or three cellulose binding carbohydrate binding module (CBM) domains surrounded by two catalytic GH domains. Most of multifunctional GHs or single domain of multifunctional GHs have been characterized (Su *et al*. 2012; Ye *et al*. 2012; Yi *et al*. 2013; Xue *et al*. 2015; Rong *et al*. 2016; Chu *et al*. 2019).

Proteomic studies revealed that *C. bescii* possesses not only an array of abundant GHs, but also some noncatalytic proteins binding to carbohydrate substrate (Lochner *et al*. 2011; Yokoyama *et al*. 2014; Poudel *et al*. 2018). Only few studies on these noncatalytic proteins were characterized. The binding properties of noncatalytic proteins Athe_0847 and Athe_0597 were investigated, and both proteins showed the highest binding affinity for the plant cell wall among the insoluble polysaccharides. The binding of these noncatalytic proteins might be necessary for efficient utilization of polysaccharides by *C. bescii* at high temperatures (Yokoyama *et al*. 2014). Tāpirins (Athe_1870) showed the binding ability to microcrystalline cellulose, switchgrass, poplar, and filter paper (Lee *et al*. 2019). Type IV pilus (T4P) was demonstrated to play a role in attachment to crystalline cellulose and xylan (Khan *et al*. 2020). CbHsp18 from *C. bescii* was reported to enhance the hydrolysis activity and thermostability of glycoside hydrolases CbCelA-TM1(GH9-CBM3C), CbXyn10A and CbCdx1A (Su *et al*. 2012). Noncatalytic protein Athe_0181 of unknown function were identified in the *C. bescii* proteins binding to the cell walls of timothy grass. It is one of the most abundant proteins in the extracellular secretome of *C. bescii* specific for growth on complex substrates (xylan, switchgrass, and Avicel), which likely have crucial roles in the deconstruction or utilization of complex substrates (Poudel *et al*. 2018). Synergistic proteins without significant hydrolytic activity on cellulose, such as swollenin and EXLX1, could act as accessory or helper agents to promote the efficiency of enzymatic hydrolysis of lignocellulose (Kim *et al*. 2014). Briefly, the current authors propose that the function of Athe_0181 may work as a synergistic protein to GHs of *C. bescii* in the hydrolysis of lignocellulose.

In this study, Athe_0181 and CbMan5C/Cel5A were expressed and purified from *Escherichia coli* BL21(DE3). Then, the lignocellulose binding capacity of Athe_0181 and the synergism of Athe_0181 to CbMan5C/Cel5A hydrolyzing lignocellulose, as well as the structure-modifying activity of Athe_0181 to microcrystalline cellulose were determined.

EXPERIMENTAL

Materials

Bacterial strains, plasmids, and regents

Plasmid pET22b (+) and pET-28a (+) were used for expression. *E. coli* BL21 (DE3) was utilized as the expression host. The components of the Luria-Bertani (LB) medium used for culture were from Oxoid Ltd. (Basingstoke, England). Additionally, 3,5-dinitrosalicylic acid (DNS), microcrystalline cellulose, and protein marker were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Isopropyl β-D-1-thiogalactopyranoside and (IPTG) and carboxymethyl cellulose (CMC-Na) were provided by Sigma Aldrich (St. Louis, MO, USA). Konjac glucomannan and cellobiose were purchased from Megazyme (Bray, Ireland). Lastly, a His-Bind Purification Kit was procured from Novagen (Beijing, China).

Methods

Protein expression and purification

The amino acid sequences of Athe_0181 (GenBank Accession No. ACM59333) and CbMan5B/Cel44A (GenBank Accession No. ACM60954) were analyzed by signalP-5.0 Server. Proteins homologous to Athe_0181 were obtained by Basic Local Alignment Search Tool (BLAST, programwww.ncbi.nlm.nih.gov/BLAST/). Athe_0181 was reported as unsuccessfully expressed in *E. coli*, probably as a result of its cytotoxicity (Yokoyama *et al*. 2014). PET22b (+) was employed as the expression plasmid in this study. Gene Athe_0181 and CbMan5C/Cel5A *via* codon optimization were synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China) and connected to the pET22b (+) and pET28a (+), respectively, and the recombinant plasmids pET22b (+)-0181 and pET28a (+)-CbMan5C/Cel5A were obtained. The recombinant plasmids were transformed into *E. coli* BL21(DE3), which were then spread on LB plates containing ampicillin at 80 μg/mL. After overnight culture at 37 °C, the monoclonal cells were inoculated into LB liquid medium (80 μg/mL ampicillin) and grown at 37 °C (200 rpm) overnight, and then were transferred to fresh liquid LB medium (80 μg/mL ampicillin). Protein expression was induced by adding IPTG at a final concentration of 0.05 to 0.5 mM when $OD600_{nm}$ of cultures reached 0.6-0.8, and then grown at 16 °C for 6 to 12 h. The *E. coli* cells were harvested by centrifugation (5000 × g for 10 min at 4 °C) and the recombinant proteins were purified as described below.

The cells were crushed by sonication and then were centrifuged at 10000 × g for 30 min at 4 °C. The crude enzyme solution was filtered through 0.45 μm membrane filter. The target proteins were purified using His-Bind Purification Kit with 1mL nickel affinity column chromatography, eluted with different concentrations of imidazole (20 to 400 mM) in 20 mM Tris-HCl buffer (150 mM NaCl, pH 7.5) at a flow rate of 1 mL/min. The molecular mass of protein and purity were determined by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) kit (Sangon Biotech, Shanghai, China). The protein concentration was assayed by a Bradford Assay Kit (Sangon Biotech, Shanghai, China). The purified proteins were used in the subsequent assays. All assays were performed in triplicate, and error bars represented the standard error of the mean.

The binding capacity of Athe_0181

Athe_0181 (300 μg/mL, final concentration) was incubated with lignocellulose containing microcrystalline cellulose, rice straw, and palm kernel meal (20 mg/mL, final concentration) in 50 mM citrate buffer (pH 5.0) for 24 h at 70 °C. After centrifugation, protein unbound to the lignocellulose in the supernatant were assayed by a Bradford Assay Kit. The amount of bound protein was calculated as by subtracting the unbound from the total amount of protein in the supernatant from the total protein (Lin *et al*. 2013).

Synergism of Athe_0181 and CbMan5C/Cel5A in hydrolysis of lignocellulose

Synergism reactions were performed with 25 to 300 μg/mL of CbMan5C/Cel5A and 25 to 300 μg/mL of Athe_0181 incubated in 50 mM citrate buffer (pH 5.0). Lignocellulose (20 mg/mL) containing microcrystalline cellulose, rice straw, and palm kernel meal were substrates. The reactions were performed at 70 °C for 4 to 48 h. The reaction mixtures with inactive protein were defined as the control. The released reducing sugars were quantified using the DNS method (Xia *et al*. 2019). Synergistic activity was calculated according to Eq. 1 (Meng *et al*. 2020),

$$\text{Synergistic activity (\%)} = (B / A - 1) \times 100 \quad (1)$$

where *A* represents the amount of reducing sugars (μg/mL) released by CbMan5C/Cel5A alone, and *B* represents the amount of reducing sugars (μg/mL) released by CbMan5C/Cel5A and Athe_0181.

Scanning electron microscopy

Microcrystalline cellulose (20 mg/mL) was treated with 300 mg/mL of Athe_0181 at 70 °C for 48 h with samples only treated with 50 mM citrate buffer (pH 5.0) as the control, and then washed by deionized water. The morphology of microcrystalline cellulose was observed by scanning electron microscopy (SEM) (Sigma-500, ZEISS, Oberkochen, Germany).

X-ray diffraction (XRD)

The XRD patterns of microcrystalline cellulose treated by the same method as SEM, were measured by a smartlab X-ray diffractometer (Rigaku Corp., Tokyo, Japan) with the CuKα radiation generated at 40 kV and 150 mA. The scans were conducted in a 2θ range, from 5° to 50°, at a scanning rate of 2° per min. The cellulose crystallinity index (*CrI*) was calculated according to Eq. 2,

$$CrI = (I_{total} - I_{am}) / I_{total} \quad (2)$$

where I_{total} is the scattered intensity at the main peak (around 2θ = 22.5°) and I_{am} is the scattered intensity due to the amorphous portion (around 2θ = 18°) (Zhe *et al*. 2018).

RESULTS AND DISCUSSION

Protein Expression and Purification

In this study, Athe_0181 was abundantly expressed in soluble form in *E. coli* BL21 (DE3) (Fig. 1A).

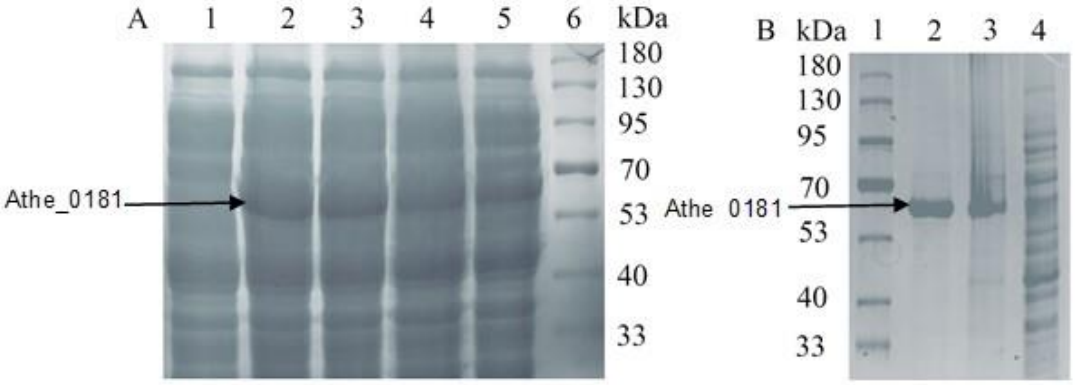


Fig. 1. SDS-PAGE analysis of the expressed and purified Athe_0181: A: the expression of Athe_0181. Lane 1: cell lysate without induction; Lanes 2 through 5: cell lysate with induction (concentration of IPTG was 0.5 mM, 0.3 mM, 0.1 mM, and 0.05 mM, respectively); and Lane 6: protein molecular mass marker. B: the purification of Athe_0181. Lane 1: protein molecular mass marker; Lane 2: the purified proteins eluted with 50 mM imidazole; Lane 3: the purified proteins eluted with 20 mM imidazole; and Lane 4: the flow through fluid *via* nickel affinity column

The theoretical molecular mass of Athe_0181 without signal sequence is 62.15 kDa, which was consistent with the results of SDS-PAGE (Fig. 1). The purification process was conducted as shown in Table 1, which shows that the yield and purification fold were 47.9% and 11.6, respectively. The purified fraction of lane 2 (Fig. 1B) was further characterized.

Table 1. Purification Process of Athe_0181

Purification Step	Total Protein (mg)	Synergistic Activity (%)	Total Synergistic Activity (%)	Yield (%)	Purification Fold
Crude Enzyme Solution	126.8 ± 2.08	7.28	923	100	1
Nickel Affinity Column	5.08 ± 0.56	87.0	442	47.9	12.0

The cellulolytic activity of Athe_0181 was investigated and no traceable amount of reducing sugar was detected when CMC-Na, cellobiose, konjac glucomannan, microcrystalline cellulose, palm kernel meal, and rice straw were incubated with Athe_0181 (data not shown). These findings indicated that Athe_0181 was definitely a noncatalytic protein to lignocellulose. Athe_0181 is annotated as extracellular solute-binding protein in databases. It contains an SBP bacterial family 1 (SBP bac 1) domain and UgpB domain (Yokoyama *et al*. 2014)). Extracellular solute-binding proteins from *Caldicellulosiruptor changbaiensis*, *Caldicellulosiruptor saccharolyticus*, and *Caldicellulosiruptor acetigenus* all showed the highest identity to Athe_0181 (83.5% amino acid sequence similarity). With the exception of the genus *Caldicellulosiruptor*, extracellular solute-binding protein from *Treponema* sp. displayed the highest identity of 44.2% to Athe_0181.

Binding Capacity and Synergistic Activity of Athe_0181

Although Athe_0181 was discovered as the plant cell wall-binding protein (Yokoyama *et al*. 2014), the binding capacity had not been characterized. It exhibited a binding capacity of 9.19, 9.77, and 13.2 μg/mg-substrate to microcrystalline cellulose, palm kernel meal, and rice straw, respectively (Fig. 2A). Athe_0181 displayed more preferential affinity to rice straw and the protein binding percentage was 88.2%.

The synergistic activity of Athe_0181 to CbMan5C/Cel5A was investigated. CbMan5C/Cel5A has been confirmed as a multimodular GH, which has a mannanase module at the N terminus and a cellulase module at the C terminus (Xue *et al*. 2015). The cellulase is capable of hydrolyzing soluble substrates such as CMC, barley β-Glucan, locust bean gum galactomannan, and 1,4-β-D-mannan (Conway *et al*. 2018). As shown in Fig. 2B, CbMan5C/Cel5A also showed hydrolysis activity to insoluble substrates, and Athe_0181 acted as helpers to promote CbMan5C/Cel5A activity. The amount of reducing sugar produced from microcrystalline cellulose, palm kernel meal, and rice straw by using a binary of 100 μg/mL of CbMan5C/Cel5A and 300μg/mL of Athe_0181 was greatly increased compared to CbMan5C/Cel5A alone, and synergistic activity reached 87.0%, 80.1%, and 73.2%, respectively. These results indicated that Athe_0181 worked as a synergistic protein to CbMan5C/Cel5A.

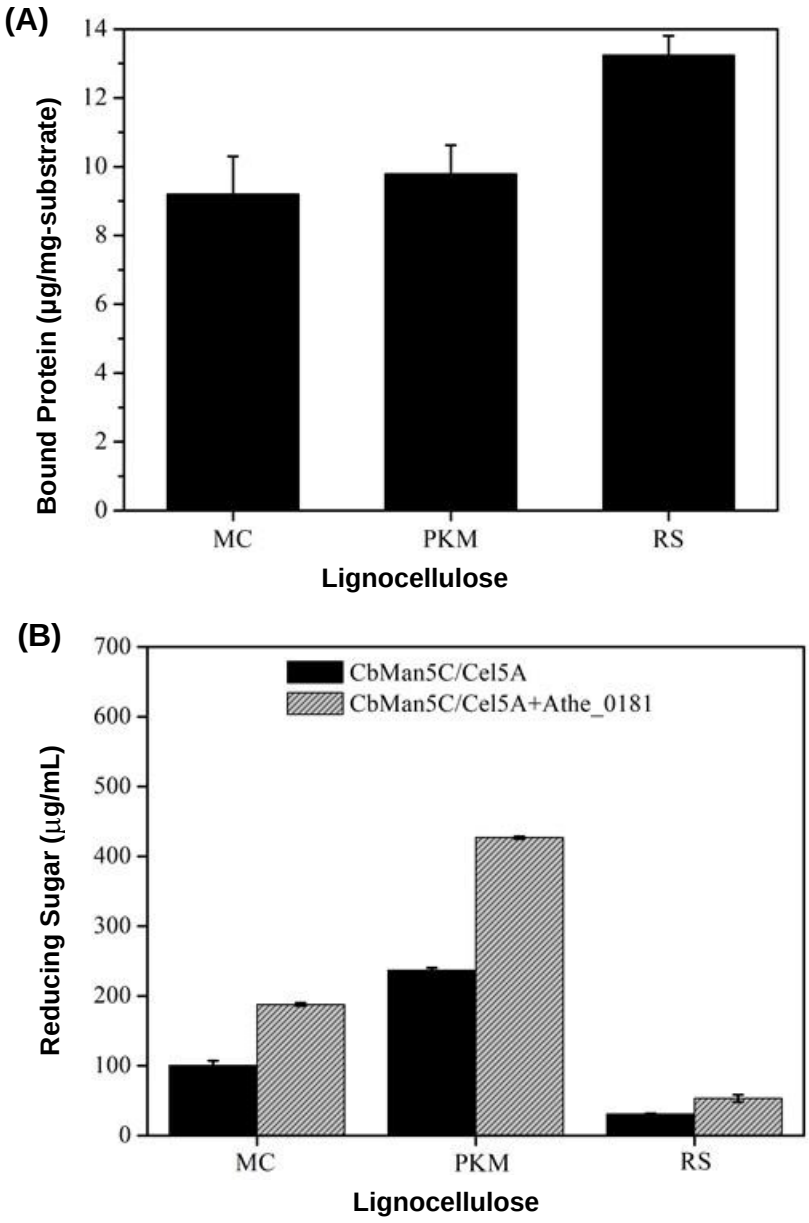


Fig. 2. The binding capacity and synergism of Athe_0181 and CbMan5C/Cel5A in hydrolysis of lignocellulose: A: the lignocellulose bound protein and B: synergism of Athe_0181 and CbMan5C/Cel5A in hydrolysis of lignocellulose (MC, PKM, and RS represent microcrystalline cellulose, palm kernel meal, and rice straw, respectively)

Synergism of Athe_0181 and CbMan5C/Cel5A in Hydrolysis of Microcrystalline Cellulose

Synergism of Athe_0181 to CbMan5C/Cel5A hydrolyzing microcrystalline cellulose was further investigated. It could facilitate the hydrolysis of microcrystalline cellulose, with a yield of 237 μg/mL of reducing sugars at 12 h, which was much more than CbMan5C/Cel5A alone (121 μg/mL) (Fig. 3), and the synergistic activity was 95.1%. The synergistic activity declined slightly with the extension of time, but the synergistic activity still achieved 53.8%, which was similar to the synergistic activity (51.5%) of swollenin POSWOI from *Penicillium oxalicum* at 48 h (Kang *et al*. 2013).

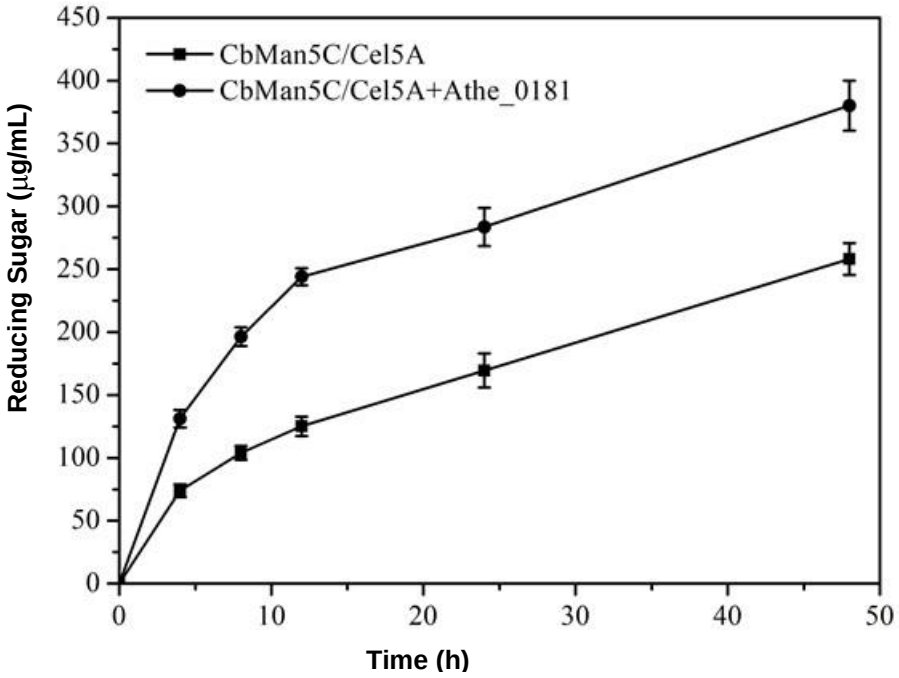


Fig. 3. Synergism of Athe_0181 and CbMan5C/Cel5A in hydrolysis of microcrystalline cellulose

Effect of Athe_0181 and CbMan5C/Cel5A Amount on Synergism in Hydrolysis of Microcrystalline Cellulose

The mixing ratio of synergistic protein to GHs is thought to be essential to exhibit synergism (Kim *et al*. 2014). Here, the relationship between the amount of Athe_0181 and the synergistic effect on CbMan5C/Cel5A hydrolyzing microcrystalline cellulose was studied (Fig. 4A). The reducing sugar yield was increased significantly with Athe_0181 concentration (0 to 50 μg/mL) ($P<0.05$), and the highest production was 299 μg/mL at an Athe_0181 concentration of 50 μg/mL and was 2.43 times of CbMan5C/Cel5A (100 μg/mL) in the absence of Athe_0181. With Athe_0181 concentration increased, no obvious change of reducing sugar yield was observed ($P >0.05$). This indicated that the synergistic effect is saturated, which is similar to the results of POSWOI and *Bs*EXLX1 (Kang *et al*. 2013; Zhang *et al*. 2021b). Athe_0181 was shown to have strong binding capacity to the microcrystalline cellulose (Fig. 2), so there may be competition for substrate sites between Athe_0181 and CbMan5C/Cel5A.

As shown in Fig. 4B, the amount of reducing sugar rose as the concentration of CbMan5C/Cel5A rose from 25 to 300 μg/mL, and in coordination with Athe_0181, the production of reducing sugar was enhanced significantly ($P <0.05$). With the assistance of 50 μg/mL of Athe_0181, the reducing sugar yield increased from 19.3 to 120 μg/mL in presence of 25 μg/mL of CbMan5C/Cel5A. The highest synergistic activity reached 523%. While the higher concentration, such as 200 μg/mL of CbMan5C/Cel5A, resulted in the synergistic activity of 112.0%, indicating that Athe_0181 was more collaborative at a lower concentration of cellulase, which was consistent with the behavior of *Bs*EXLX1 (Zhang *et al.* 2021b).

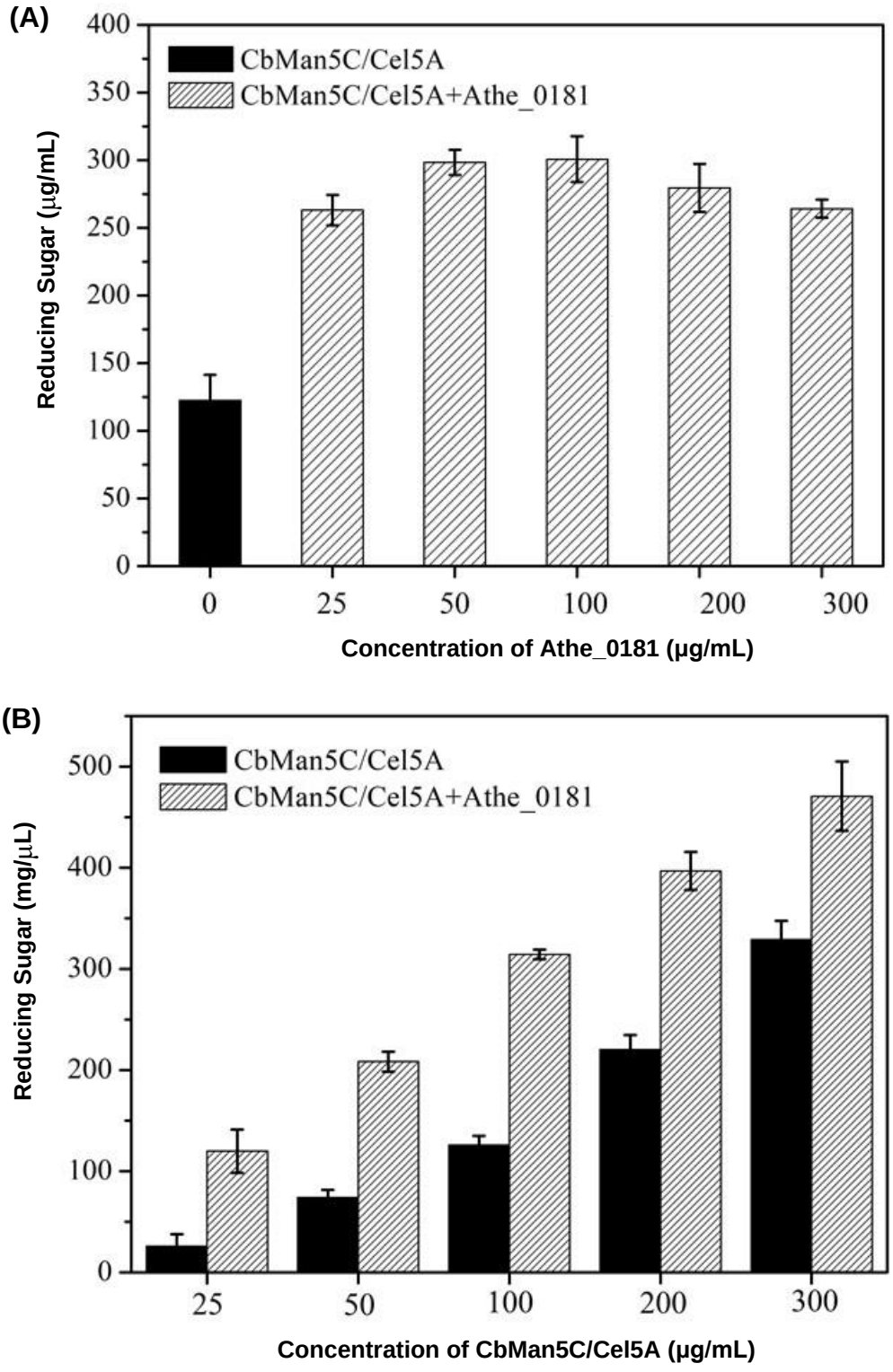
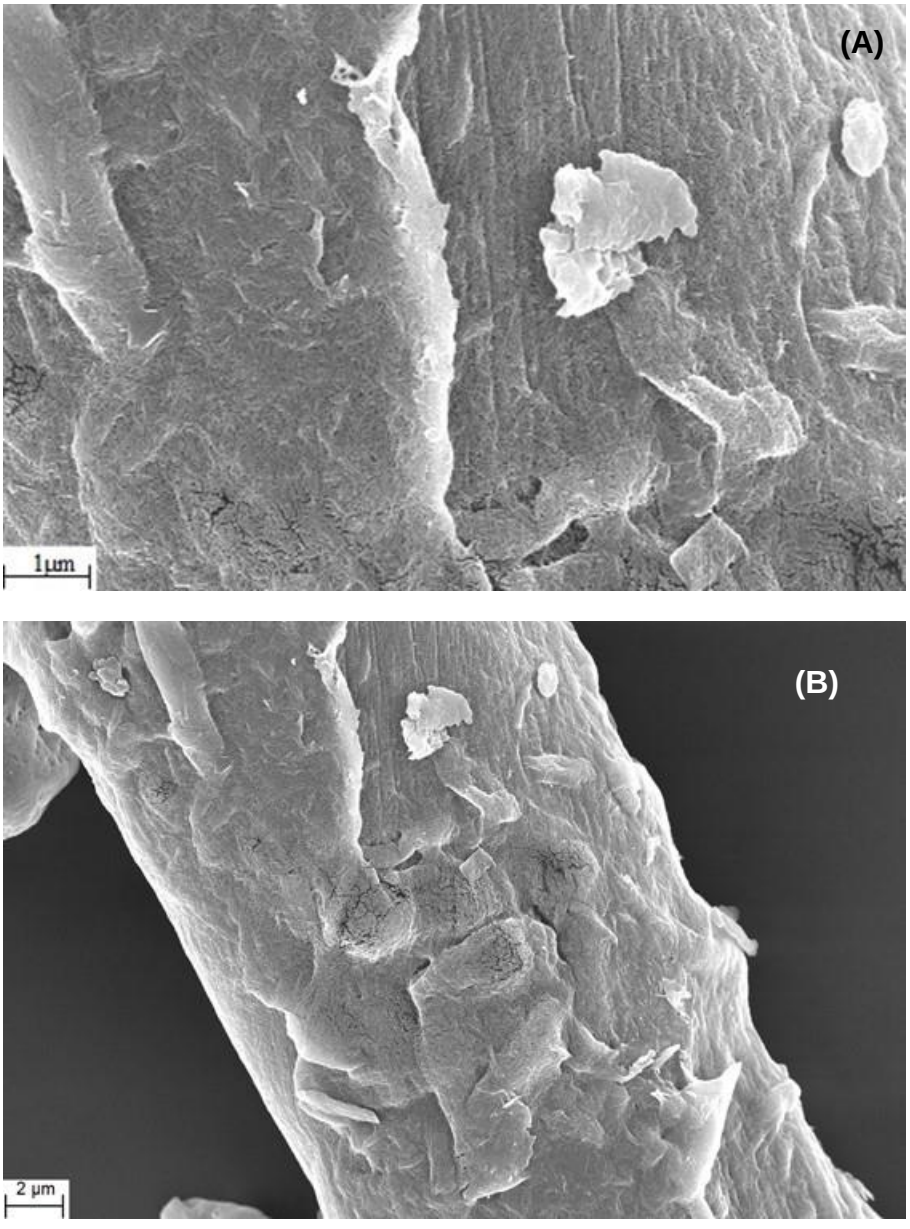
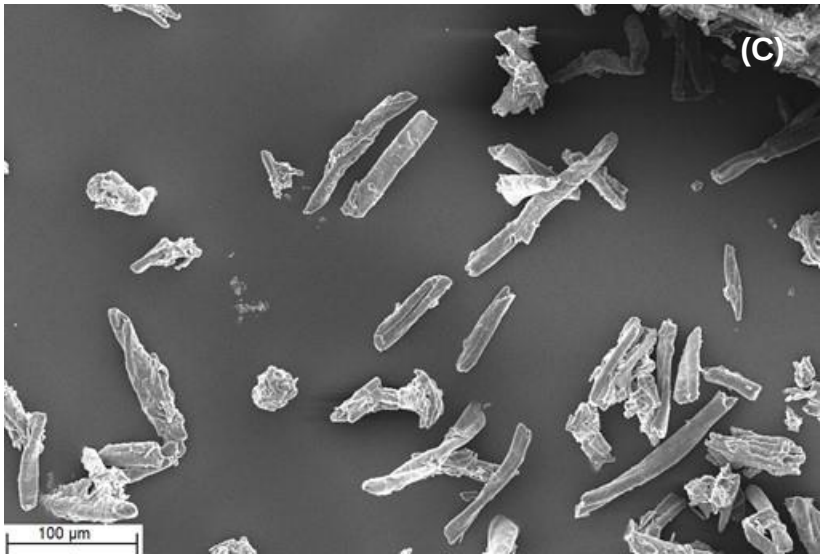


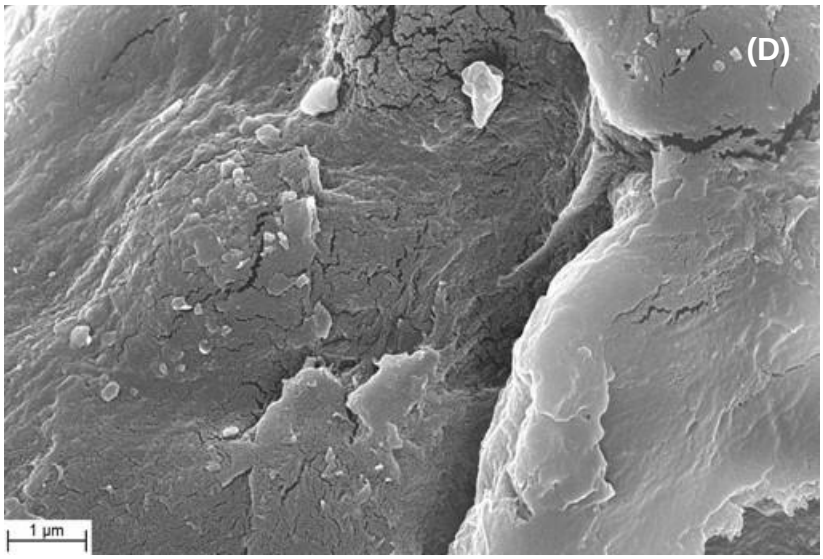
Fig. 4. Effect of Athe_0181 and CbMan5C/Cel5A amount on synergism in the hydrolysis of microcrystalline cellulose hydrolysis: A: effect of Athe_0181 amount and B: effect of CbMan5C/Cel5A amount

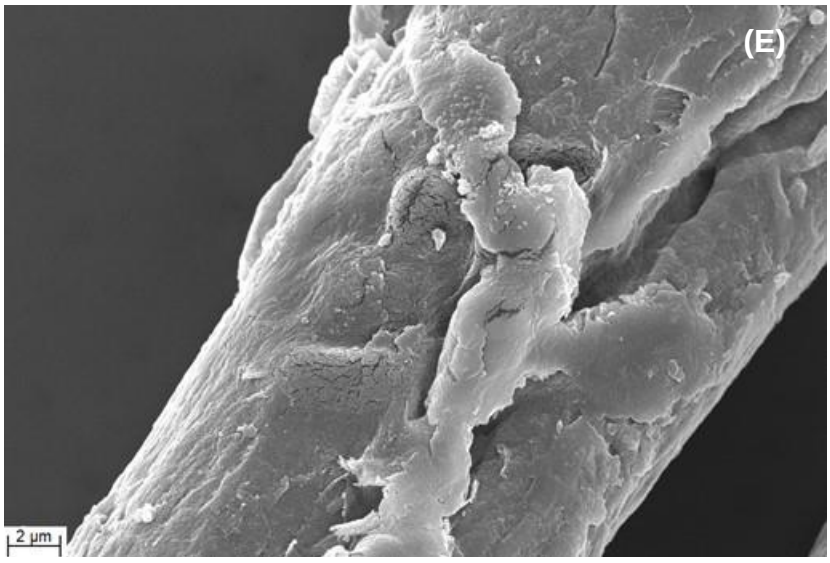
SEM Analysis of Microcrystalline Cellulose

Synergistic proteins are known to be capable of loosening or disrupting the packaging or changing morphology of the plant cell wall and polysaccharides. For example, the enlargement of fibers was observed, and microcrystalline cellulose (Avicel) was disrupted into smaller particles with the treatment of swollenin SWO and *Tl*SWO, respectively (Xiao *et al*. 2020; Zhang *et al*. 2021a). However, some synergistic proteins were reported to have no cellulose disruption activity, such as swollenin Swo2 (Zhou *et al*. 2011). In the current study, microphotographs of the Athe_0181-treated microcrystalline cellulose were taken using SEM (Fig. 5). The results showed that microcrystalline cellulose treated with Athe_0181 (Figs. 5D, E, and F) was less interconnected and dense, and the surface was rougher, compared to microcrystalline cellulose treated with citrate buffer (Figs. 5A, B, and C).




(C)
100 µm


(D)
1 µm


(E)
2 µm

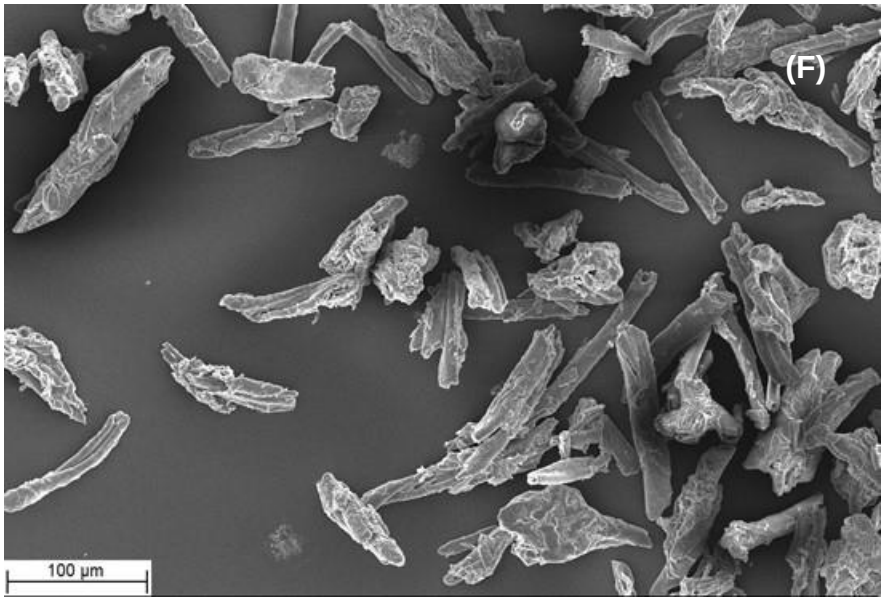


Fig. 5. The SEM images of microcrystalline cellulose (A, D: 30 KX; B, E: 10KX; C, F: 500X). A through C: citrate buffer-treated and D through E: citrate buffer containing Athe_0181-treated

XRD Analysis of Microcrystalline Cellulose

The XRD patterns of microcrystalline cellulose were analyzed (Fig. 6). Athe_0181-treated sample and the control sample presented the typical cellulose type I crystal structure (Yang *et al*. 2020). This suggested that Athe_0181 did not alter the crystal structure. The scattered intensity presented the difference between the buffer and Athe_0181-treated microcrystalline cellulose. Furthermore, I_{total} and *CrI* of microcrystalline cellulose treated with Athe_0181 was decreased and *CrI* was reduced by 1.65%.

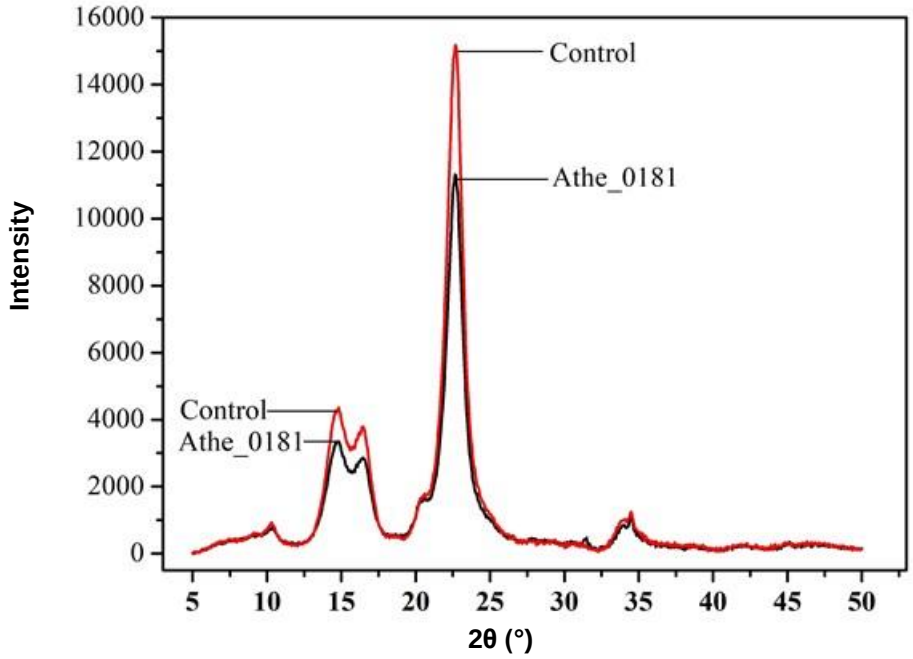


Fig. 6. The XRD patterns of microcrystalline cellulose (Control: citrate buffer-treated; Athe_0181: citrate buffer containing Athe_0181-treated)

Enhanced catalytic efficiency of GHs by synergistic proteins could be due to different reasons such as modification of the substrate structure or the direct hydrolytic activity on the substrate (Kim *et al*. 2014; Georgelis *et al*. 2014; Xiao *et al*. 2020). Athe_0181 was absent of hydrolytic activity, revealing that Athe_0181 boosting the lignocellulose degradation of CbMan5C/Cel5A did not possess hydrolytic activity. The SEM and X-ray diffraction results showed that Athe_0181 demonstrated certain structure-modifying activity, which may enhance the CbMan5C/Cel5A ability for lignocellulose hydrolysis.

CONCLUSIONS

1. The recombinant pET-22b (+) harboring the Athe_0181 gene was introduced into *E. coli* BL21 (DE3) for expression. Athe_0181 was purified *via* a nickel column, and it appeared to have no activity towards cellulosic substrates.
2. The capacity of Athe_0181 binding to the three lignocelluloses, microcrystalline cellulose, palm kernel meal, and rice straw was 9.19, 9.77, and 13.2 μg/mg-substrate, respectively. Athe_0181 worked as the synergistic protein to CbMan5C/Cel5A and synergistic activity was 87.0%, 80.1%, and 73.2%, respectively, with the three lignocelluloses as the substrates.
3. Microcrystalline cellulose was applied as the substrate, and the highest synergistic activity was observed with hydrolysis at 12 h. In the presence of 25 μg/mL of CbMan5C/Cel5A, the reducing sugar yield increased from 19.3 μg/mL to 120 μg/mL with the assistance of 50 μg/mL of Athe_0181, and the synergistic activity reached 523%.
4. The SEM and X-ray diffraction results showed that Athe_0181 could modify the structure of microcrystalline cellulose, which may result in more accessibility to the lignocellulose.
5. The functional characterization of Athe_0181 indicated that Athe_0181 may play a role in lignocellulose hydrolysis and contributed to better clarify the mechanisms of *C. bescii* in the efficient deconstruction of plant biomass.

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