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# LPMO AfAA9\_B and cellobiohydrolase AfCel6A from *A. fumigatus* boost enzymatic saccharification activity of cellulase cocktail

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**Abstract:** Cellulose is the most abundant polysaccharide in lignocellulosic biomass, where it is interlinked with lignin and hemicellulose. Bioethanol can be produced from biomass. Because breaking down biomass is difficult, cellulose-active enzymes secreted by filamentous fungi play an important role in degrading recalcitrant lignocellulosic biomass. We characterized a cellobiohydrolase (*AfCel6A*) and lytic polysaccharide monooxygenase LPMO (*AfAA9\_B*) from *A. fumigatus* after they were expressed in *Pichia pastoris* and purified. The biochemical parameters suggested that the enzymes were stable; the optimal temperature was ~60 °C. Further characterization revealed high turnover numbers ( $k_{cat}$  of 147.9 s<sup>-1</sup> and 0.64 s<sup>-1</sup>, respectively). Surprisingly, when combined, *AfCel6A* and *AfAA9\_B* did not act synergistically. Association of *AfCel6A* and *AfAA9\_B* inhibits the activity of *AfCel6A*, an outcome that needs to be further investigated. However, addition of *AfCel6A* or *AfAA9\_B* boosts the enzymatic saccharification activity of a cellulase cocktail and the activity of cellulase *Af-EGL7*. The supplementation of an enzymatic cocktail with *AfCel6A* or *AfAA9\_B* boosted the yield of fermentable sugars from complex substrates, especially sugarcane exploded bagasse, by up to 95%. The synergism between the cellulase cocktail and *AfAA9\_B* is enzyme- and substrate-specific, which suggests a specific enzymatic cocktail for each biomass.

**Keywords:** GH6 cellobiohydrolase; AA9 LPMO; lignocellulose hydrolysis; bioethanol

## 1. Introduction

Fossil fuel depletion, increasing energy consumption, growing CO<sub>2</sub> emissions, and climate change have increased the demand for renewable energy sources. In this scenario, lignocellulosic residues stand out as a new generation of renewable energy sources, including second-generation (2G) ethanol [1–5]. Lignocellulosic biomass-derived biofuels can potentially substitute fossil fuels with the advantage that they can help to reduce emission of greenhouse gases and global warming [6,7]. Every year, tons of agricultural residues, such as byproducts of sugarcane, corn, wheat, rice, and barley, are generated worldwide and have emerged as the most promising feedstock to produce biofuels by hydrolysis and subsequent fermentation [8].

The composition of the plant cell wall varies in terms of the percentage of cellulose (35–50%), hemicellulose (20–30%), and lignin (20–30%). The lignocellulosic structure of the wall is

recalcitrant and resists chemical and biological treatments. Cellulose, a crystalline homopolysaccharide, is made up of thousands of D-glucose subunits linked by  $\beta$ -1,4-glycosidic bonds, forming linear chains. The cellulose chains are bound through intra- and intermolecular hydrogen bonds, creating insoluble microfibrils [9]. The recalcitrant structure of the plant cell wall matrix makes the release of soluble sugars challenging [10].

Industrial processes that produce ethanol from cellulose require that mixtures of fungal cellulases be employed, so that soluble sugars are released for further fermentation into bioethanol [7,11]. These enzymes work synergistically to break down polysaccharides and crystalline cellulose [12,13]. First, endoglucanases (EGL, EC 3.2.1.4) hydrolyze  $\beta$ -1,4-glycosidic bonds in amorphous regions of the cellulose chains, to release cello-oligosaccharides; cellobiohydrolases (CBH; EC 3.2.1.91) act on short cellulose molecules and cello-oligosaccharides, releasing disaccharide units like cellobiose. Then,  $\beta$ -glucosidases (BG; EC 3.2.1.21) cleave cellobiose into glucose for further fermentation. Together, these enzymes are part of an enzymatic cocktail and are used to break down lignocellulose.

In contrast to cellulases, lytic polysaccharide monooxygenases (LPMO; EC: 1.14.99.53-56) degrade cellulose by an oxidative mechanism and enhance accessibility to cellulose, improving the hydrolytic performance of cellulases [14–16]. LPMOs are copper-dependent enzymes that act on crystalline cellulose and other polysaccharides in nature, to generate oxidized and mono-oxidized chain ends. In addition, LPMO is a virulence factor in fungal meningitis [17].

The fact that LPMO boosts the activity of hydrolytic enzymes during the degradation of chitin was first described in 2005 [18]. The activity of LPMO on cellulose and other biomasses has also been reported [19,20]. The copper ion in the LPMO catalytic structure is coordinated to three nitrogen atoms of the two conserved histidine residues in a histidine brace, which is essential for the activity of LPMO [21–27]. The LPMO oxidative mechanism is not fully understood, but analysis of reaction products has revealed that LPMO hydroxylates carbon C1 or C4, or both. To initiate oxidative cleavage, an enzyme, such as cellobiose dehydrogenase, or a small reductor molecule must reduce the LPMO copper center. Subsequently, the enzyme reacts with a co-substrate ( $O_2$  or  $H_2O_2$ ), to form oxygen species that can hydroxylate C1 or C4 in the glycosidic bond [28,29].

Enzymes from thermophilic microorganisms offer several advantages for industrial applications. For example, *A. fumigatus* produces thermophilic CAZymes, which have high cellulolytic activity and stability in a wide range of pH and at elevated temperatures, unlike commercial fungal cellulases [30–33].

To characterize the association of cellulases (*AfCel6A* and *Af-EGL7*) and LPMO (*AfAA9\_B*) from *A. fumigatus*, we evaluated their action on the degradation of different biomasses on a pilot scale. *AfCel6A* is a cellobiohydrolase from the glycoside hydrolase (GH) class, family 6; it acts exclusively on nonreducing ends of cellulosic polymers. *Af-EGL7* is a previously characterized endoglucanase that can potentially hydrolyze biomass [30,34].

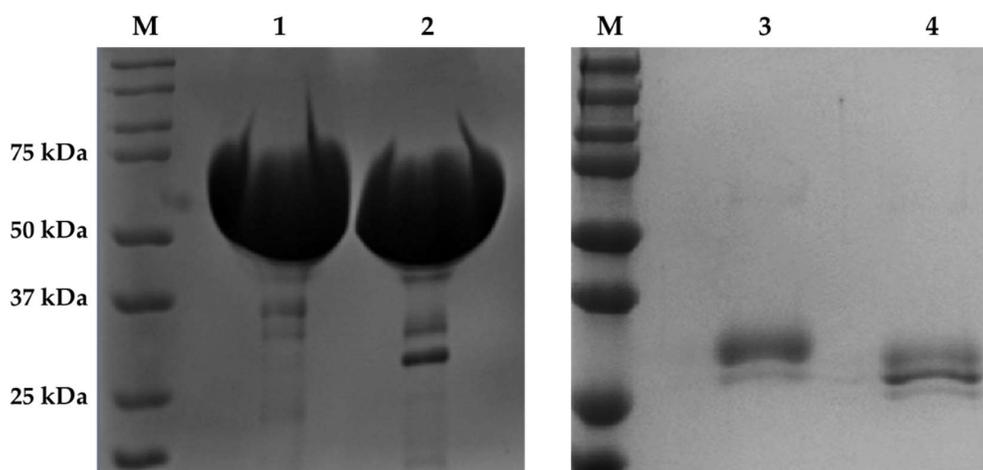
Here, we present the biochemical characterization of *AfCel6A* and *AfAA9\_B* after they are expressed in *Pichia pastoris*. We will show that supplementation of enzymatic cocktails can enhance the production of fermentable sugars, and that LPMOs have a critical role in biomass hydrolysis.

## 2. Results and Discussion

LPMO (*AfAA9\_B*) and Cellobiohydrolase GH6 (*AfCel6A*) from *A. fumigatus* and expressed in *E. coli* and *A. oryzae*, respectively, have been described [33,35]. However, to evaluate the action of the combined enzymes, we characterized and analyzed their biochemical properties after expressing them in *P. pastoris*, and we detected some differences.

### 2.1. Expression and purification of recombinant *AfCel6A* and *AfAA9\_B*

Recombinant *AfCel6A* and *AfAA9\_B* were successfully expressed in *P. pastoris* X-33. After induction for 144 h, the culture supernatants were collected, concentrated, and purified on Ni<sup>+</sup> Sepharose 6 Fast Flow resin (Ge Healthcare, Little Chalfont, United Kingdom). SDS-PAGE revealed that the purified recombinant *AfCel6A* and *AfAA9\_B* had apparent molecular masses of approximately 65 and 30 kDa, respectively (Figure 1). After Endo H treatment, the molecular mass of *AfCel6A* remained almost the same, while *AfAA9\_B* migrated as a band of approximately 26 kDa. Analyses of potential N-glycosylation sites by the NetNGlyc 1.0 program (<http://www.cbs.dtu.dk/services/NetNGlyc/>) suggested that a potential site was present at position N413 in *AfCel6A* and N159 in *AfAA9\_B*, which was confirmed by deglycosylation of the recombinant proteins by the enzyme Endoglycosidase H. The presence of N-glycans at different sites in the structure of the enzyme can influence enzymatic properties, such as secretion, folding, and stability, among others [36].



**Figure 1.** SDS-PAGE (10% polyacrylamide gel) analysis of the purified recombinant *AfCel6A* and *AfAA9\_B*. Lane M, molecular mass standards (Precision™ Protein Standards Dual Color – BioRad); lane 1, purified recombinant *AfCel6A*; lane 3, deglycosylated *AfCel6A*; lane 3, purified recombinant *AfAA9\_B*; lane 4, deglycosylated *AfAA9\_B*.

The purified *AfAA9\_B* was excised from the gel and analyzed on the LC-MS/MS Xevo TQS (Waters) system at the Multi-User Laboratory of Mass Spectrometry, which confirmed that the enzyme was LPMO (Table 1).

**Table 1.** Peptide sequences

Protein Gene	Peptide Sequence	Precursor Mz	Precursor Charge	Product Mz	Product Charge
AFUA_4G07850	ITSIAGLLASASLVAGHGFVSGIVADGK	871,675226	3	1049,562586	1
AFUA_4G07850	ITSIAGLLASASLVAGHGFVSGIVADGK	871,675226	3	992,541122	1
AFUA_4G07850	ITSIAGLLASASLVAGHGFVSGIVADGK	871,675226	3	845,472708	1
AFUA_4G07850	ITSIAGLLASASLVAGHGFVSGIVADGK	871,675226	3	746,404294	1
AFUA_4G07850	ITSIAGLLASASLVAGHGFVSGIVADGK	871,675226	3	659,372266	1
AFUA_4G07850	ITSIAGLLASASLVAGHGFVSGIVADGK	871,675226	3	602,350802	1
AFUA_4G07850	NTDPGIK	372,912411	2	630,345717	1
AFUA_4G07850	NTDPGIK	372,912411	2	529,298038	1
AFUA_4G07850	NTDPGIK	372,912411	2	414,271095	1
AFUA_4G07850	NTDPGIK	372,912411	2	317,218332	1

## 2.2. Structural analysis and predictions by circular dichroism (CD)

LPMOs comprise a group of redox enzymes that belong to the auxiliary activity (AA) class (families 9 to 16, except 12) [37] and which bear a  $\beta$ -sandwich core (presence of 8-10  $\beta$ -strands). The catalytic region of the enzyme is known as histidine brace [24,38,39], which contains many loops and accounts for the topology and substrate specificity of the active site. Specificity is due to the presence of aromatic residues and their weak interactions with polysaccharides [22,40]. Figure 2a shows the crystallized structure of LPMO *AfAA9\_B* (PDB: 5X6A), where the active site residues H1, H86, and Y175 in the histidine brace are highlighted.

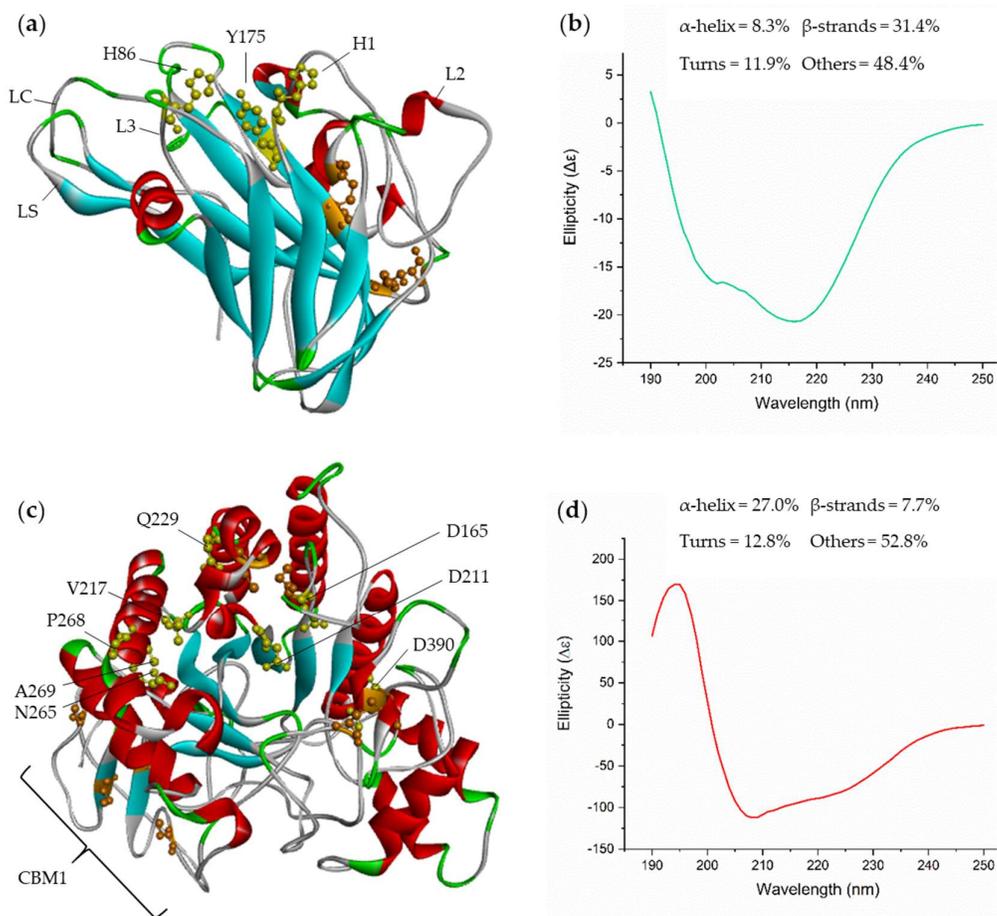
Because of its tunnel-shaped catalytic structure, *AfCel6A* acts exclusively on nonreducing ends of cellulosic polymers. The cellulosic polymers enter this catalytic structure through one of their extremities, and *AfCel6A* continuously cleaves the long chains into small cellobiose units via anomeric inversion (Figure 2c). The enzymatic core consists of a distorted  $\alpha/\beta$ -barrel motif. Few parallel  $\beta$ -strands in sandwich conformation are connected by several loops, which are rich in  $\alpha$ -helices [41–43]. As depicted in Figure 2c, *AfCel6A* contains N-terminal CBM1 (carbohydrate-binding module) as well as the main residues involved in catalysis, namely Q229, P268, V217, N265, A269 [41], D165, D211, and D390 (determined by the Pfam database [44]).

We predicted the secondary structure of both enzymes on the basis of CD spectra analysis by BeStSel [45]. This analysis showed substantial structural similarity between the enzymes and their templates from PDB:5X6A resolved by Q. Shen (unpublished) (for *AfAA9\_B*) [46] and Phyre2 web server [47] (for *AfCel6A*), as displayed in Figure 2 and Supplementary Table 1.

The CD spectrum of *AfAA9\_B* and its predicted secondary structures (Figure 2b) demonstrated that the enzyme consisted of 8.3%  $\alpha$ -helices and 31.4%  $\beta$ -strands. These values reinforced that LPMOs present a large number of  $\beta$ -strands in their cores, reflected by the well-defined negative peak at 218 nm, the small peak at 190 nm, and the approximated single band profile. Small negative peaks around 208 nm also evidenced the small number of helices [48]. Compared to the expected values based on the PDB: 5X6A structure, the percentage of  $\beta$ -strands was exactly the same, while the percentage of  $\alpha$ -helices was -4.3%. *TaLPMO9A* (PDB: 2YET)[26], an LPMO from *Thermoascus aurantiacus*, has been reported to share 71% identity with *AfAA9\_B* and to present similar proportions of  $\alpha$ -helices and  $\beta$ -strands: 30.8% and 15.0%, respectively.

*AfCel6A* presented 27.0%  $\alpha$ -helix and 7.7%  $\beta$ -strands, as estimated by BeStSel (Figure 2d). The accentuated peak at 190 nm and the two negative peaks near 208 nm and 222 nm indicated a large number of  $\alpha$ -helices. The absence of a negative peak at approximately 218 nm and a single band profile are typical of proteins with low content of  $\beta$ -strands [48]. On the basis of the proportions of  $\alpha$ -helices and  $\beta$ -strands estimated by Phyre2 [47] and the Kabsch & Sander method [49] for the modeled structure (Figure 2c), the differences were -4.3% and -1.0%, and -1.0% and -2.3%, respectively. The enzyme *Cel6A* from *Trichoderma reesei* (PDB:1QJW), which shares 69% identity, presents a similar proportion of 35.8%  $\alpha$ -helices and 8.7%  $\beta$ -strands [50]. Furthermore, a cellobiohydrolase from a different *A. fumigatus* strain that shares 99% identity with *AfCel6A* consists of 26.0%  $\alpha$ -helix and 15.4%  $\beta$ -strands, confirming that the prediction based on the CD spectrum is remarkably close.

Therefore, CD analysis of both enzymes obtained herein evidenced that their secondary structure profiles resembled the profiles described in the literature. This indicated that both enzymes were correctly folded during heterologous expression, and that their structures were maintained after they were purified.



**Figure 2.** Ribbon representation of the enzymes *AfAA9\_B* (a) and *AfCel6A* (c) and their main conserved residues and structures. Active site residues are represented in yellow, and disulfide bonds are represented in orange. *AfAA9\_B* loops are represented by LC (C-terminus), LS (short), L3, and L2. CBM1 residues are indicated by a brace in *AfCel6A*. Circular dichroism spectra obtained from 190 to 250 nm (UV-distant) for *AfAA9\_B* with Cu(II) (b) and *AfCel6A* (d) at 25°C. Both enzymes were in 20 mM sodium phosphate buffer (pH = 7.4), and the spectra were read by using a quartz cuvette with optical path of 0.1 cm. The mean spectra for each sample were normalized by subtracting the buffer spectrum.

### 2.3. Enzymatic properties of *AfCel6A* and *AfAA9\_B*

We used CM-Cellulose and 2,6-DMP as substrates to determine the enzymatic properties of *AfCel6A* and the activity of *AfAA9\_B*, respectively.

The optimal temperature for the activity of *AfCel6A* was 55–60 °C, and the enzyme retained over 54% of the maximum activity between 40 and 65 °C. At 70, 75, and 80 °C, *AfCel6A* maintained 43.5%, 30%, and 26% of the maximum activity, respectively (Figure 3a). Most characterized cellobiohydrolases, shown in Table 2, were also active at these temperatures. We studied the thermal stability of *AfCel6A* after preincubating it at 50, 60, 70, 80, or 90 °C for different times (Figure 3b). The enzyme was stable after 30 min and retained 84%, 54.4%, 48.3%, 48.2%, and 43% of the initial activity at 50, 60, 70, 80, and 90 °C, respectively. After 3 h, *AfCel6A* maintained 90%, 29%, 34%, and 26% of the initial activity at 50, 60, 70, and 80 °C, respectively. However, the enzyme was completely inactivated after 5 h at 60, 70, or 80 °C. *AfCel6A* was stable at 50 °C. It lost only 30% of its original activity after 24 h and retained 64.4% and 47.7% of its initial activity after 48 and 72 h, respectively (Figure 3c). These results showed that *AfCel6A* was

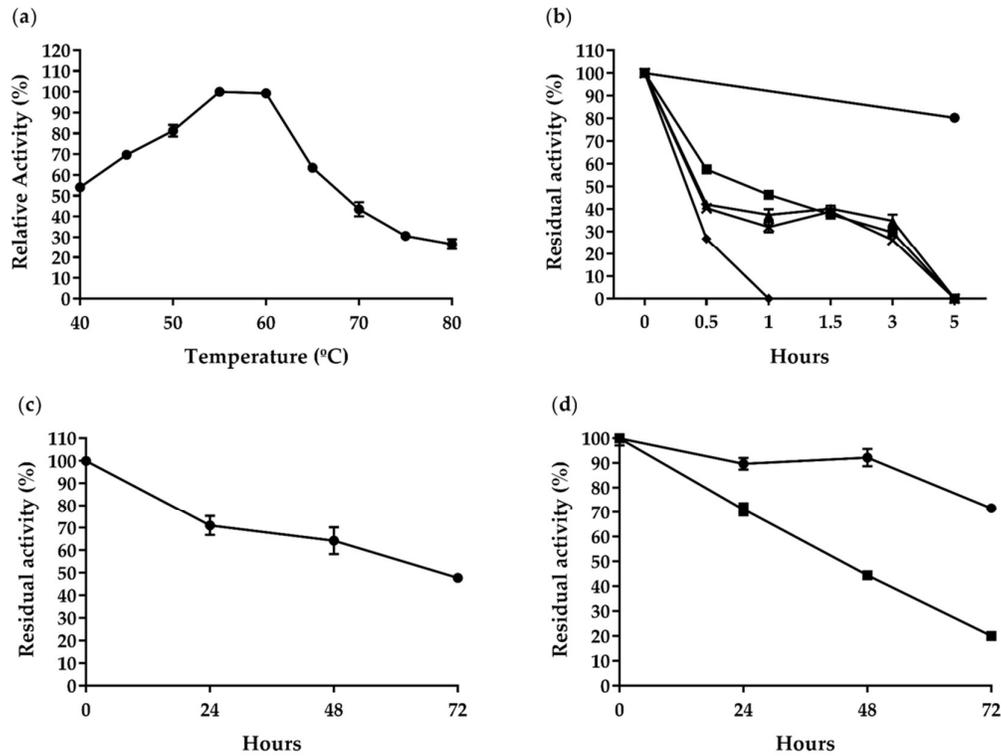
stable at high temperatures, especially at 50 °C. In another study, after expression in *A. oryzae*, AfCel6A was stable at 60 °C, but it completely lost its activity at 70 °C [33]. Therefore, AfCel6A was more stable after expression in *P. pastoris* than in *A. oryzae*.

**Table 2.** Comparison among catalytic and biochemical properties of GH6 cellobiohydrolases

Source Organism	Enzyme name	Expression System	Substrate	V <sub>max</sub>	K <sub>M</sub>	k <sub>cat</sub>	k <sub>cat</sub> /K <sub>M</sub>	Optimal T	Optimal pH	Thermal stability	pH stability	Ref
<i>Aspergillus fumigatus</i> Af293	AfCel6A	<i>Pichia pastoris</i>	CMC-Na	195.2 ± 4.65 U mg <sup>-1</sup>	7.44 ± 0.51 g/L	147.9 s <sup>-1</sup>	19.9 mL mg <sup>-1</sup> s <sup>-1</sup>	55-60 °C	pH 5.5-6.5	>70% after 24 h at 50 °C; about 40% after 90 min at 60–80 °C; more than 25% after 30 min at 90 °C	More than 70% at pH 3.0–10.0 after 72 h	This study
<i>Aspergillus fumigatus</i>	AfCel6A	<i>Aspergillus oryzae</i>	Avicel PH101	-	48.6 ± 14.8 g L <sup>-1</sup>	0.9 ± 0.1 s <sup>-1</sup>	-	70 °C	-	No loss at 60 °C after 1 h	-	[33]
<i>Aspergillus terreus</i>	AtCel6A	<i>Aspergillus oryzae</i>	Avicel PH101	-	-	-	-	50 °C	-	>90% after 1 h at 50 °C	-	[33]
<i>Talaromyces funiculosus</i>	TjCel6A	<i>Aspergillus oryzae</i>	Avicel PH101	-	21.6 ± 3.2 g L <sup>-1</sup>	0.5±0.02 s <sup>-1</sup>	-	60 °C	-	No loss at 50 °C after 1 h	-	[33]
<i>Colletotrichum graminicola</i>	CgCel6A	<i>Aspergillus oryzae</i>	Avicel PH101	-	-	-	-	40 °C	-	>90% after 1 h at 40 °C	-	[33]
	CgCel6B			-	89.0 ± 13.2 g L <sup>-1</sup>	1.8 ± 0.2 s <sup>-1</sup>	-	50 °C	-	>90% after 1 h at 50 °C	-	
<i>Trichoderma reesei</i>	TrCel6A	<i>Aspergillus oryzae</i>	Avicel PH101	-	24.3 ± 4.0 g L <sup>-1</sup>	0.6±0.04 s <sup>-1</sup>	-	70 °C	-	No loss at 50 °C after 1 h	-	[33]
	Cel6A <sup>1</sup>	<i>Pichia pastoris</i>	CMC-Na	10.7 mmol min <sup>-1</sup> mg <sup>-1</sup>	0.31 mg mL <sup>-1</sup>	-	-	60 °C	pH 5.5	90% after 30 min at 60 °C	-	[51]
	Cel6A <sup>2</sup>	<i>Pichia pastoris</i>	PASC	-	-	-	-	55 °C	pH 5.5–6.0	100% at 40 °C and 50% at 60 °C, after 30 min	No loss at pH 5.0–6.0; rapid inactivation at more alkaline pH; and some instability at more acidic pH after 30 min	[52]
<i>Magnaporthe oryzae</i> Ina72	MoCel6A	<i>Magnaporthe oryzae</i>	Cellotetraose	454.5 μg min <sup>-1</sup> mg <sup>-1</sup>	24.3 mM	-	-	40 °C	pH 9.0	-	-	[54]
			Cellopentaose	63.3 μg min <sup>-1</sup> mg <sup>-1</sup>	3.3 mM	-	-	-	-	-	-	-
<i>Schizophyllum commune</i> KMJ820	CBH II	<i>Escherichia coli</i>	pNPC	20.8 U mg <sup>-1</sup>	1.4 mM	-	-	50 °C	pH 5.0	-	-	[55]
<i>Penicillium occitanis</i> Pol 6	CBH II	-	pNPC	-	5 mM	-	-	65 °C	pH 4.0–5.0	Almost 100% at 30–50 °C; 50% at 60 °C; and complete inactivation at 70 °C, after 30 min	Stable at pH 2.0–9.0 after 24 h	[56]
<i>Talaromyces emersonii</i>	CBH II	-	CNPG <sub>3</sub>	9.1 U mg <sup>-1</sup>	4.5 mM	8.9 s <sup>-1</sup>	1.9 mM <sup>3</sup> s <sup>-1</sup>	68 °C	pH 3.8	t <sub>1/2</sub> = 38 min at 80 °C (pH 5.0)	t <sub>1/2</sub> = 38 min at pH 5.0 (80 °C)	[57]
<i>Trichoderma viride</i> CICC13038	CBH II	<i>Saccharomyces cerevisiae</i>	CMC-Na	-	-	-	-	70 °C	pH 5.0	-	-	[58]
<i>Neocallimastix patriciarum</i> J11	J11 CelA	<i>Escherichia coli</i>	Barley β-glucan	-	-	-	-	50 °C	pH 6.0	More than 70% at up to 50 °C and approximately 50% at 70 °C, after 1 h	More than 80% at pH 5.2–11.3; and approximately 70% at pH 3.0, 4.2, and 12.3, after 1 h	[59]
<i>Irpex lacteus</i> MC-2	Ex-4	<i>Pichia pastoris</i>	PASC	-	-	-	-	50 °C	pH 5.0	More than 80% at 60 °C (pH 3.0–8.0) after 1 h	More than 80% at pH 3.0–8.0 (60 °C) after 1 h	[60]
<i>Chaetomium thermophilum</i> HSAUP#2651	CBH II	<i>Pichia pastoris</i>	pNPC	-	-	-	-	50 °C	pH 4.0	No loss at 50 °C; approximately 20% at 60 °C; and complete inactivation at 80 °C, after 1 h	-	[61]

The optimal temperature for the activity of AfAA9\_B was 60 °C (data not shown). AfAA9\_B was stable at 50 and 60 °C and retained over 75% and 20% of its initial activity, respectively (Figure 3d).

Like AfAA9\_B, other LPMOs were stable at 50 and 60 °C; e.g., PMO9D\_SCYTH, PMO9D\_MALCI, MtLPMO9D, MtLPMO9J, and MtLPMO9A (Table 3).



**Figure 3.** Effects of temperature on the activity and stability of *AfCel6A* and *AfAA9\_B*. (a) Temperature-activity profiles of *AfCel6A* assayed at optimal pH for 45 min. (b) *AfCel6A* thermostability at ● 50, ■ 60, ▲ 70, × 80 and ◆ 90°C for different times. (c) *AfCel6A* thermostability at 50°C for up to 72 h. (d) *AfAA9\_B* thermostability at ● 50 and ■ 60°C for 72 h. Each value in the panel represents the mean of three experiments.

Table 3. Comparison among catalytic and biochemical properties of LPMOs

Source Organism	Protein name	Expression System	Substrate	Co-substrate	V <sub>max</sub>	K <sub>m</sub>	K <sub>cat</sub>	K <sub>cat</sub> /K <sub>m</sub>	Optimal T	Optimal pH	Thermostability	pH stability	Ref
<i>Aspergillus fumigatus</i> Af293	AfAA9_B	<i>Pichia pastoris</i> X33	2,6-DMP	§ H <sub>2</sub> O <sub>2</sub>	78.52 ± 3.33 U g <sup>-1</sup>	2.04 ± 0.24 μM	0.034 s <sup>-1</sup>	0.017 μM <sup>-1</sup> s <sup>-1</sup>	-	-	-	-	This study
				§ H <sub>2</sub> O <sub>2</sub>	1481 ± 72.19 U g <sup>-1</sup>	0.79 ± 0.12 μM	0.64 s <sup>-1</sup>	0.81 μM <sup>-1</sup> s <sup>-1</sup>	-	9	60°C: 50 % after 48 hours 50°C: almost 100% of activity after 48 hours	No loss of activity at pH 5.0-10.0	
			2,6-DMP	§ H <sub>2</sub> O <sub>2</sub>	49.26 ± 4.48 U g <sup>-1</sup>	106.3 ± 27.9 μM	0.021 s <sup>-1</sup>	1.98 × 10 <sup>4</sup> μM <sup>-1</sup> s <sup>-1</sup>	-	-	-	-	
				§ H <sub>2</sub> O <sub>2</sub>	972.5 ± 28.31 U g <sup>-1</sup>	12.15 ± 1.76 μM	0.42 s <sup>-1</sup>	0.035 μM <sup>-1</sup> s <sup>-1</sup>	-	-	-	-	
<i>Scytalidium thermophilum</i>	PMO9D_SCYTH	<i>Pichia pastoris</i> X33	2,6-DMP	§ H <sub>2</sub> O <sub>2</sub>	0.36 U mg <sup>-1</sup>	4.54 mg mL <sup>-1</sup>	2.99 × 10 <sup>2</sup> min <sup>-1</sup>	6.58 × 10 <sup>3</sup> mg <sup>-1</sup> mL min <sup>-1</sup>	-	-	-	-	[62]
				§ H <sub>2</sub> O <sub>2</sub>	14.96 U mg <sup>-1</sup>	10.6 mg mL <sup>-1</sup>	1.61 min <sup>-1</sup>	1.52 × 10 <sup>4</sup> mg <sup>-1</sup> mL min <sup>-1</sup>	60°C	7	60°C (t <sub>1/2</sub> = 60.58 hours, pH 7.0)	Above 90% after 48 hours at pH 7.0	
			2,6-DMP	§ H <sub>2</sub> O <sub>2</sub>	0.13 U mg <sup>-1</sup>	0.51 mM	1.84 × 10 <sup>3</sup> min <sup>-1</sup>	3.57 × 10 <sup>4</sup> mM <sup>-1</sup> min <sup>-1</sup>	-	-	-	-	
				§ H <sub>2</sub> O <sub>2</sub>	0.17 U mg <sup>-1</sup>	5.87 mg mL <sup>-1</sup>	1.05 × 10 <sup>2</sup> min <sup>-1</sup>	1.79 × 10 <sup>3</sup> mg <sup>-1</sup> mL min <sup>-1</sup>	-	-	-	-	
<i>Malbranchea cinnamomea</i>	PMO9D_MALCI	<i>Pichia pastoris</i> X33	2,6-DMP	§ H <sub>2</sub> O <sub>2</sub>	9.59 U mg <sup>-1</sup>	29.27 mg mL <sup>-1</sup>	0.76 min <sup>-1</sup>	2.62 × 10 <sup>2</sup> mg <sup>-1</sup> mL min <sup>-1</sup>	50°C	9	50 °C (t <sub>1/2</sub> = 144 h, pH 7.0)	Above 80% after 72 hours at pH 9.0	[63]
				§ H <sub>2</sub> O <sub>2</sub>	0.12 U mg <sup>-1</sup>	1.17 mM	1.21 min <sup>-1</sup>	1.03 × 10 <sup>4</sup> mM <sup>-1</sup> min <sup>-1</sup>	-	-	-	-	
<i>Thielavia terrestris</i>	TtLPMO9E	-	PWS	§ O <sub>2</sub>	-	49.80 g L <sup>-1</sup>	3.8 min <sup>-1</sup>	*1.85 × 10 <sup>3</sup> M <sup>-1</sup> min <sup>-1</sup>	-	-	-	-	[63]
<i>Myceliophthora thermophila</i>	MtLPMO9E	<i>Neurospora crassa</i>	cellohexao	§ O <sub>2</sub>	-	32 μM	10.1 min <sup>-1</sup>	0.30 μM <sup>-1</sup> min <sup>-1</sup>	-	-	-	-	[64,65]
				§ O <sub>2</sub>	-	230 μM	17 min <sup>-1</sup>	7.4 × 10 <sup>-2</sup> μM <sup>-1</sup> min <sup>-1</sup>	-	-	-	-	
<i>Serratia marcescens</i>	CBP21	<i>Escherichia coli</i> BL21(DE3) Star	CNW	§ H <sub>2</sub> O <sub>2</sub>	1.11 μM s <sup>-1</sup>	0.58 mg mL <sup>-1</sup>	6.7 s <sup>-1</sup>	10 <sup>0</sup> M <sup>-1</sup> s <sup>-1</sup>	-	-	-	-	[66]
				§ CNW	-	2.8 μM	-	-	-	-	-	-	
<i>Lentinus similis</i>	Ls(AA9)A	<i>Aspergillus oryzae</i> MT3568	celloetrao	§ O <sub>2</sub>	-	43 μM	0.11 s <sup>-1</sup>	2.6 × 10 <sup>3</sup> M <sup>-1</sup> s <sup>-1</sup>	-	-	-	-	[67]
<i>Aspergillus fumigatus</i> NITDGPKA3	** CAF32158.1	<i>Pichia pastoris</i> X33	2,6-DMP	§ H <sub>2</sub> O <sub>2</sub>	1.11 μM min <sup>-1</sup>	11.23 μM	0.642 min <sup>-1</sup>	5.7 × 10 <sup>-2</sup> μM min <sup>-1</sup>	-	-	-	-	[68]
<i>Myceliophthora thermophila</i>	MtLPMO9D	<i>Myceliophthora thermophila</i> CI	-	-	-	-	-	-	-	-	**** T <sub>m,app</sub> at pH 7.0 = 68°C	-	[69]
<i>Myceliophthora thermophila</i>	MtLPMO9J	<i>Aspergillus nidulans</i>	-	-	-	-	-	-	-	-	**** T <sub>m,app</sub> at pH 6.0 = 58°C	-	[70]
<i>Thermoascus aurantiacus</i>	TaLPMO9A	<i>Aspergillus oryzae</i>	-	-	-	-	-	-	-	-	**** T <sub>m,app</sub> at pH 7.0 = 69°C	-	[71]

Notations: (§) fixed concentration; (\*) K<sub>cat</sub>/K<sub>m</sub> calculated for this paper considering the molecular weight of 24.2 kDa for TtLPMO9E; (\*\*) this enzyme has no name yet, the code provided is its codifying gene; (\*\*\*) pH stability not correlated with analyzed pH values; (\*\*\*\*) apparent midpoint transition temperatures calculated based on CD (MtLPMO9D and TaLPMO9A) and ITFE (MtLPMO9J) analysis; (#) K<sub>cat</sub> estimated based on previous K<sub>cat</sub>/K<sub>m</sub> and K<sub>m</sub> values for MtLPMO9E. Kinetic studies were conducted at (a) pH = 6.0 and (b) pH = 8.0. Abbreviations: PWS – Pretreated wheat straw; CNW – Chitin nanowhisker. For more details, check the correspondent references.

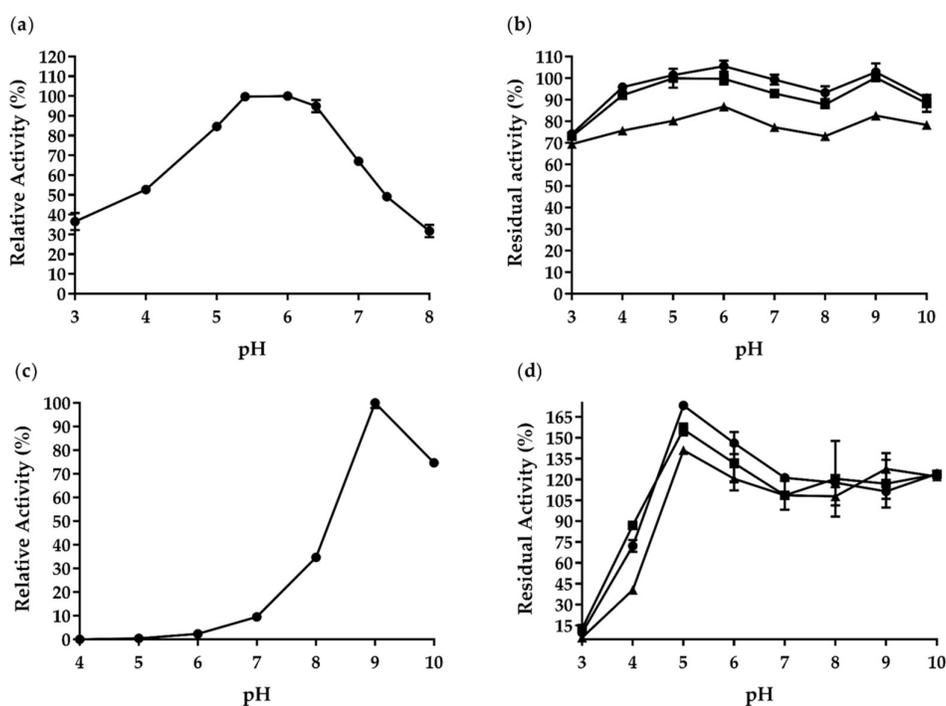
Figure 4 illustrates how pH influenced AfCel6A and AfAA9\_B. The highest activity of AfCel6A emerged at pH 5.5–6.0, but it was active in a narrow pH range (pH 4.0–7.5) and retained > 50% of maximum activity therein (Figure 4a).

Many cellobiohydrolases seem to belong to the class of acidic enzymes, with optimal pH ranging from 3.9 to 6.0; for example, CBH II from *Talaromyces emersonii* (pH 3.8), Cel6D (pH 3.9), CBH II from *Chaetomium thermophilum* (pH 4.0), CBH II from *Penicillium occitanis* (pH 3.0–5.0),

CBH II from *Trichoderma viride* (pH 5.0), J11 CelA (pH 6.0), and EX4 (pH 5.0). Only one GH6 has been classified as active at pH 9.0: MoCel6A from *Magnaporthe oryzae* (Table 2).

We also investigated the pH stability of AfCel6A (Figure 4b). Notably, AfCel6A was stable at pH ranging between 3 and 10 and retained over 70% of its original activity after 72 h. Compared to other GH6 cellobiohydrolases, AfCel6A was more stable over a wide pH range, whereas others had narrower range of pH stability—CBH II from *Talaromyces emersonii* (38 min at pH 5.0), Cel6D (over 60% activity at pH 4.0–6.0 and 47 °C and complete inactivation at pH 4.0 and 55 °C); CBH II from *Penicillium occitanis* (24 h at pH 2.0–9.0), J11 CelA (1 h), and EX4 (over 80% activity at pH 3.0–8.0 at 60 °C for 1 h).

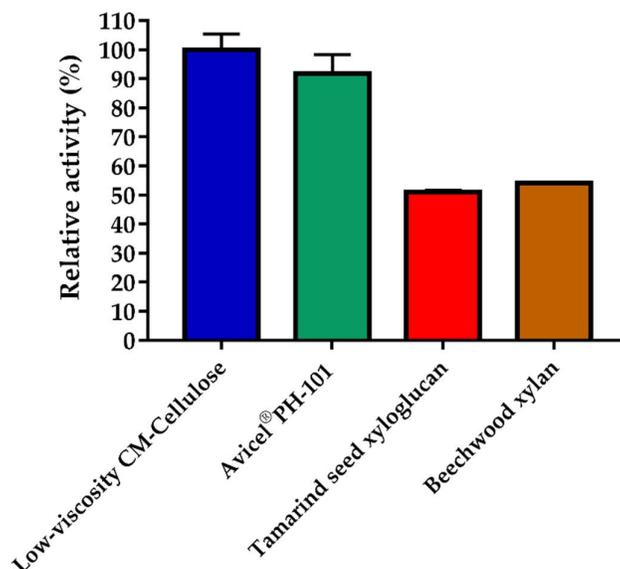
AfAA9\_B showed the highest activity at pH 9.0. At pH 10.0, it retained >74.0% of its activity (Figure 4c). The optimal pH of AfAA9\_B was pH 9.0, but this enzyme was stable at pH ranging between 5.0 and 10.0 and maintained 100% of the original activity after 72 h (Figure 4d). Compared to PMO9D\_SCYTH (pH 7.0) and PMO9D\_MALCI (pH 9.0), AfAA9\_B was more stable, whereas the former LPMOs were stable at a specific pH (Table 3).



**Figure 4.** Effects of pH on the enzymatic activity and stability of purified recombinant AfCel6A and AfAA9\_B. (a) AfCel6A pH-activity profile. (b) pH stability of AfCel6A after ● 24, ■ 48, and ▲ 72 h of preincubation at 4 °C. The enzyme activities were measured under standard conditions. (c) AfAA9\_B pH-activity profile. (d) pH stability of AfAA9\_B after ● 24, ■ 48, and ▲ 72 hours of preincubation at 4 °C. Each value in the panel represents the mean of three experiments.

#### 2.4. Substrate specificity and kinetic parameters

AfCel6A exhibited broad substrate specificity, including CM-Cellulose, Avicel®, Xyloglucan, and Birchwood xylan. This enzyme displayed higher specific activities toward CM-Cellulose ( $36.6 \pm 2.1$  U mg<sup>-1</sup>) and Avicel® ( $35.8 \pm 2.6$  U mg<sup>-1</sup>) than Birchwood xylan ( $21.1 \pm 0.1$  U mg<sup>-1</sup>) and Xyloglucan ( $19.9 \pm 0.3$  U mg<sup>-1</sup>) (Fig. 5). When CMC was the substrate, purified AfCel6A had  $K_M$ ,  $V_{max}$ , and  $k_{cat}/K_M$  of  $7.44 \pm 0.51$  g L<sup>-1</sup>,  $195.2 \pm 4.65$  U mg<sup>-1</sup>, and  $19.9$  mL mg<sup>-1</sup> s<sup>-1</sup>, respectively (Table 2).



**Figure 5. Specific activity ( $\text{U mg}^{-1}$ ) of *AfCel6A* toward CM-Cellulose, Avicel, Xyloglucan, and Xylan.** Each reaction was performed 50 mM phosphate bufer (pH 6.0) containing 0.5% (w/v) of each substrate at 55 °C for 30 min. Values are the mean  $\pm$  SD of three replicates.

We evaluated the peroxidase activity of recombinant *AfAA9\_B* toward the chromogenic substrate 2,6-DMP and the co-substrate  $\text{H}_2\text{O}_2$ , according Breslmayr et al. (2018) [72], with some modifications. Table 3 summarizes the kinetic parameters determined when the reactions were carried out at pH 6.0 or 9.0 and 50 °C.

The  $V_{\max}$  values were higher at pH 9.0 for both the substrate ( $1481 \pm 72.19 \text{ U g}^{-1}$ ) and the co-substrate ( $972.5 \pm 28.31 \text{ U g}^{-1}$ ). Because we performed the saccharification tests at pH 6.0, we also determined the kinetic parameters under these conditions. At this pH,  $V_{\max}$  was  $78.52 \pm 3.33 \text{ U g}^{-1}$  for the substrate and  $49.26 \pm 4.48 \text{ U g}^{-1}$  for the co-substrate. These results were expected because pH 9.0 was optimal for the activity of *AfAA9\_B*.

Compared to the kinetic parameters described for other LPMOs, *AfAA9\_B* had lower  $K_{\text{Mapp}}$  ( $0.79 \mu\text{M}$ ) than PMO9D\_SCYTH ( $0.51 \text{ mM}$ ), PMO9D\_SCYTH ( $0.51 \text{ mM}$ ), and PMO9D\_MALCI ( $1.17 \text{ mM}$ ), which showed that *AfAA9\_B* had higher binding affinity for 2,6-DMP (Table 3).

### 2.5. Effect of different metal ions and chemicals

Cellobiohydrolases are commonly used in many industrial processes. The effects of additives and products of cellulose hydrolysis on the activity of these enzymes must be considered during operation on an industrial scale.

Table 4 depicts how different ions and reagents influence CM-Cellulose hydrolysis by purified *AfCel6A*. At 5 mM,  $\text{MnCl}_2$  ( $189.25 \pm 2.33\%$ ), DTT ( $150.68 \pm 5.29\%$ ),  $\text{CoCl}_2$  ( $116.75 \pm 1.36\%$ ),  $\text{FeSO}_4$  ( $125.83 \pm 3.61\%$ ),  $\beta$ -mercaptoethanol ( $134.24 \pm 1.02\%$ ),  $\text{AgNO}_3$  ( $179.27 \pm 20.04\%$ ), and ascorbic acid ( $121.40 \pm 2.55\%$ ) stimulated the activity of *AfCel6A*. EDTA, DMSO, SLS, Triton X-100, Tween 20,  $\text{CaCl}_2$ ,  $\text{MgSO}_4$ , KCl, and  $(\text{NH}_4)_2\text{SO}_4$  practically did not affect the activity of *AfCel6A*. On the other hand, SDS inhibited the enzyme by approximately 50 %. The fact that  $\beta$ -mercaptoethanol and DTT boosted the activity of *AfCel6A* by 134.64% and 150.68%, respectively, suggested that the presence of sulfhydryl groups such as the ones from cysteine residues in the active site is important for enzymatic catalysis [73].

As for *AfAA9\_B*, SLS ( $115.3 \pm 0.7\%$ ), SDS ( $107.8 \pm 4.8\%$ ), Tween 20 ( $103.7 \pm 9.6\%$ ), DMSO ( $108.3 \pm 1.5\%$ ),  $\text{MgSO}_4$  ( $113.9 \pm 1.9\%$ ), and KCl ( $107.5 \pm 2.1\%$ ) did not inhibit this enzyme. DTT, EDTA,  $\text{CoCl}_2$ ,  $\text{FeSO}_4$ , and  $\text{AgNO}_3$  completely inhibited *AfAA9\_B*.  $\beta$ -mercaptoethanol,  $\text{ZnSO}_4$ , and  $\text{CuSO}_4$  decreased the activity of *AfAA9\_B* by 70%.  $\text{MnCl}_2$ ,  $\text{CaCl}_2$ , and  $(\text{NH}_4)_2\text{SO}_4$  had little effect on *AfAA9\_B*.

**Table 4.** Effects of additives on the activity of *AfCel6A* and *AfAA9\_B*

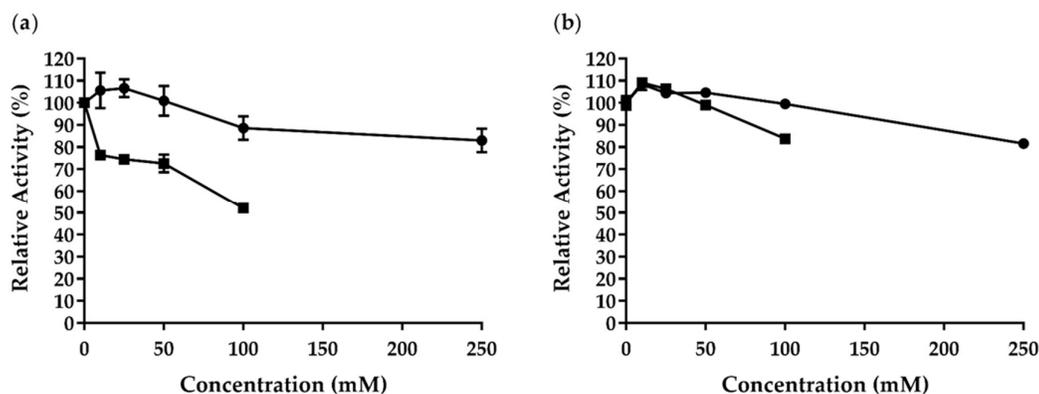
Additive	<i>AfAA9_B</i>	<i>AfCel6A</i>
	% Relative activities	
None	100.0 $\pm$ 1.0	100.0 $\pm$ 0.9
SDS	107.8 $\pm$ 4.8	53.72 $\pm$ 0.31
Tween 20	103.7 $\pm$ 9.6	93.24 $\pm$ 2.11
EDTA	0	91.88 $\pm$ 1.56
Ascorbic Acid	-	121.40 $\pm$ 2.55
DMSO	108.3 $\pm$ 1.5	101.03 $\pm$ 3.58
$\beta$ -mercaptoethanol	36.7 $\pm$ 0.6	134.24 $\pm$ 1.02
$\text{ZnSO}_4$	36.8 $\pm$ 6.1	83.67 $\pm$ 0.65
$\text{MnCl}_2$	82.2 $\pm$ 0.7	189.25 $\pm$ 2.33
$\text{CoCl}_2$	0	116.75 $\pm$ 1.36
$\text{CaCl}_2$	93.6 $\pm$ 3.3	101.1 $\pm$ 2.4
$\text{FeSO}_4$	0	125.83 $\pm$ 3.61
$\text{MgSO}_4$	113.9 $\pm$ 1.9	95.92 $\pm$ 2.13
$\text{CuSO}_4$	35.2 $\pm$ 4.6	85.08 $\pm$ 0.65
$\text{AgNO}_3$	0	179.27 $\pm$ 20.04
KCl	107.5 $\pm$ 2.1	98.87 $\pm$ 2.44
$(\text{NH}_4)_2\text{SO}_4$	88.9 $\pm$ 3.2	99.9 $\pm$ 2.1
DTT	0	150.68 $\pm$ 5.29
Triton X-100	84.8 $\pm$ 2.3	91.55 $\pm$ 1.19
SLS	115.3 $\pm$ 0.7	93.80 $\pm$ 1.60

Previously, we described that cellobiohydrolases act on short cellulose molecules and cellobiosaccharides, releasing disaccharide units, such as cellobiose [33]. Cellobiose is the major product of cellulose hydrolysis by cellobiohydrolases, whereas glucose is the final product of cellulose hydrolysis.

Product inhibition can affect lignocellulosic hydrolysis to glucose and represents a barrier to achieving the high product yields that are necessary for an efficient process [74].

We examined how different concentrations of glucose (10–250 mM) and cellobiose (10–100 mM) affected the activity of *AfCel6A* (Figure 6a). Glucose at 100 and 250 mM inhibited the activity of the enzyme by 12% and 13%, respectively. Cellobiose (100 mM) inhibited the activity of *AfCel6A* by 50%. Cellobiohydrolase from *T. reesei* (*Cel6A*) has been described as the most efficient cellobiohydrolase, with  $\text{IC}_{50}$  of 240 mM for glucose and 20 mM for cellobiose [50]. Therefore, our results showed that *AfCel6A* was more resistant to inhibition by both products because  $\text{IC}_{50}$  was higher than 250 mM for glucose and 100 mM for cellobiose.

Likewise, we investigated the effect of both sugars on *AfAA9\_B* activity (Figure 6b). Surprisingly, the enzyme retained more than 80% of its initial activity when 250 mM glucose or 100 mM cellobiose was added to the reaction. Together, these findings indicated that *AfCel6A* and *AfAA9\_B* have potential for application in enzymatic saccharification of cellulose. However, to improve the efficiency of these enzymes and to increase glucose production, synergistic association with other enzymes is required.



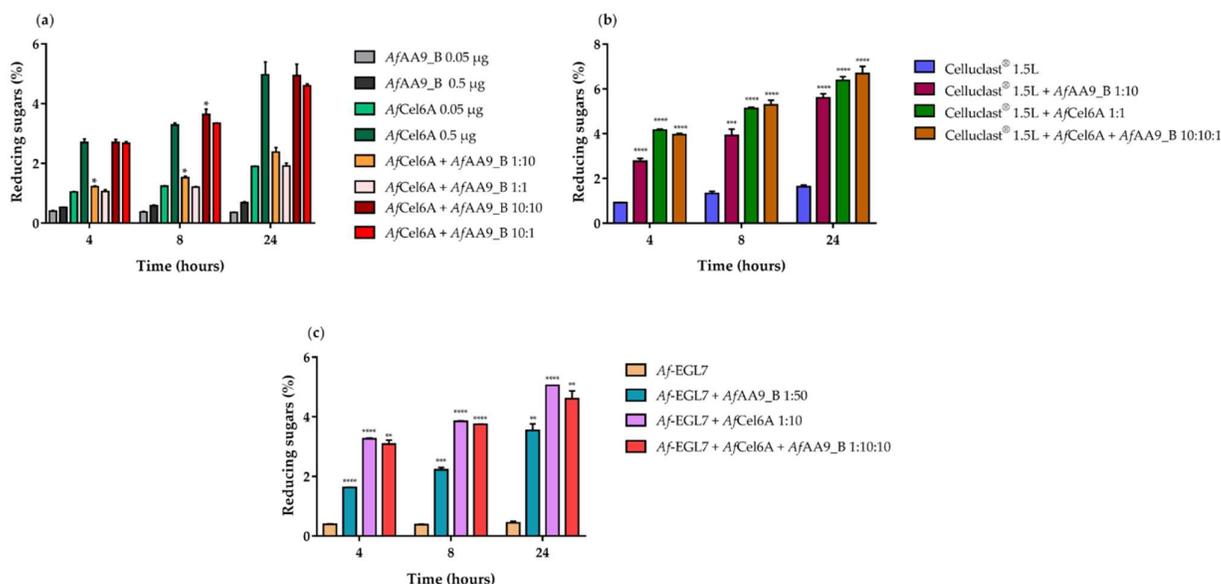
**Figure 6.** Effects of ● Glucose and ■ Cellobiose on the activity of (a) *AfCel6A* and (b) *AfAA9\_B*.

### 2.6. Synergistic action on cellulose hydrolysis

To determine the synergistic effects of *AfCel6A* and *AfAA9\_B*, we performed cellulose degradation experiments by using CMC as substrate. We conducted the reactions at different relative proportions and for different incubation times. Surprisingly, no synergistic effect between *AfCel6A* and *AfAA9\_B* was observed (Figure 7a).

We also investigated the synergistic effects between *AfCel6A* and *AfAA9\_B* and Celluclast® 1.5L at different incubation times. Hydrolysis increased over time, and the yield of reducing sugars peaked after 24 h. Compared to the cocktail alone, addition of *AfAA9\_B* or *AfCel6A* to the reaction mixture containing Celluclast® 1.5L increased the release of reducing sugars approximately 3.5 and 4.0 times, respectively. When Celluclast® 1.5L cocktail was simultaneously associated with *AfCel6A* and *AfAA9\_B* at a ratio of 1:1:10, the maximum release of reducing sugars was 4.5 times higher compared to the cocktail alone. We verified a slight synergistic degree for Celluclast® 1.5L cocktail, *AfCel6A*, and *AfAA9\_B* during CM-Cellulose hydrolysis. No inhibitory effect arose, probably because *AfAA9\_B* acted synergistically with other enzymes in Celluclast® 1.5L cocktail (Figure 7b).

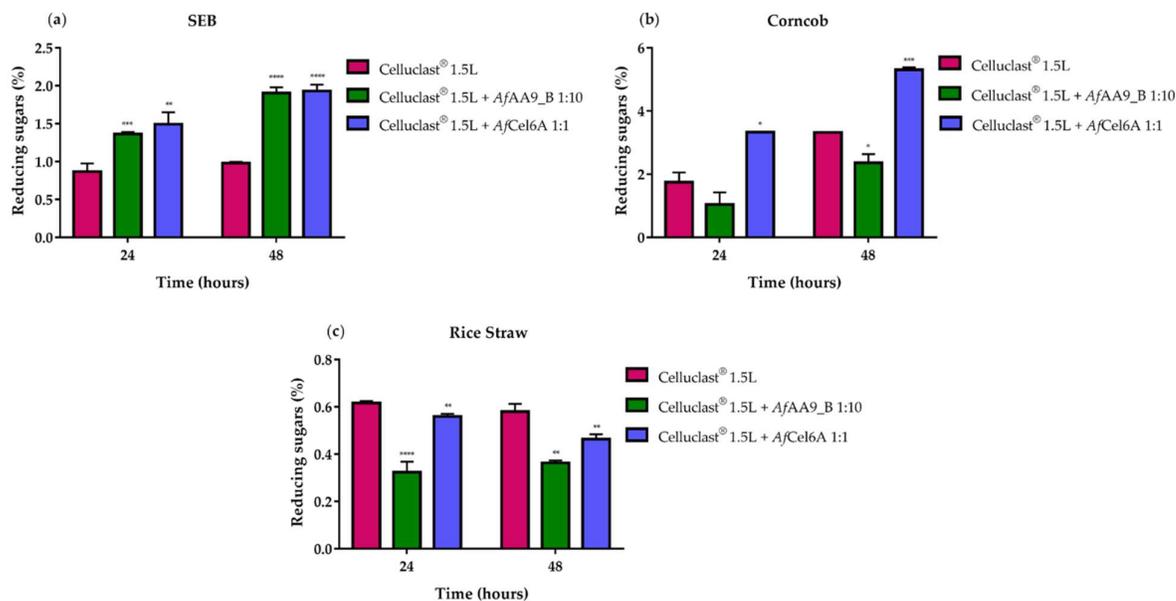
LPMOs improve the efficiency of cellulase; i.e., endoglucanases and cellobiohydrolases, during cellulose hydrolysis, and they enhance cellulase adsorption and accessibility to cellulose [75,76]. We analyzed the synergism of *AfAA9\_B* and *AfCel6A* with endoglucanase *Af-EGL7*, which had been previously characterized[30]. The combination of *Af-EGL7* and *AfAA9\_B* increased the release of reducing sugars by 8.0 times, whilst the combination of *Af-EGL7* and *AfCel6A* increased hydrolyses by 11.5 times compared to *Af-EGL7* alone. When the three enzymes were associated at an *Af-EGL7/AfAA9\_B/AfCel6A* ratio of 1:10:10, 12.5 times more reducing sugars was released (Figure 7c). Thus, *AfAA9\_B* acted synergistically with *Af-EGL7*, but not with *AfCel6A*.



**Figure 7. Synergistic action on 1% (w/v) CM-Cellulose of (a) *AfCel6A* and *AfAA9\_B*; (b) *AfCel6A*, or *AfAA9\_B*, or both with Celluclast<sup>®</sup> 1.5 L cocktail; and (c) *AfCel6A*, or *AfAA9\_B*, or both with *AfEGL7*.** All reactions were incubated in 50 mM sodium phosphate buffer (pH 6.0) at 1000 rpm and 50 °C for 4, 8, or 24 h. At the end of each reaction, the measured reducing sugars were plotted as a function of the relative proportions among the added enzymes. Asterisks indicate significant difference ( $p < 0.05$ ) in relation to the control system (*AfCel6A*, *AfEGL7*, or cocktail alone).

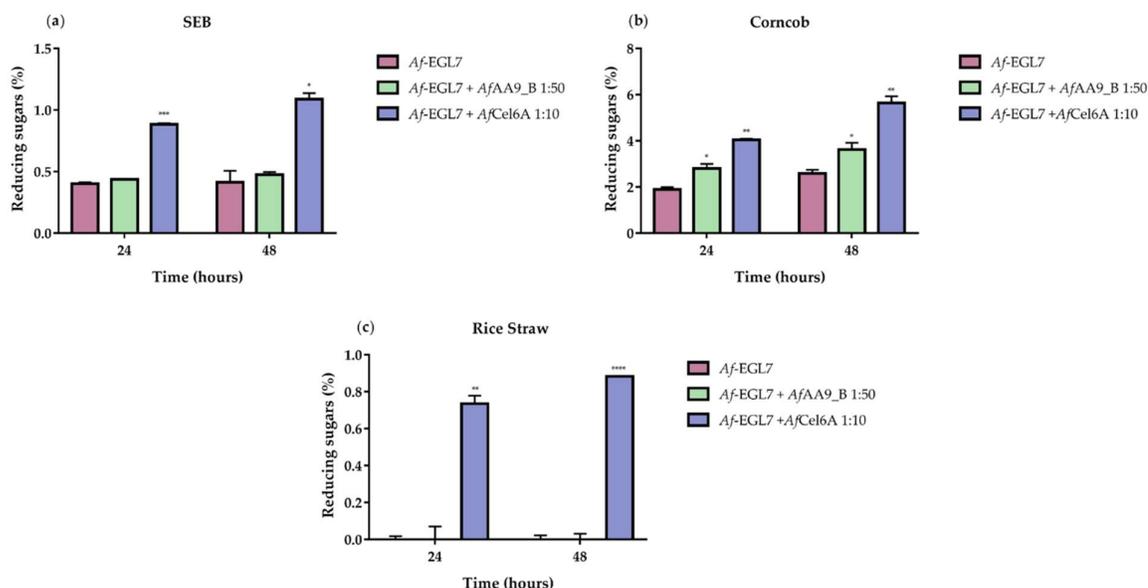
The efficiency of synergy among enzymes depends on the relative amount of crystalline to amorphous cellulose that is accessible within the substrate [77]. To evaluate how these enzymes acted on lignocellulosic biomass, we analyzed the associations of the enzymes in complex biomass, including SEB, rice straw, and corncob. SEB and corncob hydrolyses depended on time, but reducing sugars released from rice straw did not increase when the reaction time was changed from 24 to 48 h. Bernardi *et al.* (2019) [30] observed the same profile when they accomplished rice straw hydrolysis by a cocktail under similar conditions.

As shown in Figure 8a, compared to Celluclast 1.5L cocktail alone, addition of *AfCel6A* or *AfAA9\_B* increased SEB hydrolysis by ~ 70% and ~ 95% after 24 and 48 h, respectively. Similarly, association between commercial cellulases and *AfCel6A* boosted corncob hydrolysis by ~ 90% and ~ 70% after 24 and 48 hours, respectively. On the other hand, addition of *AfAA9\_B* seemed to affect hydrolysis negatively (Figure 8b). The same inhibitory effect of LPMOs has been observed on rice straw, while addition of *AfCel6A* almost did not impact the release of reducing sugars (Figure 8c). The divergent results among the three agricultural residues pointed to the substrate-dependence and substrate specificity of *AfCel6A* and *AfAA9\_B* synergism with cellulases [78].



**Figure 8. Effect of Celluclast® 1.5L cocktail supplementation with *AfCel6A* or *AfAA9\_B* on hydrolysis of (a) SEB, (b) Corncob, and (c) Rice straw.** All reactions were incubated in 50 mM sodium phosphate buffer (pH 6.0) containing 1% (w/v) of each biomass at 1000 rpm and 50 °C for 24 and 48 h. At the end of each reaction, the measured reducing sugars was plotted as a function of the relative proportions between the recombinant enzymes and commercial cellulases. Asterisks indicate significant difference ( $p < 0.05$ ) in relation to the cocktail alone.

Compared to *Af-EGL7* alone, the association between *Af-EGL7* and *AfCel6A* increased the amount of reducing sugars released from the three biomasses: ~ 163%, ~ 118%, and ~ 88% for SEB (Figure 9a), corn cob (Figure 9b), and rice straw (Figure 9c), respectively, after 48 h. The combination of *Af-EGL7* and *AfAA9\_B* also improved hydrolysis of SEB and corn cob, but it had no effect on rice straw degradation.



**Figure 9. Combined activities of *Af*-EGL7 and *Af*Cel6A or *Af*AA9\_B on hydrolysis of (a) SEB; (b) Corncob; and (c) Rice straw.** All reactions were incubated in 50 mM sodium phosphate buffer (pH 6.0) containing 1% (w/v) of each biomass for 24 and 48 h at 50 °C and 1000 rpm. At the end of each reaction, the measured reducing sugars were plotted in function of the relative proportions between the recombinant enzymes. Asterisks indicate significant difference ( $p < 0.05$ ) in relation to the *Af*-EGL7 alone.

### 3. Materials and Methods

#### 3.1. Strains, Culture Conditions, and Vectors

Mycelia of *Aspergillus fumigatus* Af293 (kindly donated by Professor Sérgio Akira Uyemura - University of São Paulo, Ribeirão Preto, Brazil) were obtained for RNA extraction. Fresh conidia ( $2 \times 10^6$  per mL) were inoculated in YNB minimal medium (1× salt solution, 0.1% (v/v) trace elements, and 0.05% (w/v) yeast extract) containing 1% (w/v) fructose and incubated under shaking at 200 rpm and 37 °C for 16 h. The mycelia were harvested, washed, and transferred to YNB medium containing 1% (w/v) sugarcane exploded bagasse (SEB) at 200 rpm and 37 °C for 24 h.

*Escherichia coli* DH10β was used to clone and to propagate the recombinant vectors. The strain was kept in Luria–Bertani medium supplemented with the appropriate antibiotic.

*Pichia pastoris* strain X-33 (Invitrogen, Carlsbad, CA, USA) was used to produce the heterologous proteins. The employed growth conditions are described in the EasySelect™ *Pichia* Expression Kit manual (Invitrogen, Carlsbad, CA, USA).

The plasmids pPICZB and pPICZαA (Invitrogen, Carlsbad, CA, USA) were used to clone, to sequence, and to express *Af*AA9\_B and *Af*Cel6A, respectively.

Xyloglucan from tamarind seed and xylan from beechwood were acquired from Megazyme (Megazyme International, Bray, Co., Wicklow, Ireland). Avicel® PH-101 and low-viscosity CM-Cellulose (CMC) were purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA).

Biomasses (rice straw and corncob) were provided by Professor Maria de Lourdes Teixeira de Moraes Polizeli (University of São Paulo, Ribeirão Preto, Brazil). Sugarcane exploded bagasse (SEB) was provided by Professor João Atilio Jorge (University of São Paulo, Ribeirão Preto, Brazil).

#### 3.2. RNA Extraction, cDNA Synthesis, and Gene Amplification

Total RNA from *A. fumigatus* mycelia was isolated by using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA); the manufacturer's instructions were followed. cDNA was synthesized by using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

Table 1 describes the specific primer sequences obtained for amplification and cloning of *AfAA9\_B* and *AfCel6A* into the vectors pPICZB and pPICZαA, respectively:

**Table 5.** Primer sequences used to amplify and to clone genes

Primer name	Sequence (5'-3')
<i>AfAA9_B</i> Fw	CAAAAAACAAC <b>T</b> AATTATTCGAAACGAGGAATTCCATGACTTTGTCCAAGATCAC
<i>AfAA9_B</i> Rv	CAGATCCTCTTCTGAGATGAGTTTTTGTCTAGAGCGTTGAACAGTGCAGGAC
<i>AfCel6A</i> Fw	GAGAAAAGAGAGGCTGAAGCTGAATTCCAGCAGACCGTATGG
<i>AfCel6A</i> Rv	ATCCTCTTCTGAGATGAGTTTTTGTCTAGAAAGGACGGGTTAGC

\*The overlapping regions between the vector and the insert are in bold.

The amplification reactions were performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MS, USA), and the PCR product was analyzed by electrophoresis and purified from 1% (w/v) agarose gel by using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany).

### 3.3. Production and purification of enzymes

The *AfAA9\_B* and *AfCel6A* ORFs (Open Reading Frames) with and without predicted signal peptides, respectively, were cloned into the corresponding vectors pPICZB and pPICZαA (previously digested with the restriction enzymes *EcoRI* and *XbaI*) by the circular polymerase extension cloning (CPEC) method [79]. Both CPEC reactions were carried out with Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The thermocycling conditions were as follows: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 2 min 30 s; and 72 °C for 10 min. The cloning products were transformed to *E. coli* DH10β, and the resistant transformants were selected with zeocin (50 μg mL<sup>-1</sup>). Next, the recombinant vectors pPICZB/*AfAA9\_B* and pPICZαA/*AfCel6A* were linearized with *PmeI* and transformed into competent *P. pastoris* X-33 cells by electroporation according to the EasySelect™ Pichia Expression Kit manual (Invitrogen).

Zeocin-resistant *P. pastoris* transformants were selected to produce the enzymes. The recombinant yeasts were cultivated in buffered glycerol-complex medium (BMGY) at 240 rpm and 30 °C. For heterologous expression of *AfAA9\_B*, *P. pastoris* cells were resuspended in buffered methanol-complex medium (BMMY). Methanol (1% (v/v)) was added to the medium at 24-h intervals for six days, and the supernatant was harvested from the grown culture. The supernatant containing secreted recombinant enzyme (*AfAA9\_B*) was concentrated 10 times by using an Amicon Ultra-15 Centrifugal Filter—10-kDa cutoff (Millipore, Burlington, MS, USA). Protein expression was verified by SDS-PAGE.

*AfCel6A* was expressed as described above, but 1.5% (v/v) methanol was added.

To purify the enzymes, the concentrates were resuspended in 20 mM sodium phosphate buffer containing 500 mM NaCl (pH 7.4) and loaded onto Ni<sup>2+</sup> Sepharose 6 Fast Flow resin (Ge Healthcare, Little Chalfont, United Kingdom). An imidazole gradient from 0 to 500 mM was applied to the columns to elute the recombinants His6-tagged *AfAA9\_B* and His6-tagged *AfCel6A*. The fractions were collected, and the enzymes were analyzed by 10% (w/v) SDS-PAGE, stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO, USA). Fractions containing the recombinant enzymes were mixed and buffer-exchanged by using an Amicon Ultra-15 Centrifugal Filter - 10 kDa cutoff (Millipore) to remove excess imidazole.

To coordinate copper to the active site of *AfAA9\_B*, the purified recombinant enzyme was incubated with CuSO<sub>4</sub> at 1:3 molar ratio and 4 °C for 30 min. Then, the *AfAA9\_B* solution was dialyzed against 20 mM sodium phosphate buffer containing 500 mM NaCl (pH 7.4) under

shaking at 4 °C for 48 h, to remove traces of non-coordinated Cu<sup>2+</sup>. The concentration of purified AfAA9\_B was determined by the Greenberg method [80].

The AfAA9\_B band from the SDS-PAGE gel was manually excised, reduced, alkylated, digested with trypsin, purified (Promega, Madison, WI, EUA - V5111), and analyzed by mass spectrometry according to a previously described method [81].

### 3.4. Glycosylation

N-glycosylation sites were predicted by employing NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>), and O-glycosylation was analyzed by using NetOGlyc 4.0 (<http://www.cbs.dtu.dk/services/NetOGlyc/>). The deglycosylation of recombinant AfAA9\_B and AfCel6A was accomplished by using Endoglycosidase H (Endo H, New England Biolabs, Ipswich, MA, USA) in non-denaturing conditions, as per the manufacturer's procedure. The resulting enzymes were further analyzed by SDS-PAGE.

### 3.5. Structural Analysis by Circular Dichroism (CD)

Circular dichroism (CD) spectra of the enzymes were obtained between 190 and 250 nm (far-UV) on a JASCO-810 spectropolarimeter; quartz cuvettes with optical path of 0.1 cm were employed. AfAA9\_B (0.021 mg mL<sup>-1</sup>) and AfCel6A (0.0026 mg mL<sup>-1</sup>) were diluted in 20 mM sodium phosphate (pH 7.4), and the readings were performed in quadruplicate at scanning speed, band width, and D.I.T. of 50 nm min<sup>-1</sup>, 3 nm, and 1 s, respectively. All the spectra were corrected for the buffer contributions and converted from millidegrees (mdeg) to  $\Delta\epsilon$  in M<sup>-1</sup> cm<sup>-1</sup> according to the following equation:  $\Delta\epsilon = \theta \cdot [(0.1 \cdot \text{MRW}) / (d \cdot c \cdot 3298)]$ , where  $\theta$  is the ellipticity value originally given by equipment (millidegrees), MRW is the mean residual weight of the enzyme, d is the optical path (cm), and c is the concentration of the enzyme (mg mL<sup>-1</sup>). All the secondary structures of the enzymes were predicted by using the BeStSel web server [45], and the results were compared with structures modeled on the Phyre2 [47] and Discovery Studio [82] web servers.

### 3.6. LPMO activity assay

The activity of purified AfAA9\_B was analyzed as reported by Breslmayr *et al.* (2018) [72]. The assay consisted of a reaction mixture containing 1 mM 2,6-dimethoxyphenol (2,6-DMP) (Sigma-Aldrich, St. Louis, MO, USA), 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and recombinant purified AfAA9\_B in 50 mM sodium phosphate buffer (pH 8.0). For the blank, the enzyme was denatured by incubation at 99 °C for 30 min before the reaction mixture was added. After 5 min at 30 °C, absorbance was read at 469 nm to calculate the peroxidase activity of LPMO.

### 3.7. AfCel6A activity assay

The activity of AfCel6A was determined by measuring reducing sugars from the reaction by the 3,5-dinitrosalicylic acid (DNS) method [83]. Briefly, the reaction mixture consisting of 1% CM-Cellulose (w/v) in 50 mM sodium phosphate buffer (pH 6.0) was incubated at 55 °C for 30–45 min. The enzymatic action was stopped by adding an equal volume of the DNS reagent. The mixture was boiled for 5 min and cooled, and absorbance was measured at 540 nm. One unit of AfCel6A was defined as the amount of enzyme that released 1  $\mu$ mol of reducing sugar from the substrate per minute. Each assay was carried out in triplicate. The concentration of the enzyme was determined by the Greenberg method [80].

### 3.8. Enzymatic properties of AfAA9\_B and AfCel6A

The optimal pH for the activity of AfAA9\_B was measured at pH ranging from 4.0 to 8.0 in McIlvaine buffer (citric acid-Na<sub>2</sub>HPO<sub>4</sub>) and at pH 9.0 and 10.0 in 100 mM Glycine-NaOH buffer at 30 °C. The relative activity was calculated with respect to the maximum activity of 100%; the

aforementioned method was followed. pH stability was estimated by measuring the residual enzymatic activity after the enzyme was incubated without substrate in the aforementioned buffers at pH ranging from 3.0 to 10.0 and 4 °C for up to 72 h. To determine the thermal stability of *AfAA9\_B*, the enzyme was preincubated without substrate at 50 and 60 °C for up to 72 h. To measure the residual activity, the activity of the enzyme with no preincubation was considered 100%.

The optimal pH for the activity of *AfCel6A* was measured from 3.0 to 8.0 in McIlvaine buffer (citric acid- $\text{Na}_2\text{HPO}_4$ ) at 55 °C. The optimal temperature was examined between 40 and 80 °C. The relative activity was calculated with respect to the maximum exhibited activity of 100%; the aforementioned method was followed.

The pH stability of *AfCel6A* was estimated by measuring the residual enzymatic activity under standard conditions after the enzyme was incubated without substrate in McIlvaine (citrate-phosphate) buffers pH 3.0–8.0 and in 100 mM Glycine-NaOH buffers pH 9.0 and 10.0 at 4 °C for up to 72 h. To determine the thermal stability of *AfCel6A*, the enzyme was preincubated without substrate at temperatures ranging from 50 to 90 °C for different times. For residual activity measurement, the activity of enzyme with no preincubation was considered 100%.

### 3.9. Effect of additives

The effects of various metal ions on the activity of *AfAA9\_B* and *AfCel6A* were determined by adding  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{NH}_4^+$ ,  $\text{K}^+$ , or  $\text{Ag}^+$  at a final concentration of 5 mM to the reaction mixture. The effects of EDTA, SDS, Tween 20, Triton X-100, SLS,  $\beta$ -mercaptoethanol, DTT, and DMSO were also tested. For *AfCel6A*, the effect of ascorbic acid addition was also evaluated. Control reactions (100% activity) were performed without any additive. The relative activity was estimated as compared to the controls.

### 3.10. Glucose and cellobiose effects on the activity of *AfCel6A* and *AfAA9\_B*

The glucose (10–250 mM) and cellobiose (up to 100 mM) effects on the activity of *AfAA9\_B* and *AfCel6A* were determined in the presence of increasing concentrations of both sugars by using the chromogenic substrates 2,6-DMP and 4-nitrophenyl  $\beta$ -D-cellobiose (Sigma-Aldrich, St. Louis, MO, USA), respectively.

### 3.11. Kinetic assays

The kinetic parameters of *AfAA9\_B* ( $K_M$ ,  $V_{max}$ , and  $k_{cat}$ ) were determined for the substrate 2,6-DMP (0.1 to 10 mM) and the co-substrate  $\text{H}_2\text{O}_2$  (1 to 500  $\mu\text{M}$ ). The reactions were performed in 50 mM sodium phosphate buffer (pH 6.0) and 100 mM glycine-NaOH buffer (pH 9.0) at 50 °C. The parameters were calculated by Michaelis-Menten nonlinear regression.

The kinetic parameters of *AfCel6A* were determined when CM-Cellulose (0.5–30  $\text{mg mL}^{-1}$ ) was used as substrate. The reactions were performed in 50 mM sodium phosphate buffer (pH 6.0) as previously described. The parameters were calculated by the Michaelis-Menten nonlinear regression graphical method.

### 3.12. Combined assays

*AfAA9\_B* and *AfCel6A* enzymatic assays were carried out concomitantly with the recombinant endoglucanase *Af-EGL7* as previously described<sup>30</sup>. The assays were performed by adding 1  $\mu\text{g}$  of *Af-EGL7* to 50  $\mu\text{g}$  of *AfAA9\_B* (1:50) or 10  $\mu\text{g}$  of *AfCel6A* (1:10) per each gram of substrate. The reaction mixtures consisted of CM-Cellulose (1% (w/v)) in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM ascorbic acid in a final volume of 1 mL. The reactions were performed in a thermomixer (Eppendorf) at 50 °C and 1000 rpm for 4, 8, or 24 h.

In the same way, *AfAA9\_B* and *AfCel6A* were combined at different concentration proportions (10:1, 1:1, 10:10, or 1:10), where the minimum and maximum enzyme loading corresponded to 5 and 50  $\mu\text{g}$  of added enzyme per gram of CM-Cellulose, respectively.

Finally, the effect of the simultaneous association of the three recombinant enzymes on the degradation of CM-Cellulose was evaluated. While the concentration of *Af-EGL7* was 1  $\mu\text{g g}^{-1}$ , the concentrations of both *AfAA9\_B* and *AfCel6A* were 10  $\mu\text{g}$  per gram of CM-Cellulose, generating the ratio 1:10:10. The reactions were carried out as described above.

The degradation efficiencies were assessed by estimating the released reducing sugars by the DNS method. The reported results represent the mean  $\pm$  SD calculated from at least three experimental replicates.

### 3.13. Synergistic activity with Celluclast® 1.5L

The synergistic activity of *AfAA9\_B* and *AfCel6A* during enzymatic hydrolysis was investigated in combination with Celluclast® 1.5L, a commercial cellulase cocktail from *Trichoderma reesei*.

Therefore, 0.05 FPU of Celluclast® 1.5L cocktail was associated with 50  $\mu\text{g}$  of *AfAA9\_B* (ratio 1:10) or 5  $\mu\text{g}$  of *AfCel6A* (ratio 1:1) per each gram of CM-Cellulose (1% (w/v) in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM ascorbic acid. The reactions were conducted at 1000 rpm and 50 °C for up to 24 h in a final volume of 1 mL.

The effect of the simultaneous association between commercial cellulases and the two recombinant enzymes from *A. fumigatus* on the degradation of CM-Cellulose was also evaluated. While the Celluclast® 1.5 L cocktail loading was fixed in 0.05 FPU  $\text{g}^{-1}$ , the concentrations of *AfCel6A* and *AfAA9\_B* were 5 and 0.5  $\mu\text{g}$  of enzyme added per gram of CM-Cellulose, respectively. The reactions were carried out as described above.

The percent hydrolysis yields were determined by estimating the released reducing sugars by the DNS method [84]. The reported results represent the mean  $\pm$  SD calculated from at least three experimental replicates.

### 3.14. Lignocellulosic Biomass saccharification

Enzymatic hydrolyses of some agro-industrial residues were carried out as described by Bernardi et al. (2019) with some modifications [30]. Saccharification was accomplished in 50 mM sodium phosphate buffer (pH 6.0) containing 1% (w/v) of one of the following biomasses: SEB (sugarcane exploded bagasse), rice straw, or corncob.

Different associations between the enzymes were used during biomass saccharifications. *Af-EGL7* (18  $\mu\text{g g}^{-1}$ ) was combined with *AfAA9\_B* (900  $\mu\text{g g}^{-1}$ ) or *AfCel6A* (180  $\mu\text{g g}^{-1}$ ). Similarly, a fixed concentration of Celluclast 1.5L cocktail (0.9 FPU  $\text{g}^{-1}$ ) was associated with *AfAA9\_B* (900  $\mu\text{g g}^{-1}$ ) or *AfCel6A* (90  $\mu\text{g g}^{-1}$ ). The reactions were conducted at 1000 rpm and 50 °C for up to 48 h in a final volume of 1 mL. DNS was added to stop the reactions and to measure the released reducing sugars. The reported results represent the mean  $\pm$  SD calculated from at least three experimental replicates.

### 3.15. Reproducibility of the Results

All the data are the mean of at least three independent experiments and show consistent results.

## 4. Conclusions

Novel cellobiohidrolase and LPMO from *Aspergillus fumigatus* were characterized after they were expressed in *P. pastoris*. Supplementation of a cellulase cocktail with both enzymes improved the yield of saccharification of different biomasses, especially SEB. However, *AfAA9\_B* did not have a positive effect on *AfCel6A* activity. On the other hand, *AfAA9\_B* acted

synergistically with endoglucanase *Af*-EGL7. These different synergistic effects are important to understand the action of LPMOs with cellulases and would help to design new commercial enzymatic cocktails. Considering the reduction of costs in lignocellulose conversion, we can conclude that supplementation of Celluclast® 1.5L with *Af*Cel6A or *Af*AA9\_B suffices to increase the hydrolytic activity, so the composition of cellulase cocktails may need to be reconsidered.

**Supplementary Materials:** Supplementary materials can be found at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1).

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## Abbreviations

SEB	Sugarcane Exploded Bagasse
CD	Circular dichroism
2,6-DMP	2,6-Dimethoxyphenol

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