Recombinant Expression of Lytic Polysaccharide Monooxygenase and its Functional Characterization

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Lytic polysaccharide monooxygenases (LPMOs) are a class of copper-dependent enzymes that can act on crystalline polysaccharides directly, which plays a critical role in cellulose degradation. In addition to reports on its structure and mechanism of action, it is important to study the auxiliary activity 9 (AA9) characteristics from different resources to support the mechanism research. The gene encoded *To*LPMO9A was cloned from *Trichoderma orientalis* EU7-22 and first heterologously expressed in *Pichia pastoris* GS115. Both metal ions and reducing agent concentrations showed an important effect on *To*LPMO9A. The *To*LPMO9A exhibited maximal activity at 60 °C and a 6.0 pH. In addition, *To*LPMO9A showed substrate specificity. The matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis showed that *To*LPMO9A cleaved the glycosidic bonds at C_1 and C_4/C_6 position *via* oxidation. Concerning the synergistic effects on enzymic activity, *To*LPMO9A exhibited promotion with endo-glucanase or exo-glucanase, but inhibition with β-glucosidases. In conclusion, *To*LPMO9A could be a good choice for enzyme cocktails and provide theoretical support for subsequent action mechanisms and broader applications.

Keywords: Lytic polysaccharide monooxygenases; Trichoderma orientalis EU7-22; Substrate specificity; Regioselectivity; Synergistic cooperation

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INTRODUCTION

Lignocellulose is the richest renewable resource and widely distributed form of crop and forestry resources on the planet (Jönsson *et al.* 2013). It can provide renewable resources for the production of biofuels, chemicals, and polymers. However, due to the lack of effective technologies, burning is still the traditional method of utilization, which not only causes resource waste but aggravates the environmental pollution problem as well. Therefore, it is important to find ways to utilize it (Rodionova *et al.* 2017). Biochemical conversion of lignocellulose preserves the original carbohydrate structure in the form of monomeric sugars (as opposed to thermochemical conversion, which destroys carbohydrates). Enzymatic techniques are usually considered to be the key point of saccharification technology (Horn *et al.* 2012). Despite a lot of work that has been done over the past decade, the enzymatic hydrolysis efficiency of lignocellulosic biomass remains a critical limiting step in many biorefining methods (Klein-Marcuschamer *et al.* 2012). The limiting factor is the heterogeneity of plant cell walls (mainly cellulose,

hemicellulose, and lignin) (Chundawat *et al*. 2011), and the inaccessibility and recalcitrance of its components. Thus, the enzymatic hydrolysis process is inefficient and costly (Lynd *et al.* 2008).

The classic scheme for cellulose degradation involves three enzymes, endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidases (Horn *et al.* 2006; Payne *et al.* 2011). These enzymes work synergistically, as the endoglucanase generates new reduced and non-reducing chain ends for exoglucanase and releases cellobiose, which is converted to glucose by β-glucosidase (Kostylev and Wilson 2012; Wood and McCrae 1979). Unlike the traditional way of cellulase mentioned above, LPMOs directly cleave glycosidic bonds in an oxidative manner to generate non-reducing chain ends and reducing chain ends, which provides more accessible sites for cellulase. This synergistic reaction can greatly improve the hydrolysis efficiency (Dimarogona *et al*. 2013; Gouvêa *et al.* 2019; Zhang *et al*. 2019). It has completely changed the enzymatic processing of polysaccharides (Chylenski *et al*. 2019).

Due to its glycoside hydrolase activity, the AA9 family was defined as the GH61 (glycoside hydrolase) family. However, compared to other endocellulases derived from the same genus, the hydrolytic activity of the GH61 family is notably lower than other enzymes (Karlsson *et al.* 2001). Also, the crystal structure of GH61 family enzymes is different from the glycoside hydrolases structure. The GH family structure does not contain the clustering of conserved catalytic acidic side chains, which constitutes the classic catalytic mechanism of this class of proteins (Karkehabadi *et al.* 2008; Harris *et al.* 2010). In 2010, the GH61 family was first demonstrated as a cellulose-boosting enzyme to meaningfully improve hydrolysis efficiency (Harris *et al.* 2010). Since then, additional studies have demonstrated that there are both oxidized and non-oxidized products in the hydrolysate. Thus, the GH61 family is considered as a polysaccharide monooxygenase (Quinlan *et al*. 2011; Westereng *et al*. 2011; Beeson *et al.* 2012). In 2012, the GH61 family was reclassified as lytic polysaccharide monooxygenases (LPMO). In 2013, LPMO and lignin-degrading enzymes were classified into one group and constitute new carbohydrate active enzymes named "Auxiliary Activities". In addition, the LPMOs are distributed in seven Auxiliary Activity families in CAZy database (www.cazy.org), with various origins, including eukaryota, bacteria, virus and archaea. LPMO-encoding genes are highly abundant in nature, and LPMOs are predicted to play a significant role in global carbon flux (Frandsen and Lo Leggio 2016). Therefore, there are several reviews on LPMO (Frommhagen *et al*. 2018b; Zhang 2020; Zhou and Zhu 2020), such as the substrate specificity, regioselectivity, electron-donating systems and the application. Besides, the GH61 family was reclassified into the AA9 family (Levasseur *et al.* 2013).

Currently, there is limited public data that addresses the possible relationship between the structural characteristics of LPMOs and the regioselectivity on C1/C4/C6 (Frandsen and Lo Leggio 2016; Vaaje-Kolstad *et al.* 2017). Therefore, more LPMO-substrate interactions must be found to improve the understanding of the structure-function relationship of LPMOs. A large number of biochemical, structural, functional, and regiospecific data show that the research of LPMO in fungi mainly focuses on the AA9 family (Payne *et al*. 2011; Phillips *et al.* 2011; Aachmann *et al*. 2012; Beeson *et al.* 2012; Kittl *et al*. 2012; Bey *et al*. 2013; Wu *et al*. 2013; Vu *et al*. 2014).

In this study, the AA9 LPMO, the gene encoding *To*LPMO9A, was first cloned from *Trichoderma orientalis* EU7-22 and was heterologous expressed in *Pichia pastoris* GS115. The enzymatic properties, substrate specificity, and regioselectivity of

*To*LPMO9A were investigated. Meanwhile, the synergistic actions with three cellulases were studied.

EXPERIMENTAL

Methods

Strains and enzymes

The *T. orientalis* EU7-22 was used as the source of the *To*LPMO9A gene. *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, USA) was used to construct and amplify the recombinant plasmids, and *P. pastoris* GS115 (Invitrogen) was used for the heterologous expression of the recombinant *To*LPMO9A. Commercial cellulase was purchased from Novozymes (Nanjing, China). The recombinant enzymes EGII (endo-β-1,4-glucanase), CBHII (exo-β-1,4-glucanase), and BGLI (β-glucosidases) from *T. orientalis* EU7-22 were expressed in *P. pastoris* and preserved in the laboratory. The culture environment of the plate was 30 °C, and the liquid medium was incubated in the rotary shaker at 30 °C and 180 rpm.

Cloning of ToLPMO9A gene

The frozen spore powder of *T. orientalis* EU7-22 was stored at -70 °C and inoculated into the potato dextrose agar (PDA) plate and incubated for 4 days. The strain colony was then injected into the PDA liquid medium and incubated for 24 h. The mycelia suspensions above were collected and incubated in the inducing medium (Xue *et al.* 2020). After induction culture for 48 h, the total RNA was isolated using an RNA prep pure plant kit (DP432, Tiangen, Beijing, China), and then it was reverse-transcribed into cDNA by polymerase chain reaction (PCR). The *To*LPMO9A gene was cloned from *T. orientalis* EU7-22 cDNA with a pair of primers by PCR. The forward primer was 5′-CCGGAATTCCGGACACATCAACAACATTG-3′, and the reverse primer was 5’-AAGGAAAAAAGCGGCCGCCTAGCTAAGGCACTGGGCATAG-3′ (the restriction endonuclease sites *Eco*RI and *Not*I were underlined).

The PCR product of the *To*LPMO9A gene and the expression vector pPIC9K (Invitrogen) were both digested by the *Eco*RI and *Not*I. The gene was then ligated to the pPIC9K to generate pPIC9K-*To*LPMO9A plasmids. The recombinant plasmid was transformed into *E. coli* DH5α, and the correct transformants were identified by PCR and DNA sequencing.

Expression of ToLPMO9A in P. pastoris and enzyme purification

The pPIC9K-*To*LPMO9A recombinant plasmid was extracted by the TIANprep midi plasmid kit (Tiangen, Beijing, China). The plasmid pPIC9K-*To*LPMO9A was linerized by *Bgl* I and then electrotransformed into *P. pastoris* GS115. After 3 days of culture on minimal dextrose (MD) medium (1.34% yeast nitrogen base without amino acids, 2% glucose) plates, the transformants were verified by PCR with a sequencing primer, 5’AOX1 (5'-GACTGGTTCCAATTGACAAGC-3'), and 3’AOX1 (5'-GGCAAATGGCATTCTGACAT-3'). The positive recombinants were inoculated into the BMGY medium until the culture reached an optical density of 2.0 to 6.0 at 600 nm (OD_{600}). The yeast cells were collected by centrifugation (4 °C, 6000 × g, 10 min) from the culture medium. The cells were resuspended in the BMMY medium, and methanol was added

daily, while keeping the concentration at 1%. The BMMY medium was centrifuged. The fermentation broth was collected by centrifugation (12000 × g, 5 min) to collect the fermentation broth, which was stored at -20 °C for later use.

The fermentation supernatant was concentrated in a 10 kDa Millipore ultrafiltration centrifuge tube and loaded on a dextran G-50 gel filtration column. Afterwards, the culture supernatant was eluted with a phosphate buffer at a flow rate of 0.6 mL/min. The recombinant protein expression was verified with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separation gel concentration and concentrated gel were 13% and 5%, respectively.

Substrate specificity

Five kinds of substrate (microcrystalline cellulose, CMC, corn cob, filter paper, beechwood xylan) were used for the experiments for substrate specificity of *To*LPMO9A.

The components in the reaction system were composed of 0.5% (w/v) substrate, 10 mmol/L NH_4Ac-HAc (pH5.0), 1 mmol/L ascorbic acid, 1 mmol/L $CuSO_4$, and 5 μmol/L *To*LPMO9A enzyme. The reaction system was incubated at 50 °C and 200 rpm for 48 h, and the reaction was terminated by a boiling water bath.

The supernatant of all incubations was obtained by centrifugation (4 °C, 6000 × g, 10 min). The enzyme activity was further determined by measuring the reducing sugar concentration of the reaction solution using the reported method (Miller 1959). All reactions were performed in triplicate.

Effect of reducing agent and metal ions concentration

To study the effect of reducing agents on *To*LPMO9A, different ascorbic acid concentrations were added to the enzymatic reaction system from 1 mmol/L to 10 mmol/L.

To study the effect of metal ions on *To*LPMO9A, five metal ions (Cu^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, and Fe^{3+}) were added to the enzymatic reaction system at the terminal concentration of 1 mmol/L and 5 mmol/L.

The components in the reaction system were the same as above, and the substrate used in the reaction was microcrystalline cellulose.

Effects of temperature and pH on the stability of ToLPMO9A

To study the effect of temperature on *To*LPMO9A, the enzyme activity was measured at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C, and the highest enzyme activity was 100%.

To study the effect of pH on *To*LPMO9A, the reaction was incubated with a buffer with a range of 3.0 to 9.0, and the highest enzyme activity was 100%.

The components in the reaction system were the same as above, and the substrate used in the reaction was microcrystalline cellulose.

Mass spectrometry

The oxidation product type was identified by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS). The substrate used in mass spectrometry was phosphoric acid swollen cellulose (PASC), which was prepared from Avicel PH-101 (Sigma, St. Louis, MO, USA) following instructions from Wood (1988). The reaction solution for enzymatic hydrolysis and the test conditions followed previous reported Vaaje-Kolstad *et al*. (2010).

The analysis was completed on a MALDI-TOF 5800 (AB SCIEX, Singapore). For the MALDI-TOF-MS analysis, the instrument operated in a linear mode and acquired spectra in the range of 0 to 7000 m/z.

Enzyme synergistic hydrolysis

To study the synergistic effect of *To*LPMO9A enzymes, three former studied cellulases were introduced in this research: EGII, CBHII, and BGLI. They were cloned from EU7-22 and heterologously expressed in *P. pastoris* GS115.

The supernatant of all incubations was obtained by centrifugation (4 °C, 6000 × g, 10 min). The experiment of synergistic hydrolysis followed the method from Zhu *et al*. (2018). The synergistic system was incubated at 60 °C, pH 6.0 and 200 rpm for 48 h, and the reaction was terminated by a boiling water bath.

To further understand the synergistic mechanism, three formerly investigated enzymes in the cellulase degradation system were selected in this study. As a parameter, DS (degree of synergism) was employed to describe the effect of synergism, as shown in Eq. 1,

$$DS = \frac{C_{ab}}{C_a + C_b} \quad (1)$$

where *DS* means the degree of synergism, C_{ab} is the reducing sugar concentration obtained from the combined system with both *To*LPMO9A and cellulase, C_a is that from the simple system with *To*LPMO9A only, and C_b is that from the simple system only containing cellulase.

RESULTS AND DISCUSSION

Cloning of *To*LPMO9A Gene

Using *T. orientalis* EU7-22 as a template, the genes were amplified by PCR with the primers given above. The results on agarose gel electrophores is shown in Fig. 1.

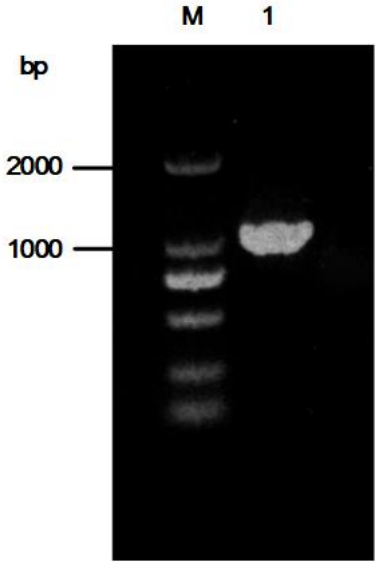

Fig. 1. Cloning of the gene encoding *To*LPMO9A from *T. orientalis* EU7-22; Lane M: DNA marker; Lane 1: cDNA (1044 bp)

Figure 1 indicates that the full length of the *To*LPMO9A gene cDNA sequence including the removed signal peptide sequence was 981 bp. The obtained sequence was compared with the existing *To*LPMO9A in the entire genome sequence of EU7-22 by DNAMAN, and the similarity was 100%, which proved that the cDNA sequence of *To*LPMO9A in EU7-22 was successfully cloned.

Expression of *To*LPMO9A in *P. pastoris*

The *To*LPMO9A expressed in *P. pastoris* was analyzed by SDS-PAGE. The theoretical molecular weight of *To*LPMO9A was 33 kDa, while the molecular weight was approximately 68 kDa (Fig. 2) due to the over glycosylation of *To*LPMO9A in *P. pastoris.*

The result of function domain analysis shows that *To*LPMO9A have the carbohydrate-binding module (CBM) domain, which have been reported for both LPMO9s and LPMO10s (Forsberg *et al*. 2016; Courtade *et al*. 2018; Chalak *et al*. 2019; Laurent *et al*. 2019).

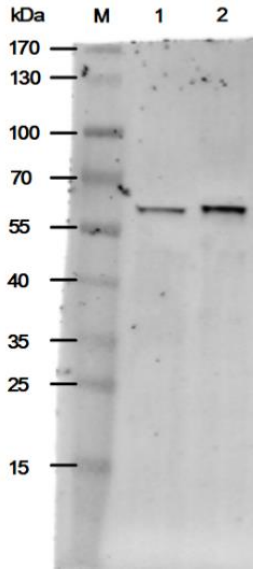

Fig. 2. SDS-PAGE of *To*LPMO9A; Lane M: protein marker; Lane 1: unpurified*To*LPMO9A; and Lane 2: purified *To*LPMO9A

Substrate Specificity

There are multiple excurrent loops in LPMOs that could shape the binding surface of the substrate to recognize and bind polysaccharides. The substrate specificity of AA9 was determined by the loops, which contained various hydrophilic and aromatic residues related to the substrate-binding of LPMO (Aachmann *et al.* 2012; Wu *et al.* 2013; Borisova *et al*. 2015).

When *To*LPMO9A acted on different substrates, the reducing sugar yield of the reaction system was also different. As shown in Fig. 3, *To*LPMO9A can lead to the catalytic degradation of four substrates: microcrystalline cellulose, CMC, corn cob, and xylan. Furthermore, the reducing sugar yield was the highest when microcrystalline cellulose was used as the substrate, followed by the corn cob. In addition, *To*LPMO9A was active with xylan, which was the same as previous research (Jagadeeswaran *et al*. 2016; Ladevèze *et al*. 2017; Simmons *et al.* 2017).

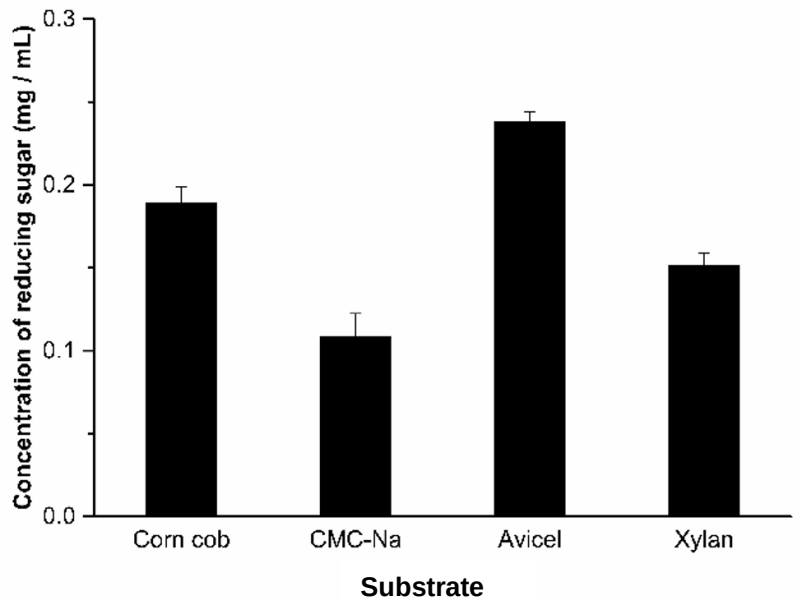

Fig. 3. Reducing sugar yields from cellulosic specimens treated with *To*LPMO9A

Effect of Reducing Agent and Metal Ions Concentration

In 2010, a study that revealed the oxidative mechanism behind LPMO activity showed that LPMO requires electrons (Vaaje-Kolsatand *et al.* 2010). To catalyze the oxidation of polysaccharides, LPMO relies on reducing agent, which provides electrons to reduce copper in the active site and activate molecular oxygen (Frandsen and Lo Leggio 2016). The effect of the reducing agent concentration on *To*LPMO9A was investigated. There was an increase in enzymatic activity when the ascorbic acid concentration increased from 1 to 10 mmol/L (Fig. 4a). Moreover, when the ascorbic acid concentration reached 10 mmol/L, the conversion amount of reducing sugar increased by 6.46 times.

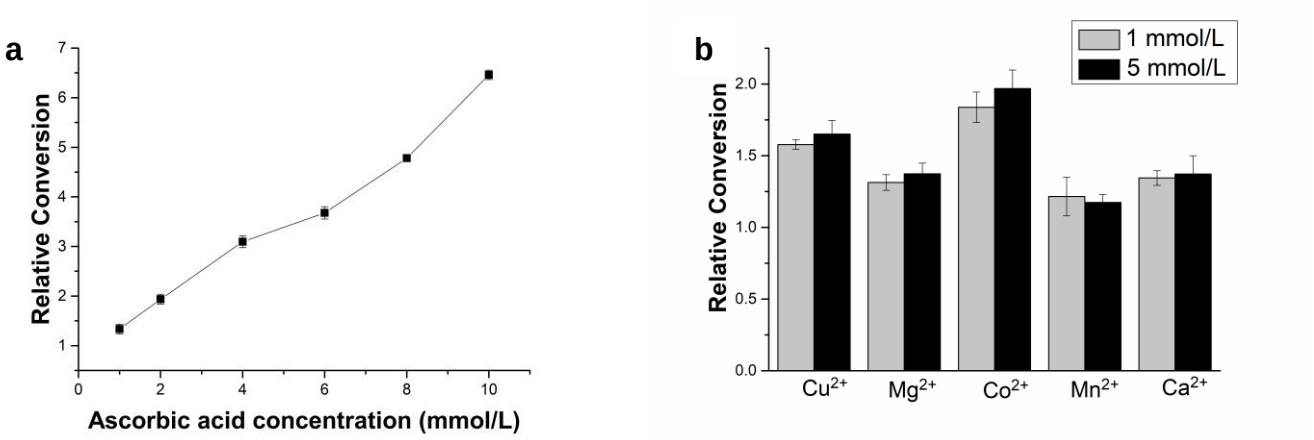

Fig. 4. Effect of reducing agent and metal ions on enzyme activity: (a) effect of reducing agent on *To*LPMO9A activity; (b) effect of metal ions on *To*LPMO9A activity

The effect of the metal ion concentrations on *To*LPMO9A was investigated. Except for the reaction with Fe^{3+}, the enzyme activity increased in all reactions with various metals (Fig. 4b). In particular, the metal ions Co^{2+} and Cu^{2+} notably promoted substrate degradation at low concentrations. At a concentration of 5 mmol/L, the increasing amount

of reducing sugar was 1.65 and 1.97, respectively, indicating that *To*LPMO9A is a metal-dependent enzyme. The results in this research are consistent with the previous report (Naghshehechi 2014).

Effects of Temperature and pH on the Stability of *To*LPMO9A

The relative enzyme activity of *To*LPMO9A at different temperatures is shown in Fig. 5a. The results show that the optimal reaction temperature of *To*LPMO9A was 60 °C. In the range of 30 °C to 60 °C, the relative enzyme activity of *To*LPMO9A gradually increased with an increase of reaction temperature. At a temperature higher than 60 °C, the relative enzyme activity of *To*LPMO9A decreased rapidly, while at 90 °C, *To*LPMO9A was almost completely inactivated.

There have been few studies on the effect of pH on LPMO. According to previous studies, the pH preference of different sources of LPMO is different. For example, LPMO from *Pestalotiopsis sp.* has the highest activity at pH 5.5 (Patel *et al.* 2016), and the LPMO from *Gloeophyllum trabeum* is the most active at pH 7.0 (Hegnar *et al.* 2018). The optimum pH was 6.0 for *To*LPMO9A, which is consistent with the preference pH of *T. orientalis* EU7-22 (Fig. 5b).

Regioselectivity Identification by Mass Spectrometry

The oxidization acting sites by AA9 LPMOs are located on either C1 (Frommhagen *et al.* 2016, 2017; *Liu et al.* 2018), C 4 (Frandsen *et al.* 2016; Liu *et al.* 2017) or both the C1 and C4 position (Vu *et al.* 2014; Song *et al.* 2018), and occasionally the C6 position (Chen *et al.* 2018).

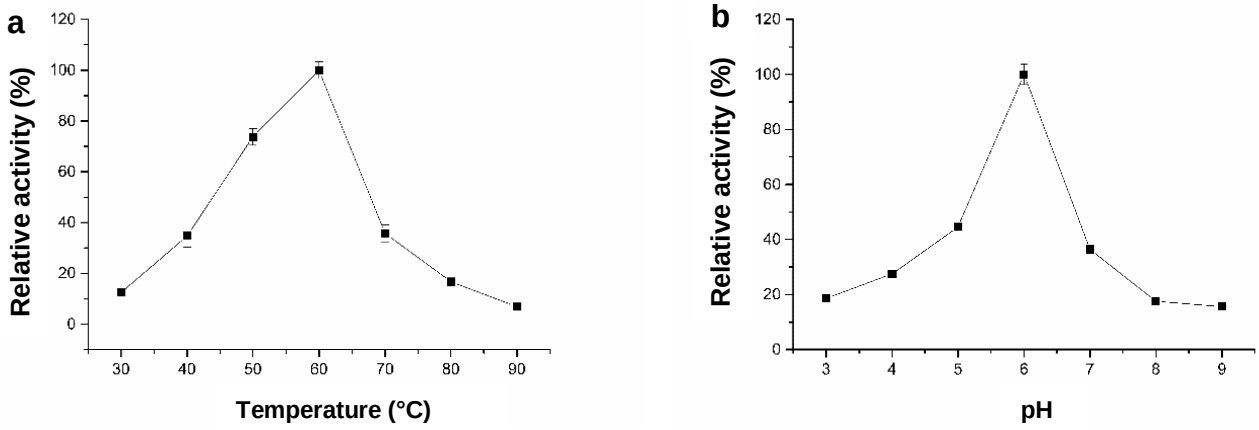

Fig. 5. Effect of temperature and pH on enzyme activity: (a) effect of temperature on *To*LPMO9A activity; (b) effect of pH on *To*LPMO9A activity

Figure 6 shows the results of the MALDI-TOF-MS analysis on the PASC degradation products by *To*LPMO9A. According to the mass spectrometry analysis, the product contained sodium adducts of oligosaccharides with the degree of polymerization (DPs) ranging from 3 to 6, and the *m*/*z* ratios of 527, 689, 851, and 1013. In addition to the various non-oxidized oligosaccharides, the mass spectrometry results also contained oxidized oligosaccharides. The oxidation peaks at the *m*/*z* values of 543, 705, 867, and 1029 correspond to the sodium adducts of C1 oxidized oligosaccharides (*m*/*z* + 16). The peaks at *m*/*z* of 525, 687, 849, and 1011 correspond to the sodium adducts of C4 oxidized

oligosaccharides (C4-ketoaldose, C6-hexodialdose, *m*/*z*-2). Accordingly, *To*LPMO9A cleaved the polysaccharide chain of cellulose at both the C1 and C4/C6 positions. More detailed information on its cleavage pattern is reported in the references below (Frommhagen *et al.* 2018a).

For LPMO application in the renewable biomass industry, more enzymatic and structural characterizations of LPMO from different sources are needed to clarify the substrate specificity and oxidative regioselectivity.

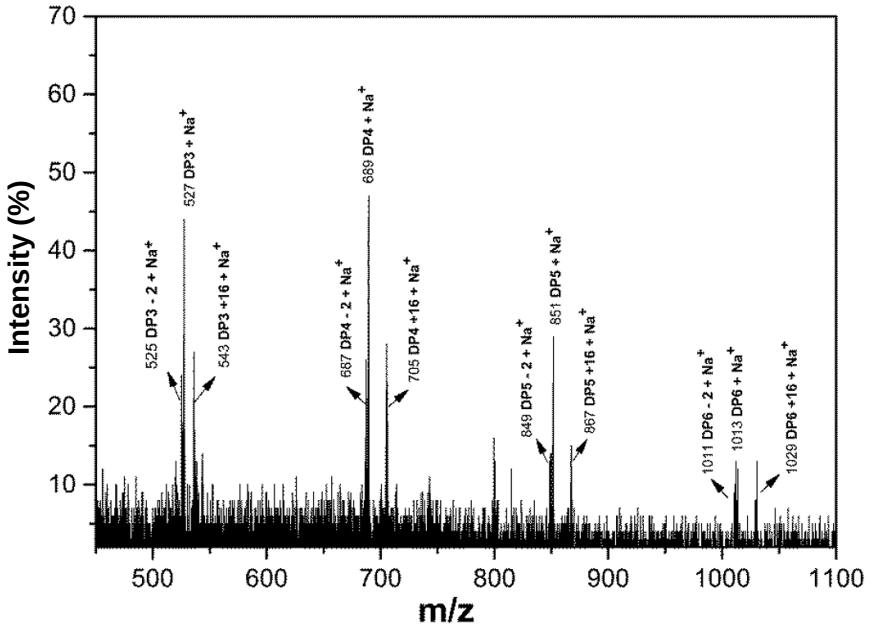

Fig. 6. MALDI-TOF-MS analysis of the degradation products by *To*LPMO9A

Synergistic Hydrolysis by *To*LPMO9A with Three Cellulases

The LPMOs have received widespread attention since their discovery due to their unique oxidation mechanism and their effective contribution to the cellulase activity (Vaaje-Kolstad *et al*. 2010; Aachmann *et al.* 2012).

The degradation ability of three enzymes were increased to varying degrees after the addition of *To*LPMO9A (Fig. 7). The initial reducing sugar concentration was 0.950 mg/mL and 0.096 mg/mL for the EGII and CBHII enzymes, respectively. The reducing sugar concentration increased to 2.56 and 0.32 mg/mL for the system with *To*LPMO9A and the DS were 2.27 and 1.16, respectively. For BGLI enzymes, the initial reducing sugar concentrations were 0.47 mg/mL, but with the addition of *To*LPMO9A, the reducing sugar concentrations were 0.55 mg/mL and the DS was 0.9. Therefore, for both EGII and CBHII, *To*LPMO9A exhibited positive synergistic effects, but the negative effect was shown in the synergism of *To*LPMO9A and BGLI.

It is speculated that this synergy is due to the oxidative cleavage of polysaccharide crystalline regions by LPMOs, which can bring more accessible sites for glycoside hydrolases (Vermaas *et al*. 2015) as EGII and CBHII enzymes. Concurrently, the EGII and CBHII enzymes degraded the polysaccharide chains and unlocked new binding sites to contribute to the LPMO reaction (Bissaro *et al.* 2017). In the following experiments, it is possible to adjust the ratio of enzymes and substrates so as to obtain more suitable operating conditions for industrial application. Therefore, AA9 can meaningfully increase the EGII and CBHII enzyme activity, and it can improve the synergy between AA9 and EGII/CBHII. However, as noted previously, the oxidization at the C1 position by AA9 produces gluconic acid that inhibits glucosidase activity (Hemsworth *et al.* 2015). Thus, the main interactions between BGL and *To*LPMO9A finally resulted in inhibition.

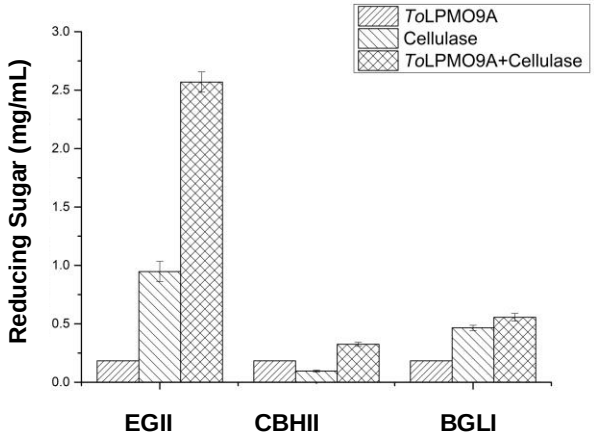

Fig. 7. Synergistic hydrolysis of PASC by *To*LPMO9A with three cellulases

CONCLUSIONS

1. An LPMO gene (*To*LPMO9A) was cloned from *T. orientalis* EU7-22 and it was heterologously expressed in *Pichia pastoris* GS115 successfully. *To*LPMO9A exhibited maximal activity at 60 °C and pH 6.0.
2. Both metal ions (Co^{2+}, Cu^{2+}) and reducing agent (ascorbic acid) concentrations showed a noteworthy effect on *To*LPMO9A. The *To*LPMO9A could degrade microcrystalline cellulose, corn cob, CMC and xylan, and the enzymatic activity on them were different. The MALDI-TOF-MS analysis showed that the *To*LPMO9A cleaved the glycosidic bonds at C1 and C4/C6 positions oxidatively.
3. The *To*LPMO9A exhibited positive synergistic effects with EGII or CBHII, and DS were 2.27 and 1.16, while *To*LPMO9A exhibited inhibitory effects with BGLI.

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