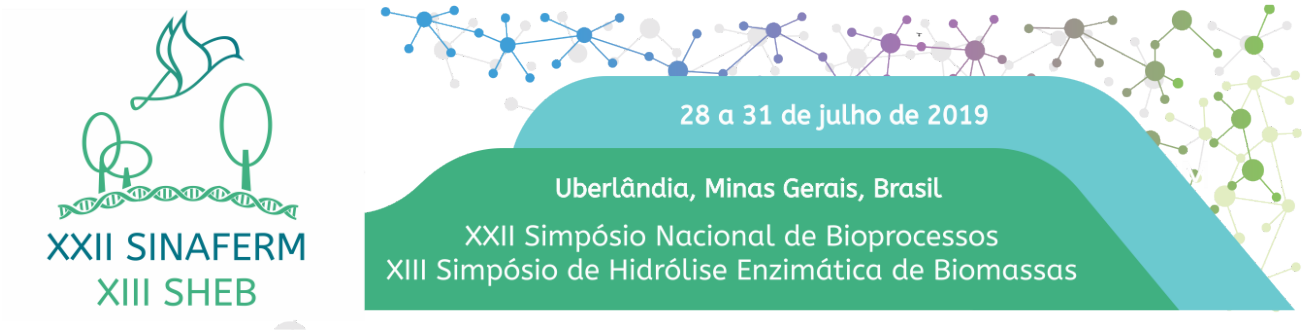

HETEROLOGOUS EXPRESSION OF A NEW LYTIC POLYSACCHARIDE MONOOXYGENASE FROM *Hahella ganghwensis* AND THEIR FUNCTIONAL CHARACTERIZATION

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ABSTRACT

The powerful class of oxidative enzymes, lytic polysaccharide monooxygenases (LPMOs) - also named Auxiliary Activity (AA) - are able to oxidize recalcitrant polysaccharides on lignocellulosic biomass. In this work, we successfully expressed three catalytic domains from bacterial LPMOs in the yeast Komagataella phaffii: domain MdAA10.1-SD (from Moritella dasanensis), domain VmAA10.2-SD (from Verrucosispora maris), and domain HgAA10.1-SD (from Hahella ganghwensis). Heterologous expression was analyzed by SDS-PAGE, Western-Blot, and Dot-Blot, while functional activity of these proteins was investigated by a combination of mass spectrometric and chromatographic methods. The recombinant LPMO catalytic domain HgAA10.1-SD from H. ganghwensis, a C1-oxidizer, was able to promote an oxidative cleavage of phosphoric-acid swollen cellulose (PASC) substrate in the presence of ascorbic acid as an electron donor, showing its potential for cellulose depolymerization.

1. INTRODUCTION

The copper-dependent LPMOs are essential for the deconstruction of recalcitrant lignocellulosic biomass. They are able to boost biomass degradation through synergism with hydrolytic enzymes (Bissaro et al., 2018). Currently, there are seven classes of LPMOs, also named Auxiliary Activity (AA) according to the classification of the Carbohydrate-active enzymes database (CAZy; http://www.cazy.org) (AA9, AA10, AA11, AA13, AA14, AA15, and AA16). Their mode of action is copper-based and differs mainly on substrate specificity and regioselectivity. AA10 family, formerly CBM33, have been shown to act on chitin and cellulose, the two most abundant polysaccharides in nature, and are found mainly in bacteria (Bissaro et al., 2018). In the context of green chemistry and circular bioeconomy, more attention should be given to rationalizing the customization of enzymatic cocktails in the lignocellulosic biorefinery industry, as protein loading and pretreatment

are the bottlenecks for achieving a sustainable and economic process for biomass degradation (Monclaro & Filho, 2017). Based on this, in-depth knowledge about LPMOs is essential for a rational application in lignocellulosic biorefineries. In the present work, we investigated the heterologous expression and functional characterization of three new bacterial LPMOs, using the methylotrophic yeast *Komagataella phaffii* as an expression host.

2. MATERIAL AND METHODS

The genes encoding only the single AA10 catalytic domain of LPMOs from *V. maris* (*Vm*AA10.2-SD; GenBank AEB43663.1), *M. dasanensis* (*Mc*AA10.1-SD; GenBank WP_017222644.1), and *H. ganghwensis* (*Hg*AA10.1-SD; GenBank WP_020410109.1) were artificially synthesized (GenOne, Brazil). The gene constructions were obtained with native gene sequence, with the α-factor secretion signal, and with a histidine tag in the C-termini of the protein. The methylotrophic yeast *K. phaffii* was used as expression host (Rodrigues et al., 2017). pGAPZαA was the selected plasmid for *Vm*AA10.2-SD and *Mc*AA10.1-SD catalytic domains, and pPICZαA for *Hg*AA10.1-SD catalytic domain. The recombinant plasmids were transformed into *Escherichia coli* TOP10 through electroporation. For DNA plasmidial extraction, PureLink Genomic DNA Kit (Thermo Scientific) was used. *K. phaffii* transformants resistant to zeocin were analyzed using a colony PCR procedure in order to confirm the presence of the gene in the genome of the yeast. Based on the positive result of this PCR, 1-5 transformants were screened for protein production in BMGY (*Vm*AA10.2-SD and *Md*AA10.1-SD) or BMMY (*Hg*AA10.1-SD) culture media. The cells were harvested by centrifugation and stored at 4°C. The supernatants were analyzed by SDS-PAGE, *Western* blot, and dot blot protocols. *Hg*AA10.1-SD was purified using a one-step purification protocol using a Ni-NTA Purification System (Thermo Scientific). *Hg*AA10.1-SD fractions were pooled, dialyzed against water, and concentrated to 2 mL using a VivaSpin® 500 (MWCO 3 kDa, GE Healthcare). The molecular weight and purity of the enzyme was analyzed by MALDI-TOF MS. Enzyme sample and sinapinic acid (20 mg/mL prepared in 30% acetonitrile) at 1:3 proportion (v:v) were added to a MPT 384 *target plate ground steel* BC (Bruker Daltonics). Analysis was performed in an UltrafleXtreme MALDI-TOF/TOF instrument (Bruker Daltonics) operated in Linear Positive mode. *Hg*AA10.1-SD was assayed for activity on *phosphoric-acid swollen cellulose* - PASC (0.2%) using ascorbic acid (1mM) as an electron donor, at 50°C and 14000 rpm for 48h. LPMO reaction products on PASC were analyzed by HILIC-UHPLC-ESI-MS in a Shimadzu Nexera X2 UHPLC system (Kyoto, Japan) equipped with an Acquity UPLC BEH Amide column and VanGuard BEH Amide precolumn (Waters, Milford, USA), coupled to a ESI-Q-TOF mass spectrometer (maXis 4G TM MS, Bruker Daltonics, Germany) operated in negative ion mode. The LC-MS method was based on previous studies (Boulos & Nyström, 2016).

3. RESULTS AND DISCUSSION

The LPMO from *V. maris* (*Vm*AA10.2-SD) presents a C-terminal CBM2 domain and have a theorical molecular mass of 21.33 kDa; *Md*AA10.1-SD and *Hg*AA10.1-SD are single-domain AA10 LPMOs and have theorical molecular mass of 18.79 kDa and 18.71 kDa, respectively (Figure 1A). We selected

only the catalytic domain (AA10) from these LPMOs, and the three enzymes were successfully expressed in *K. phaffii,* as confirmed by immunodetection using antibodies raised against the poly-HIS tag. For *Vm*AA10.2-SD and *Md*AA10.1-SD, dot blot was performed (Figure 1B); for *Hg*AA10.1-SD, *Western* blot was performed and showed a band with higher molecular mass (~28 kDa) compared to the theorical one (Figure 1C), likely due to glycosylation. Through MALDI-TOF MS, the purity of the enzyme and the molecular mass could be confirmed as 28.04 kDa (Figure 1D).

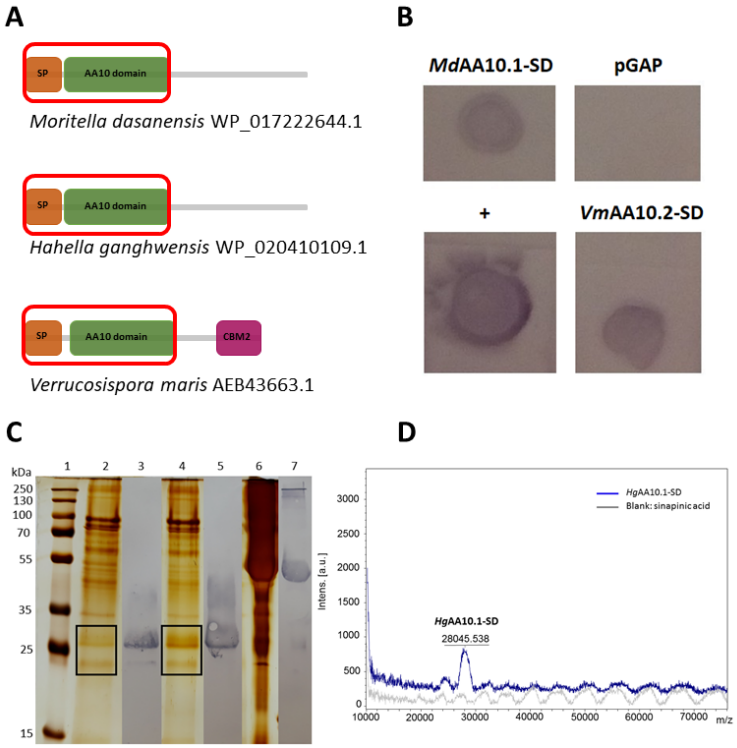

Figure 1. Characterization of three recombinant catalytic domains of LPMOs from the bacteria M. dasanensis (MdAA10.1-SD), V. maris (VmAA10.2-SD), and H. ganghwensis (HgAA10.1-SD). A) Representation of the modularity of the enzymes. In red: the signal peptide (SP) and catalytic domain of AA10 LPMOs that were synthesized. B) Dot blot of supernatant of K. phaffii transformants producing MdAA10.1-SD and VmAA10.2-SD LPMOs: (+) - Positive Control; pGAP - Empty vector, negative control. C) SDS-PAGE and Western blot of HgAA10.1-SD. 1 - PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific); 2 and 4 – Supernatant from K. phaffii transformants producing HgAA10.1-SD LPMO (colonies 2 and 10, respectively); 3 and 4 – immunodetection using antibodies against the poly-HIS tag (colonies 2 and 10, respectively); 6 and 7 – Positive control. D) MALDI-TOF MS of purified HgAA10.1-SD and a blank sample corresponding to sinapinic acid matrix.

HILIC-UHPLC-ESI-MS analysis suggests that *Hg*AA10.1-SD displayed oxidative activity on PASC (Figure 2). Native and C1-oxidized cello-oligosaccharides were released in the presence of ascorbic acid, suggesting C1-regioselectivity. Even though ions having *m/z* values 16 units superior to native oligosaccharides (*e.g.* DP2 [m/z 341.108] and $DP2_{ox}$ [m/z 357.103]) may correspond to both C1- (aldonic acid form) and C4-oxidized (gemdiol form) oligosaccharides, they most likely correspond to

the C1-oxidized products, for two main reasons: 1) they display a chromatographic behavior similar to glucuronic acid (a carboxylic acid analogous to aldonic acids); 2) the LC-MS method employed favors the detection of C1-oxidized products in the aldonic acid form [M+16Da] and the C4-oxidized products in the ketoaldose form [M-2Da] (Boulos & Nyström, 2016). In the absence of ascorbic acid, small amounts of native oligosaccharides were released by *Hg*AA10.1-SD activity on PASC, suggesting a possible *K. phaffii* endogenous endoglucanase contaminant in the preparations, or a low hydrolytic activity of the heterologous enzyme. In summary, this work showed for the first time that the *Hg*AA10.1-SD LPMO from *H. ganghwensis* can be secreted by *K. phaffii* cells in its functional form. The production of this enzyme could be optimized for different applications, such as saccharification of lignocellulosic biomass, as a technology for increasing forage fiber utilization, production of cellulose nanofibers, among other industrial applications.

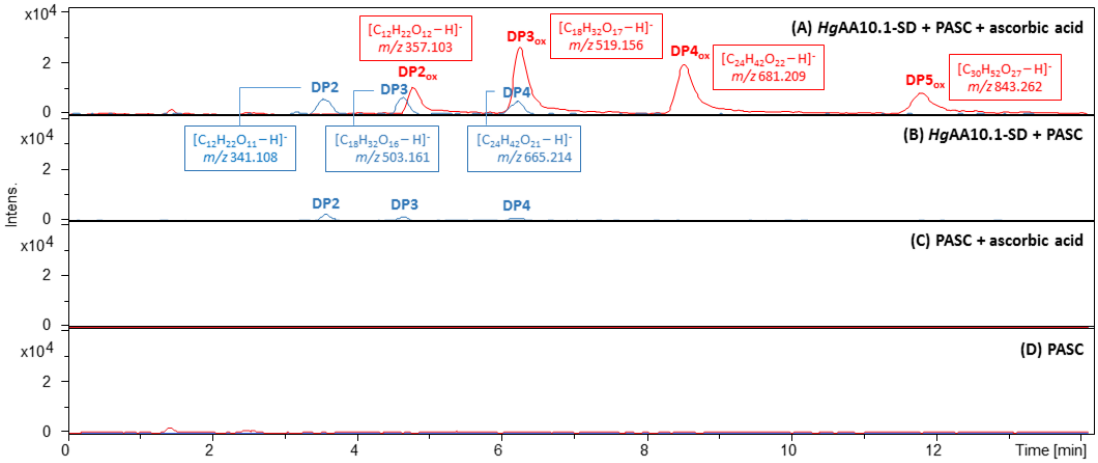

Figure 1. HILIC-UHPLC-ESI-MS of the HgAA10.1-SD (1µM) cleavage products on PASC (0.2%) using ascorbic acid (1mM) as an electron donor (A). Assays in the absence of enzyme and/or ascorbic acid were also performed (B, C and D). Lines in blue refer to the extracted ion chromatograms (EIC) of ions corresponding to native cello-oligosaccharides (DP2 – DP4). Lines in red refer to the EIC of ions corresponding to oxidized cello-oligosaccharides ($DP2_{ox}$ – $DP5_{ox}$), having m/z values 16 units superior to their native counterparts.

5. REFERENCES

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