TWO α-ARABINOFURANOSIDASES PRODUCED BY *CHRYSOPORTHE CUBENSIS* AND THE POSSIBLE SYNERGISM BETWEEN THEM

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ABSTRACT

To investigate the potential of two α-arabinofuranosidases produced by the fungus *Chrysoporthe cubensis* COAD 3356 to increase the sugar production from lignocellulosic biomass and possible synergism between them in this process, these enzymes were identified, characterized, and applied to the alkaline pretreated sugarcane bagasse saccharification. The two α-arabinofuranosidases identified were named α-Ara1 and α-Ara2 and classified on GH51 and GH54/CBM42 families, respectively. The sugarcane bagasse saccharification experiment showed that the supplementation of a commercial cellulase-rich cocktail with α-Ara2 (15 U/g) resulted in an increase of 1.6, 3.9, and 6.1 times in the release of glucose, xylose, and arabinose, respectively. The supplementation with α-Ara1 in the same conditions did not result in an increase in sugars released. However, the simultaneous use of both α-arabinofuranosidases (7.5 U/g each) promoted similar results to those obtained with α-Ara2 alone. Therefore, the results indicate a combined action between GH51 and GH54 α-arabinofuranosidases, that reduced enzyme load and maintained the GH54 effect. This probable synergism is a potential target for future studies that aim to reduce the costs of enzymatic hydrolysis and make second-generation ethanol more competitive in the biofuel market.

Keywords: Glycoside hydrolase family 51. Glycoside hydrolase family 54. Enzyme supplementation. Enzymatic hydrolysis.

1 INTRODUCTION

Brazil is the largest producer of sugarcane in the world and responsible for the largest production of first-generation bioethanol from sugarcane. In this scenario, sugarcane bagasse is an agro-industrial residue available in large quantities and at a low cost, which makes it an excellent biomass for second-generation ethanol production[1]. Three main steps are required to convert lignocellulosic biomass into 2G bioethanol: biomass pretreatment, saccharification, and fermentation. The conversion of polysaccharides into fermentable sugars occurs due to the action of enzymes in the biomass during saccharification. This step represents one of the biggest technological challenges of the process due to the high cost and low efficiency of the enzymes.[2]

Since lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin, some studies have focused on improving the hydrolysis of the hemicellulosic fraction (xylan) to facilitate the access of cellulases to cellulose and thus increase the efficiency of saccharification. Therefore, in addition to adequate pretreatment of biomass, the combined action of different types of enzymes such as cellulases, hemicellulases and accessory enzymes is necessary.[3,4,5] Most commercial enzyme mixtures used in biomass hydrolysis, such as Multifect® CL, have a high activity of cellulases and xylanase, but little or no hemicellulase, including the α-arabinofuranosidase activities. In this context, α-arabinofuranosidases have gained prominence as a target for supplementation of enzymatic preparations intended for application in the hydrolysis of lignocellulosic biomass.[4,5,6]

α-L-Arabinofuranosidases (EC 3.2.1.55) are hemicellulases classified into six different glycosyl hydrolases (GH) families, namely, GH2, GH3, GH43, GH51, GH54, and GH62. They are responsible for the release of α-1,2-, α-1,3-, and α-1,5-L-arabinofuranosyl residues present in hemicelluloses, such as arabinoxylan and other cell wall polysaccharides.[7,8] Previously, two α-arabinofuranosidases were predicted in a study of the bioinfosecretome of *Chrysoporthe cubensis*, which produced α-arabinofuranosidases of GH51 and GH54 families when cultivated in wheat bran or sugarcane bagasse.[9,10] While α-arabinofuranosidases are effective in boosting xylose production, their potential to enhance cellulose conversion to glucose is still debated because some studies reported an increase in glucose production with the addition of these enzymes, and others have found no effect or observed that their production of xylooligosaccharides inhibits cellulase, thereby reducing process efficiency.[6] Therefore, to better understand the role of α-arabinofuranosidases in biomass saccharification, this study aimed to relate the molecular characteristics of two α-arabinofuranosidases from *C. cubensis* with their effects on the alkaline pretreated sugarcane bagasse saccharification.

2 MATERIAL & METHODS

The fungus *Chrysoporthe cubensis* COAD 3356 was obtained from the mycological collection of the Forest Pathology Laboratory, Federal University of Viçosa, MG, Brazil. The microorganism was maintained on potato dextrose agar (PDA) plates at 28 °C and subcultured periodically.[11] To induce the production of α-arabinofuranosidases, *C. cubensis* was grown under solid-state fermentation using wheat bran and sugar beet flour (1:1) as a carbon source.[2] Then, the target enzymes were partially purified using different chromatographic methods.[6]

The purified enzymes were digested with trypsin and analyzed by LC-MS/MS analysis, using a nano UHPLC system Dionex® Ultimate 3000 coupled to a hybrid quadrupole-orbitrap mass spectrometer Q-exactive™ with a nanospray ion source. The database searching was done using the software PEAKS Studio version 8.5. Protein identification was performed by comparison of raw MS/MS2 spectra against the in-house *C. cubensis* database, derived from in-silico digestion of the bioinfosecretome of the fungus *C. cubensis*.[9] Multiple alignments of protein sequences of microbial α-arabinofuranosidases with known structures were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).[6]

Sugarcane bagasse (SCB) was obtained at the sugar and ethanol plant in Jatiboca (Urucânia, Minas Gerais, Brazil). After the pretreatment stage using 1.5% NaOH,[12] the alkaline pretreated SCB was submitted to the enzymatic saccharification in microtubes with a working volume of 2 mL, using 15 FPase units of the commercial cocktail Multifect® CL per gram of biomass in 50 mmol/L sodium acetate buffer, pH 5.0 as positive control. The supplementation of the commercial cocktail was performed using 15 U/g of α-Ara1 or α-Ara2 isolated or in combination, where 7.5 U/g of each was used. The negative control was the same assay but using a sodium acetate buffer instead of the enzyme. The liquid fractions were collected after 72h, submitted to a boiling bath for denaturation of the enzymes, and centrifuged for 5 min at 16,000 g. The supernatants were analyzed by high-performance liquid chromatography.[6] For statistical analysis of the sugar release results, an analysis of variance (ANOVA) was used, followed by a Tukey test with a significance level equal to 5% (α = 0.05) using the GraphPad Prisma 6.01 software. The analyses were performed in triplicate and the standard deviation was calculated for all tests.

3 RESULTS & DISCUSSION

After the mass spectrometry and bioinformatics analyses (Tabe 1), the α-Ara1 was identified as α-L-arabinofuranosidase (EC 3.2.1.55) of the GH51 family with a molecular weight of 62 kDa (Table 1). Its C-terminal catalytic domain Alpha-L-AF_C is responsible for the hydrolysis of α-arabinofuranosidic bonds present in non-reducing ends of arabinan polymers.[13]

Table 1 Results of the identification and molecular characterization of α-arabinofuranosidases from *Chrysoporthe cubensis* carried out by mass spectrometry and bioinformatics analyses.

Characteristics	Enzymes	
	α-Ara1	**α-Ara2**
Databank Code	g6428	g11408
Molecular Mass (kDa)	62.0	52.9
Unique Peptides Number	4	5
Functional Domain	Alpha-L-AF_C (PF06964)	ArabFuran-catal (PF09206) / AbfB (PF05270)
Classification	GH51	GH54 / CBM42

Regarding the α-Ara2, it was identified as α-L-arabinofuranosidase of the GH54 family with a molecular weight of 52.9 kDa and presented a catalytic domain, known as ArabFuran-catal, and a noncatalytic domain, called AbfB (PF05270), corresponding to CBM42 (Table 1). The catalytic domain is responsible for catalyzing the hydrolysis of α-1,2, α-1,3-, and α-1,5-L-arabinofuranoside bonds present in hemicelluloses containing arabinose, such as arabinoxylan and arabinan.[14] On the other hand, the CBM42 is a protein domain whose function is to assist the catalytic domain in binding to their substrates, thereby improving catalytic efficiency.[15]

To investigate how these molecular differences between the two target enzymes influence the saccharification efficiency of lignocellulosic biomass, both a-arabinofuranosidases from *C. cubensis* were used, together or not, to supplement a commercial cocktail Multifect® CL in an alkaline pretreated sugarcane bagasse hydrolysis (Figure 1).

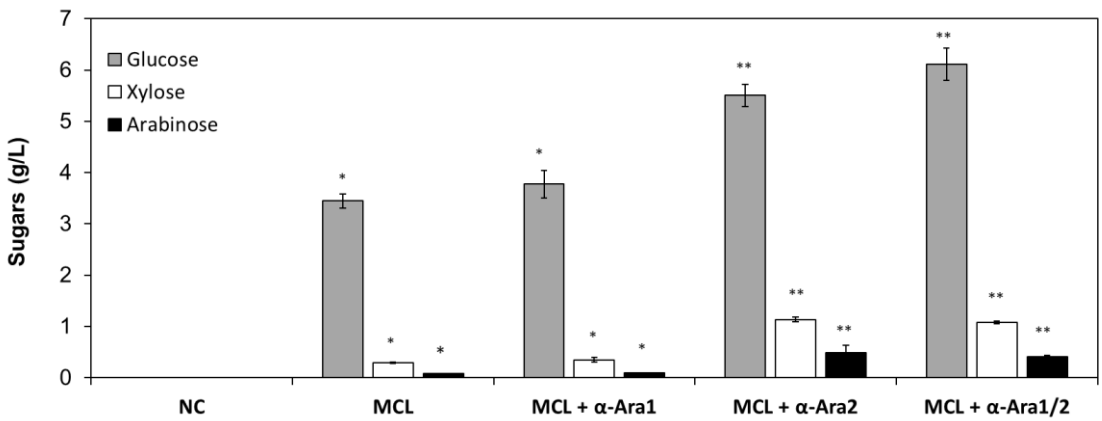

Figure 1 Production of glucose, xylose, and arabinose after 72h of the alkaline pretreated sugarcane bagasse saccharification using commercial cocktail Multifect CL (MCL) supplemented or not with α-Ara1, α-Ara2 or both target enzymes. The bars referring to the same sugar followed by the same symbol showed no significant difference in the analysis of variance (ANOVA) with Tukeys test, with α = 0.05.

The saccharification of alkaline pretreated sugarcane bagasse using only Multifect® CL (15 FPU/g) released 3.45 ± 0.14 $g.L^{-1}$ of glucose, 0.29 ± 0.01 $g.L^{-1}$ of xylose, and 0.08 ± 0.01 $g.L^{-1}$ of arabinose. The Multifect® CL supplementation with 15 U/g of α-Ara1 did not result in any significant improvement in the glucose (3.77 ± 0.27 $g.L^{-1}$), nor xylose (0.35 ± 0.05 $g.L^{-1}$) or arabinose (0.09 ± 0.01 $g.L^{-1}$) release after 72 h of hydrolysis. However, the supplementation with 15 U/g of α-Ara2 increased the glucose, xylose, and arabinose release by 1.6, 3.9, and 6.1 times, resulting in 5.51 ± 0.21 $g.L^{-1}$,1.13 ± 0.05 $g.L^{-1}$, and 0.49 ± 0.14 $g.L^{-1}$, respectively. These results indicate that the α-Ara1 had difficulty hydrolyzing complex biomass like the sugarcane bagasse. At the same time, the α-Ara2 increased the sugar release and its superior hydrolysis of hemicellulose may also have benefited the release of glucose from cellulose. GH51 enzymes exclusively break down small substrates, such as short-chain arabino-oligosaccharides. In contrast, GH54 enzymes demonstrate a broader capability by hydrolyzing small and polymeric substrates, such as arabinoxylans.[14] Since the CBM42 binds to L-arabinofuranosidic residues present in the substrate, increasing the access of the enzyme to insoluble polysaccharides, the better performance of α-Ara2 on alkaline pretreated sugarcane bagasse saccharification can be partially attributed to the presence of this protein domain. When α-Ara1 and α-Ara2 were applied together the glucose, xylose, and arabinose released were 6.11 ± 0.31 $g.L^{-1}$, 1.08 ± 0.03 $g.L^{-1}$, and 0.41 ± 0.03 $g.L^{-1}$, respectively. These results were similar to those presented by α-Ara2, even with the load of this enzyme reduced by half. Although there is a lack of synergy between enzymes from crude extracts from the same fungi [16], the results obtained in the present study show that there may be a combined action between α-Ara1 and α-Ara2 of *C. cubensis* on hydrolysis of lignocellulosic biomass.

4 CONCLUSION

The fungus *C. cubensis* produced two active α-arabinofuranosidases of the GH51 and GH54 families. Despite the promising results presented by α-Ara2, the supplementation using both target enzymes (α-Ara1 and α-Ara2) showed to be an interesting target for the biotechnology industry, especially to increase biomass saccharification efficiency with reduced enzyme load.

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