Aspergillus awamori endoglucanases promote faster lignocellulosic biomass liquefaction in high-solids enzymatic hydrolysis

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Abstract

Endoglucanases are necessary to improve high-solids enzymatic hydrolysis of lignocellulosic biomass by promoting liquefaction and decreasing the medium viscosity, alleviating one of the processes' major hindrances. In this study, endoglucanases produced by a particular strain of *Aspergillus awamori* were evaluated to speed up biomass liquefaction in reactions with 30% solids. Firstly, *A. awamori* crude supernatant (Aa) was assessed as a supplement to commercial enzymes, decreasing the media viscosity in 10-fold and improving glucose release by 20% after 24 h. Afterward, Aa was fractionated by size-exclusion chromatography and an endoglucanases-rich fraction was identified by liquid chromatography-mass spectrometry. This fraction was then supplemented to the most efficient commercial enzyme and its performance compared with the unfractionated Aa, resulting in the same improvement on medium viscosity and glucose release in 6 h. These data indicate that *A. awamori* endoglucanases have a powerful effect on the viscosity decrease during high-solids enzymatic hydrolysis.

Keywords: *Aspergillus awamori*; Endoglucanase; Enzymatic hydrolysis; High-solids loading; Liquefaction

1. Introduction

Enzymatic hydrolysis is the leading technology studied and commercially applied in biorefinery processes to convert cellulose into glucose. However, the enzymatic conversion of lignocellulosic biomass still has technical drawbacks for broader industrial implementation, including the economic feasibility of the hydrolysis process (Chandel, Garlapati, Singh, Antunes, & da Silva, 2018). In this sense, high-solids enzymatic hydrolysis has been pointed out to significantly increase the process economics by increasing the final product concentration, impacting both capital and operational costs, with a reduction in equipment volumes alongside the costs for downstream steps (A. S. A. Da Silva et al., 2020). In high-solids enzymatic hydrolysis, there is no free water in the slurry at the onset of the hydrolysis process, usually observed in solids loading higher than 15% (w/w) dry solids (A. S. A. Da Silva et al., 2020). However, regardless of its advantages compared to low-solids enzymatic hydrolysis, the industrial implementation of a high-solids process poses difficulties regarding mass transfer limitations, enzyme inhibition, and the impaired rheological properties of the reaction medium, which collectively hinder the hydrolysis process (González Quiroga, Costa, & MacIel Filho, 2010; Knutsen & Liberatore, 2010; Kristensen, Felby, & Jørgensen, 2009). In this scenario, the initial phase of high-solids enzymatic hydrolysis is challenging as the medium's lack of free water hampers an adequate mixing and increases energy expenditure (A. S. A. Da Silva et al., 2020; Humbird, Mohagheghi, Dowe, & Schell, 2010). This initial phase of enzymatic hydrolysis, known as "liquefaction," is responsible for the most pronounced changes in the biomass structure. Indeed, during this phase, the viscosity of the medium decreases due to the enzymatic breakdown of the fibers enabling a better medium flow and water release (Jørgensen, Vibe-Pedersen, Larsen, & Felby, 2007). Thus, an in-depth rheological analysis of the liquefaction phase, coupled with a better understanding of the enzymes that can be advantageous to improve liquefaction, is crucial for improving high-solids enzymatic hydrolysis.

The enzymatic hydrolysis of cellulose into glucose requires the activity of cellobiohydrolases (EC 3.2.1.74 and 3.2.1.91), endoglucanases (EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21). Endoglucanases catalytic attack occurs mostly internally on the amorphous part of the cellulose fiber, known as the cellulose's hydrophilic fraction (Chami Khazraji & Robert, 2013; Oksanen, Pere, Paavilainen, Buchert, & Viikari, 2000). The hydrolysis of the internal β-1,4 cellulose linkages results in the breakdown of cellulose and the loss of the fibers' water retention ability, culminating in the medium liquefaction (Szijártó, Siika-aho, Sontag-Strohm, & Viikari, 2011). Moreover, identifying new enzymes that better promote liquefaction is crucial for establishing high-solids processes in an industrial context.

A previous study from our research group indicated that endoglucanases from the culture supernatant of *Aspergillus awamori* (Aa) would possibly be responsible for the superior liquefaction observed during the hydrolysis of hydrothermally pretreated sugarcane bagasse (A. S. da Silva et al., 2016). The authors showed that the enzyme cocktail

containing Aa enzymes promoted higher solids solubilization during the first hours of hydrolysis when compared to commercial cocktails in high-solids enzymatic hydrolysis (A. S. da Silva et al., 2016). Indeed, thermostable endoglucanases from *A. awamori* have been reported, with a molecular mass in the range of 32 and 43 kDa (Nguyen & Quyen, 2010; Singh, Shukla, Khare, & Nain, 2011). However, no study has, so far, correlated the *A. awamori* endoglucanases to more efficient biomass liquefaction.

Thus, considering the importance of discovering new enzymes capable of leveraging biorefineries' implementation, this study evaluated the role of the *A. awamori* enzymes in the biomass liquefaction of high-solids enzymatic hydrolysis. Experiments were conducted with the crude fungus culture supernatant as well as an endoglucanases-rich fraction obtained via size-exclusion chromatographic fractionation of the culture supernatant. The study was divided into three stages: (1) a comparison of saccharification and liquefaction promoted by commercial enzymes and the lab-made cocktail containing *A. awamori* enzymes; (2) an evaluation of liquefaction by using *A. awamori* enzymes as a supplement in commercial cocktails; and (3) a study of *A. awamori* supernatant fractionation, aiming to separate proteins with different enzymatic activities, to identify the fraction rich in endoglucanases, and finally to evaluate the effect of this fraction on the liquefaction of pretreated sugarcane bagasse.

2. Materials and Methods

2.1 Source of materials and determination of the biomass chemical composition and enzymes

Hydrothermally pretreated sugarcane bagasse (190 ° C, 12 min), with 45.85% (±2.22%) of glucan, 1.8% (±0.08%) of xylan, 31.7% (±0.49%) of lignin and 16.37% (±3.53%) of ashes, was kindly supplied by Inbicon A/S (Kalundborg, Denmark). The chemical composition characterization was carried out according to the analytical protocol described by Sluiter et al. (2012).

The commercial enzymatic cocktail Cellic® CTec 2 used was acquired from Sigma-Aldrich (St. Louis, MO, EUA), Celluclast 1.5 L and Novozymes 188 used was kindly donated by Novozymes Latin America (Araucária, Brazil). *Trichoderma reesei* and *Aspergillus awamori* supernatant were obtained according to Silva et al. (2016). After production, the supernatants were filtered using glass fiber filters. The resulting filtrate was concentrated six times by ultrafiltration with polyethersulfone membrane modules of 1 m² of filtration area in the following conditions: cutting diameter of the membrane of 30 kDa, 0.5 bar of pressure, and 25 °C. After concentration, the *T. reesei* supernatant was stored at -20 °C for 48 h and lyophilized using the Christ Delta 2-24 LSC plus equipment (Osterode am Harz, Germany), producing a vacuum equivalent to the pressure of 1.8 bar.

2.2 Measurement of enzymatic activities and total protein content

The β-glucosidase activity was determined using the methodology described by IUPAC (Ghose, 1987) with

modifications, as 500 µL of the enzyme, properly diluted in 0.05M, pH 4.8 sodium citrate buffer, were reacted with 500 µL of a 15 mM cellobiose solution for 30 min at 50°C. The glucose concentration was then determined using the YSI 2700 Select Biochemistry Analyzer (Yellow Springs, OH, USA). One unit of β-glucosidase (BGU) was defined as the amount of enzyme that converted 1 µmol of cellobiose into glucose per minute at 50 ºC.

Filter paper activity (FPase) was measured according to the adapted IUPAC methodology described by Adney and Baker (1996) using 3,5-dinitrosalicylic acid (DNS) reagent (Sigma-Aldrich, Saint Louis, MO, EUA) prepared according to Teixeira et al. (2012). One filter-paper unit (FPU) was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugars (glucose equivalent) per minute, using an enzyme dilution providing 2 mg of glucose after a 60-minute assay reaction.

CMCase experiments were based on the methodology described by IUPAC (Ghose, 1987) and quantification of sugar release was determined using DNS reagent, prepared as described above. One unit of CMCase was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugars (glucose equivalent) per minute, using an enzyme dilution providing 0.5 mg of glucose after a 30-minute assay reaction. The enzymatic activities of the enzymes' formulations used in this study are presented in the Supplementary Table S1.

Total protein concentrations were determined using the bicinchoninic acid method kit from Sigma-Aldrich (Saint Louis, MO, EUA). The assay was incubated at 60 °C for 15 minutes.

2.3 Fractionation of *Aspergillus awamori* enzymes

Aspergillus awamori filtrate was eluted in a size-exclusion column to obtain protein fractions. The column was 65 cm high and 3 cm inner wide and was filled, according to Teixeira et al. (2010), with a Sephadex G-75 resin and sodium acetate buffer 0,05 M pH 5,0, containing sodium chloride 0.15 M. According to the manufacturer, GE Healthcare (Chicago, Illinois EUA), Sephadex G-75 resin separates the proteins with a molecular mass between 3 and 80 kDa. A flow of 20 mL/h was set, using a peristaltic pump (P-1, GE Healthcare, Chicago Illinois EUA). Ninety-five fractions of 4 mL were automatically collected in an AKTA fraction collector (GE Healthcare, Chicago, Illinois). The protein elution was monitored with a spectrophotometer at 280 nm.

2.4 Enzymatic activity of the protein fractions

The fractions which contained proteins were individually tested for enzymatic activities in micro assays adapted from Filho et al. (1993) to estimate the activity of endoglucanases, β- glucosidase and xylanase enzymes, using carboxymethylcellulose (CMC) (Sigma- Aldrich St. Louis, MO, EUA) (1%), 4-nitrophenol β-D-glucopyranose (pNPG) (Sigma-Aldrich St. Louis, MO, EUA), xylan beechwood (Sigma-Aldrich St. Louis, MO, EUA) (1%) as the substrate for each test, respectively. Then, to determine the relative activity of CMCase and xylanase, 100 µL of CMC or xylan was

combined to 50 µL of the collected fractions, incubated at 50 °C for 30 minutes. Afterward, 300 µL of DNS reagent (prepared as described before) was added, the tubes were boiled for 10 minutes, cooled in an ice bath, and then read in a spectrophotometer at 540 nm. For the β-glucosidase assays, 100 µL of the fractions was used along with 50 µL of pNPG and 350 µL of distilled water. The tubes were incubated for 10 minutes at 50 °C and the reaction was stopped by adding 1 mL of sodium carbonate (1M) to the reaction media. The samples were read in a spectrophotometer at 410 nm. After separating the 95 fractions and the analysis of enzymatic activities, the fractions were grouped according to the peaks of protein and enzyme activity observed, resulting in four main fractions.

2.5 Osmotic dialysis and lyophilization of the protein fractions

The cellulose membranes from Sigma-Aldrich (St. Louis, MO, EUA), D-5692, were affixed with two clips and filled with 10 mL of the four main fractions obtained from the size-exclusion chromatography and placed in a beaker, set with a strand and duct tape. The beaker was filled with 3 L of distilled water and placed inside a cold chamber under stirring, being the water changed every 30 min, for a total of 4 h.

After the dialysis, the four fractions were stored at -20 °C for 48 h and lyophilized using the Christ Delta 2-24 LSC plus equipment (Osterode am Harz, Germany), producing a vacuum equivalent to the pressure of 1.8 bar. The lyophilized samples were then analyzed in a liquid chromatography – mass spectrometer (LC-MS) and the selected samples were used in enzymatic hydrolysis assays.

2.6 Liquid chromatography - mass spectrometry analysis of the fractions of *Aspergillus awamori* supernatant

The four lyophilized fractions were re-suspended in 100 µL of urea/thiourea 7.0 M/2.0 M, respectively, to establish a denaturing environment for the proteins. Following that, protein concentration was determined using a Qubit 2.0® kit (Invitrogen, Carlsbad, CA, EUA). Samples of 80 µg of each fraction were taken. The digestion protocol was followed according to Nogueira et al. (2012), with some modifications, using 10 mM DTT at 30 °C for 1 h for the reducing reaction, 40 mM of iodoacetamide for 30 min in the dark at room temperature for the alkylation reaction and the trypsin enzyme (Promega, Madison, WI, USA) in a 1:50 (m/m) proportion for the digestion, which occurred at 35 °C for 18 h. After digestion, formic acid was added to reach a final concentration of 1% and the peptides were cleaned using reversed-phase chromatography with microcolumns Poros 20 R2 (PerSeptive Biosystems, Framingham, EUA), as described by Larsen et al. (2002), the peptides were then re-suspended in 0.1% solution of formic acid and measured with Qubit 2.0®.

The peptides were analyzed with an EASY II-nanoLC system (ThermoScientific, Waltham, MA, EUA) coupled with an LTQ Orbitrap Velos spectrometer (ThermoScientific, Waltham, MA, EUA). The peptides' elution happened through solvent gradient (0.1% formic acid, 95% acetonitrile) from 5-35% for 45 min, 35-95% for 10 min, and 95% for 5 min, in

a constant flow of 250 nL/min. The total time of analysis was 60 min. MS1 spectrum was obtained in positive mode, while the MS2 spectrum was obtained by data-dependent acquisition. Each full scan event constitutes an interval between 350-1800 m/z, resolution of 60.000 FWHM (for 400 m/z). To obtain the MS2 spectrum, the ten most intense ions were selected and fragmented using collision-induced dissociation (CID) with a normalized collision energy of 35, the minimum intensity of $1x10^4$, m/z isolation window 2, and dynamic exclusion list of 60 s. Chromatograms and spectra were visualized using the software Xcalibur v.2.1. The database search was done with Proteome Discoverer 2.1 (ThermoScientific, Waltham, MA, EUA) using the database of *Aspergillus awamori* in Uniprot (http://uniprot.org/). Only proteins identified with at least one unique peptide, high confidence (FDR < 0.01 at peptide and protein level), and present in two replicates were considered in this study.

2.7 Electrophoresis gel

A 12% SDS-PAGE was prepared according to Laemmli (1970). Samples containing 50 μg of protein were mixed to the sample buffer (200 mM of Tris-HCl, pH 6,8), 8% of SDS, 0.4% of bromophenol blue, 40% of glycerol, 400 mM of beta-mercaptoethanol in 1:3 proportion (buffer:sample). The current was adjusted to 15 mA (for each plate) and subsequently to 20 mA after the aligned samples reached the separating gel. After the run, the gel was stirred for approximately 2 min. The water was removed, and the gel coloring solution was added (2.5 g of Coomassie blue, 450 mL of methanol, 100 mL of glacial acetic acid, and 450 mL of water). The solution was left overnight with the gel. The gel coloring solution was removed on the following day, and a destaining solution was added (450 mL of methanol, 100 mL of glacial acetic acid, and 450 mL of water).

2.8 Enzymatic hydrolysis

Enzymatic hydrolysis assays were carried out using 30% (w/w) of hydrothermally pretreated sugarcane bagasse. The biomass (1.5 g of dry mass) and the pertinent enzyme cocktail were added with an enzyme load of 20 FPU/g of cellulose and a minimum proportion of FPU:BGU of 1:3 to sodium citrate buffer 50 mM pH 4,8 in 25 mL flasks with a lid, with a total reaction mass of 5 g. For the experiments with the endoglucanase-rich fraction from *A. awamori* supernatant reduced assays were conducted, where the biomass (0.75 g of dry mass) and the pertinent enzyme cocktail were added to sodium citrate buffer 50 mM pH 4,8 in 25 mL flasks with a lid, with a total reaction mass of 2.5 g. The total amount of proteins loaded in each reaction for this set of experiments was 9.71 mg for CT2Aa and 8.25 mg for fraction F3.

All enzymatic hydrolysis assays were conducted in duplicate at 50 °C and 200 rpm for 24 h. Independent flasks were set for the analysis of each reaction time. The reactions were stopped after 3, 6, 9, and 24 h by submersion of the flask in boiling water for 5 minutes. Samples were taken after distilled water was added to the flasks to dilute the sample four times, considering the initial reaction volume, and its content was vigorously mixed. This step was necessary due to the

difficulty to aliquot a homogenous liquid fraction given that the sugars formed during high-solids hydrolysis may concentrate in different locations of the sample. After that, glucose quantification was carried out by high-performance liquid chromatography (HPLC). All the results were statistically compared using Tukey's test ($p<0.05$).

The yield of cellulose conversion into glucose was calculated with Equation 1 (Eq. 1):

$$Glucose\ yield\ (\%) = \frac{(C_{glucose} - C_{glucose0})}{1.111 \left(\frac{W_t}{V_{h0}}\right) F_{ins0}\ F_{glucan}} \times 100 \qquad \text{Eq. 1}$$

Where $C_{glucose}$ is the glucose concentration in the hydrolysate (g/L); $C_{glucose0}$ is the initial glucose concentration in the hydrolysis experiment; W_t is the total mass in the hydrolysis assay (g); V_{h0} is the initial liquid volume; F_{ins0} is the solid mass fraction of insoluble solids in the hydrolysis assay; F_{glucan} is the glucan mass fraction in insoluble solids. V_{h0} is the volume that corresponds to the initial mass (g) of liquid that was added in the hydrolysis assay.

In parallel, hydrolysis assays were conducted to provide samples for the viscosity analysis as explained above, but using 50 mL flasks with lid, 3 g of bagasse in a total reaction mass of 10 g. Each flask's entire reaction medium was transferred to plastic recipients with lid and reserved at 4 °C for viscosity analysis.

2.9 Glucose quantification

The analyses were carried out either with a 2700 SELECT biochemical analyzer or by a Shimadzu (Shimadzu Corporation, Kyoto, Japan) HPLC system equipped with a refractive index detector (Model RID-10A) The chromatography column used was Aminex HPX-87H (300 mm X 7.8 mm X 9 mm, Bio-Rad Laboratories, Hercules, CA, USA), equipped with a Carbo-H precolumn (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase used was 5 mM sulfuric acid at a flow rate of 0.6 mL/min with an oven temperature of 55 °C and a detector temperature of 55 °C.

2.10 Viscosity measurements

Viscosity analyses were carried out according to Stickel et al. (2009), using an Anton Paar rheometer, model MCR 302 (Anton Paar GmbH, Graz, Austria) equipped with plate-plate geometry with 20 mm diameter and 4 mm gap at 50 °C and with Peltier system and thermostabilized water bath Tecnal TE 2015 (Piracicaba, Brazil) to control sample temperature. The samples' viscosity was determined through rotation testing concerning the shear rate, as controlled shear rate (CSR) type. The shear rate interval ranged between 1-100 s^{-1}. Samples were placed in a water bath at 50 °C for 5 minutes before each analysis, homogenized and put in the rheometer, covering all the equipment base and filling the plates' gap. Data were extracted using Rheoplus/32 software (Anton Paar GmbH, Graz, Austria). Flow behavior was analyzed according to Equation 2 from the Newtonian model (Eq. 2):

$$\tau = \eta\gamma \qquad\qquad\qquad \text{Eq. 2}$$

In which τ is shear stress (Pa), η represents viscosity (Pa.s) and γ is the shear rate (s^{-1}).

3. Results and Discussion

3.1 Effect of the different enzymatic cocktails in biomass liquefaction

Hydrothermally pretreated sugarcane bagasse, obtained from Inbicon A/S (Kalundborg, Denmark), was subjected to enzymatic hydrolysis with 30% of solids (w/w) using three enzymatic cocktails: Cellic® CTec2 (CT2), a blend of Celluclast 1.5L and Novozymes 188 (CEL188), and the lab-made enzymes cocktail resulting from the mixture of *T. reesei* and *A. awamori* supernatants (TrAa) (Gottschalk, Oliveira, & Bon, 2010; A. S. da Silva et al., 2016). CT2 has high total cellulase and β-glucosidase activities besides lytic polysaccharides monooxygenases (LPMOs) to intensify the hydrolysis of crystalline cellulose. Celluclast 1.5L is a commercial *T. reesei* cellulase usually supplemented with Novozymes 188, an *Aspergillus niger* cocktail rich in β-glucosidase. The lab-made preparation was composed of *T. reesei* supernatant, rich in total cellulases activity, supplemented with *A. awamori* supernatant, rich in β-glucosidase (Gottschalk et al., 2010; A. S. da Silva et al., 2016).

The enzymatic cocktails' comparative performance was first evaluated regarding their ability to release glucose, and the results are presented in Figure 1. The use of CT2 and TrAa resulted in similar performances, producing, respectively, 115 g/L and 114 g/L of glucose within 24 h of hydrolysis, which corresponded to 52% glucose yield. This result agrees with the data reported in the previous study from our group, in which the CT2 cocktail had only a slightly better performance when compared with TrAa in 24 h of enzymatic hydrolysis of 20% hydrothermally pretreated bagasse (A. S. da Silva et al., 2016). However, in this same study, TrAa showed a better performance within 6 h of hydrolysis when compared to CT2. This result was not observed in the present study, which could be related to higher initial solids loading (30% w/w). The hydrolysis time course of CEL188 plateaued within 9 h of hydrolysis and resulted in the lowest glucose accumulation in 24 h, of 54 g/L, corresponding to 24% glucose yield (Figure 1). Cara et al. (2007) reported similar results when using the CEL188 cocktail, reaching a glucose concentration of around 50 g/L within 24 h of enzymatic hydrolysis of liquid hot water-pretreated olive tree pruning biomass at 30% of solids. The low hydrolysis performance of CEL188 could be related to product inhibition, as an inhibitory effect by glucose on CEL188 was observed by Kristensen et al. (2009) when 50 g/L of exogenous glucose was added in the hydrolysis medium of 20% filter paper. Additionally, CEL188 has been reported to be prone to product inhibition resulting from increased solids loading, a phenomenon not observed in saccharification assays with CT2 (Weiss, Felby, & Thygesen, 2019).

The corresponding results for the rheological analysis are presented in Figure 2. In the absence of enzymes (control experiment), the pretreated sugarcane bagasse showed a shear-thinning flow behavior, with the viscosity decreasing with the increase of shear rate, which was also observed for other lignocellulosic materials (Stickel et al., 2009; Wiman, Palmqvist, Tornberg, & Lidén, 2011). At a shear rate of 1 s^{-1}, the measured viscosity was approximately 1000 Pa.s,

similar to that reported for pretreated switchgrass at 30% solids loading (Cruz et al., 2013). The enzyme preparations tested promoted different results, as clearly seen by the effect of the 1 s^{-1} shear rate on viscosity for the samples taken at 3, 6, 9, and 24 h. The medium viscosity decreased from 1340 to 104 Pa.s for CEL188; 408 to 13.9 Pa.s for CT2; and 598 to 2.2 Pa.s for TrAa, for measurements done between 3 and 24 h of hydrolysis at a shear rate of 1 s^{-1} (Figure 2). Results indicated that the liquefaction stage with TrAa was more prominent in the first 9 h of hydrolysis as the viscosity decreased two orders of magnitude within this time interval compared to that from 9 to 24 h (Figure 2). These results agree with a previous report that identified the first 8-10 h of hydrolysis as the most significant for the decrease in the reaction medium viscosity (Wiman et al., 2011). The prominent viscosity decrease promoted by TrAa action after 6-9 h was also perceived visually, presented in the Supplementary Figure S1, as the medium was mostly formed by a thick liquid with good flowability, containing only a few nodes compared to the presence of coarse material at the beginning of the hydrolysis.

TrAa promoted a higher reduction on viscosity than CT2 and CEL188, clearly observed in 9 and 24 h of enzymatic hydrolysis (Figure 2). Even though CT2 provided a higher glucose release in the first 9 h of hydrolysis (Figure 1), viscosity reduction was not the highest. Indeed, TrAa liquefied more efficiently the medium, indicating that liquefaction is not necessarily translated into higher saccharification and vice versa. After 24 h of hydrolysis, the medium with TrAa was around 15 times more flowable than CT2, although the same amount of glucose was released for both preparations. Thus, TrAa is a cocktail that effectively reduces the viscosity and produces high concentrations of glucose, being advantageous for the high-solids hydrolysis at industrial scale, since this could represent a significant economy in mixing power, biomass handling, and pumping (Knutsen & Liberatore, 2010). This lack of correlation between liquefaction and saccharification was also observed in a study that evaluated the enzymatic hydrolysis of wheat straw hydrothermally pretreated using different purified enzymes. According to the authors, the weak correlation between liquefaction and saccharification is explained because these two phenomena are related to different enzymatic classes (Skovgaard et al., 2014).

According to the literature, the main enzymes responsible for liquefaction are endoglucanases (Szijártó et al., 2011). Consequently, it is possible to infer that the efficiency observed in the liquefaction promoted by TrAa could be related to a mixture of endoglucanases present in this enzymatic cocktail as both *T. reesei* and *A. awamori* are reported as endoglucanase producers (Gottschalk et al., 2010; S. Petrova, Bakalova, Benadova, & Kolev, 1995; Singh et al., 2011). However, CEL188, which is derived from *T. reesei*, promoted unsatisfactory medium liquefaction. Thus, it was hypothesized that the TrAa liquefaction effect would probably be related to the endoglucanases of *A. awamori*, as suggested in the previous work from our group (A. S. da Silva et al., 2016). Thus, to test this hypothesis, Aa supernatant

was supplemented to the commercial enzyme preparations to test its ability to promote better liquefaction in high-solids enzymatic hydrolysis.

3.2 Effect of *Aspergillus awamori* culture supernatant on improving the liquefaction ability of Celluclast + Novozyme 188 and Cellic® CTec 2

The commercial enzymatic cocktails CT2 and CEL188 were supplemented with the same volume of Aa supernatant used in the lab-made cocktail TrAa, resulting in the mixtures CT2Aa and CEL188Aa. The relation between the total units of cellulases and beta-glucosidase (FPU:BGU ratio) for CT2Aa in comparison to CT2 changed only slightly, from 1:51 to 1:54, due to the high β-glucosidase activity background of CT2. For CEL188Aa, the FPU:BGU ratio doubled, from 1:3 to 1:6 in comparison to CEL188; although the increase in β-glucosidase activity could impact glucose yields, this should not impact the medium viscosity, as there is no reported correlation between β-glucosidase activity and viscosity decrease in enzymatic hydrolysis. The results related to the glucose release and the dynamic viscosity of the hydrolysis reaction media are shown in Figure 3.

As presented in Figure 3A, the Aa supplementation resulted in a positive effect in the glucose released from 3 h onwards for CEL188Aa and 6 h for CT2Aa. Moreover, an increase in glucose production, of 40 and 20 g/L for CT2Aa and CEL188Aa, respectively, was observed in comparison to the results obtained with CT2 and CEL188 after 24 h of hydrolysis. No noticeable change in viscosity was observed for Aa supplementation of CEL188 and CT2 up to 6 h of enzymatic hydrolysis (Figure 3B). However, after 9 h of hydrolysis, CT2Aa promoted better liquefaction (Figure 3C), and with a similar performance observed for the TrAa preparation. Additionally, Aa supplementation resulted in a 10-fold increase in the media flowability after 24 h of hydrolysis for both enzyme mixtures (Figure 3D).

The effect of endoglucanases from other *Aspergillus* sp. on liquefaction and glucose production has also been reported, resulting in the reduction of carboxymethylcellulose viscosity (Dobrev & Zhekova, 2012). In other studies, the supplementation of Celluclast 1.5 L with a purified endoglucanase from *A. fumigatus* increased the release of sugars during the enzymatic hydrolysis of several lignocellulosic materials, including pretreated sugarcane bagasse (Bernardi et al., 2018; Bernardi, Yonamine, Uyemura, & Dinamarco, 2019).

Interestingly, the effect of Aa supplementation observed in both commercial cocktails was different. The glucose release by CEL188Aa was increased from the hydrolysis onset onwards in comparison to CEL188, and liquefaction improvements were observed at a later hydrolysis stage, indicating a delayed product inhibition effect, as discussed above, by the addition of more units of β-glucosidase from Aa. Regarding the performance of CT2Aa, the increase in glucose release was only observed after 9 h, when pronounced liquefaction resulted in the decrease of mass transfer limitation, facilitating the access of enzymes to the substrate. Corroborating our observations,

11

Weiss et al. (2019) have shown that CEL188 is highly susceptible to product inhibition, different from CT2, which is mainly affected by mass transfer limitation.

Thus, aiming to better correlate the positive effect of endoglucanases from *A. awamori* supernatant in the high- solids enzymatic hydrolysis, the fungus culture supernatant was subjected to a protein fractionation process for the isolation of an endoglucanase-rich fraction.

3.3 *Aspergillus awamori* culture supernatant fractionation and proteins identification

The *A. awamori* culture supernatant was subjected to fractionation by size-exclusion chromatography to isolate an endoglucanase-rich fraction. Sephadex G75, which excludes proteins with a molecular mass higher than 80 kDa, was chosen for protein fractionation as *A. awamori* endoglucanases usually present molecular mass of around 32 and 43 kDa (Nguyen & Quyen, 2010; Singh et al., 2011). Ninety-five fractions were obtained after the elution of the Aa supernatant sample through the size-exclusion chromatography column and were monitored in the spectrophotometer to identify protein peaks qualitatively. After this, the fractions that indicated proteins' presence were subjected to three assays to semi-quantitively identify the enzymatic activities related to endoglucanases, β-glucosidases, and xylanases. Figure 4 presents the protein profile and the relative enzymatic activities measured after the *A. awamori* supernatant's fractionation using the Sephadex G-75 resin. The estimation of protein at 280 nm and measurement of enzymes activities in the eluted samples allowed the identification of fractions F1, F2, F3, F4, and F5. Tubes corresponding to these fractions were pooled and reserved for electrophoresis and mass spectrometry analysis.

The eluted samples of F1 contained proteins active for the hydrolysis of *p*-Nitrophenyl-β-D-glucopyranoside (pNPG) and xylan, indicating the presence of β-glucosidases and xylanases. The F2 peak showed residual activities of β-glucosidase, xylanase, and endoglucanase. The F3 peak showed xylan and carboxymethylcellulose (CMC) activity, indicating the presence of xylanases and endoglucanases, while F4 showed a residual activity towards xylan. No enzyme activity was detected in F5. Likely, the high absorbance at 280 nm of F4 and mostly F5 fractions does not correspond to proteins' presence, but rather to the elution of dark pigments from the supernatant.

Due to the absence of enzymatic activity, F5 was disregarded for further analysis. Electrophoresis analyses were conducted for fractions F1, F2, F3, and F4 and it is presented in the Supplementary Figure S2. The most abundant proteins from F1 and F2 had molecular masses higher than 35 kDa, while in F3, the most abundant proteins had a molecular mass ranging from 35 to 14.8 kDa. Finally, this analysis confirmed the initial hypothesis that the high absorbance measured at 280 nm for F4 was not related to proteins, but probably to the presence of pigments, as only very light stains were observed in the gel for this sample. The overall results from the electrophoresis analysis showed the fractionation's efficiency and indicated the presence of endoglucanases within molecular masses lower than 35 kDa.

Each fraction of the *A. awamori* supernatant was assessed by LC-MS and the detailed data are presented in the Supplementary Table S2. A total of 85, 29, 30, and 5 proteins were identified in the fractions F1, F2, F3, and F4, respectively. Nevertheless, only a few proteins of each fraction were associated with the enzymatic hydrolysis of cellulose and xylan, as showed in Table 1. Fraction F1 contained three proteins with β-glucosidase and one with β-xylosidase activity. In the fraction F2, only one β-glucosidase was identified, and, in the fraction F3, four proteins with endoglucanase, one with xylanase, two with β-xylosidase, and one with β-glucosidase activities were identified. Regarding the residual activity in xylan observed in F4, two xylanases were identified in this fraction's data; however, they were not listed in the final analysis, as they were only identified in one replicate, probably as a result of the low abundance of this protein, and thus, not meeting the parameters set for protein identification in mass spectrometry analysis. Also, one of the five proteins identified in F4 was an alpha-L-arabinofuranosidase axhA, also identified in F3. However, the pretreated biomass used in this study had no detectable residues of arabinose in its composition, indicating that these enzymes have probably no effect in our experiments.

In F1, three β-glucosidases were identified, which correlated well to the enzymatic activity against *p*NPG detected in this fraction (Table 1). The β-glucosidase (A0A401KS37) identified had an estimated molecular mass of 94 kDa, which differs from other β-glucosidases reported for *A. awamori*, of 180 kDa and 116 kDa (Nishida et al., 2018; S. D. Petrova, Andreev, Bakalova, Benadova, & Kolev, 2002). Besides the β-glucosidases, a β-xylosidase and a xyloglucanase, enzymes involved in xylans' hydrolysis, were identified in F1 (Sporck et al., 2017). The presence of xylanases has been associated with beneficial effects in high-solids enzymatic hydrolysis (Hu et al., 2015); however, the low amount of xylose in the hydrothermally pretreated sugarcane bagasse used in this study (1.8%) indicates that those enzymes would have a minor impact, if any, in our experiments. Although β-glucosidases and xylanases are relevant enzymes for the enzymatic hydrolysis, F1 was not considered for further evaluation, as no endoglucanase was identified in this fraction.

In F2, only one protein was associated with the enzymatic hydrolysis of cellulose (Table 1), a β-glucosidase, which was the same identified in F1 and F3, probably due to the high abundance of this protein. The same happened with a glucoamylase, present in all the fractions. The abundance based on the exponentially modified protein abundance index (emPAI) of a glucoamylase in F2 was higher than the other identified proteins, indicating that F2 was mainly composed of amylases. As F2 contained no endoglucanases, this fraction was not considered for further experiments.

In F3, three proteins were associated with the hydrolysis of xylans and five were related to the hydrolysis of cellulose, being four endoglucanases (Table 1), which correlates well to the highest relative activity in xylan and CMC measured in this fraction (Figure 4). These endoglucanases were exclusively detected in this fraction; endoglucanase A had the highest quantitative value based on emPAI, being the second most abundant protein in this fraction after an

uncharacterized protein. Additionally, a defined band in 25 kDa was observed in the electrophoretic gel, the same molecular mass of the endoglucanase A found in the LC-MS analysis. Another enzyme with a high emPAI number was a β-xylosidase, with a molecular mass of 34 kDa, which also corroborated the band observed in the electrophoretic gel. Proteins corresponding to the other endoglucanases could not be associated with the data from the gel, which could be explained by their low abundance in the sample and the low sensitivity of the electrophoresis in comparison to LC-MS analysis. Another class of enzyme identified in F3 was feruloyl esterases. This result agrees with a previous study that observed ferulic acid esterase activity in *A. awamori* culture supernatant (Gottschalk et al., 2010). Feruloyl esterases have been associated with the enhancement of biomass degradation, mainly due to its synergy with xylanases (Katsimpouras, Antonopoulou, Christakopoulos, & Topakas, 2016). However, as discussed, the low xylan content in the biomass used in this study suggests a low influence of those enzymes in our experiments.

Comparatively, Singh et al. (2011) purified a thermostable endoglucanase from *A. awamori* culture supernatant with a molecular mass of 43 kDa, similar to endoglucanase-4 and endoglucanase E1 identified here. Another study isolated an endoglucanase from *A. awamori* with similar molecular mass (26-28 kDa) to the endoglucanase A presented in Table 1 (Dobrev & Zhekova, 2012). The presence of low molecular mass endoglucanases in the *A. awamori* supernatant is probably an advantage as it could facilitate its access into the lignocellulosic structure and improve the cellulose hydrolysis, as it is observed in low-molecular-mass xylanases during biomass hydrolysis (Sutay Kocabaş, Güder, & Özben, 2015). As four endoglucanases were identified in F3, we selected this fraction to evaluate its effect in liquefaction during high-solids enzymatic hydrolysis of pretreated sugarcane bagasse in comparison to the complete supernatant.

3.4 Effect of the endoglucanase-rich fraction of *Aspergillus awamori* in liquefaction

Table 2 shows the supplementation effect of Cellic® CTec 2 with F3 (CT2F3) on the glucose production over a 24 h period and the viscosity of the medium after 6 h of hydrolysis, comparing with the use of Cellic® CTec 2 alone and supplemented with the unfractionated supernatant of *A. awamori* (CT2Aa).

Both CT2Aa or CT2F3 resulted in similar glucose production within 3 h and 6 h, with a statistically significant increase of glucose release, of approximately 10 g/L, compared to the performance of CT2. This increase is in agreement with the hypothesis proposed by Silva et al. (2016), who observed better solubilization of solids in the first 6 h of hydrolysis using the lab-made enzymatic cocktail TrAa, indicating that the effect observed was due to the presence of endoglucanases in the supernatant of *A. awamori*. At 9 h and 24 h of enzymatic hydrolysis, the performance of CT2Aa continued to be superior compared to CT2, while CT2F3 produced the same amount of glucose as CT2. These results indicate that the supplementation with the fraction F3 of the enzymatic supernatant of *A. awamori* increased glucose release only in the initial enzymatic hydrolysis stages. In contrast, the supplementation with the whole supernatant resulted in continuous

improvement of the enzymatic hydrolysis at high-solids content. One explanation could be due to the losses in enzymatic activity and protein denaturation that inevitably occur during fractionation, and thus, the effect observed in Aa could not be reproduced in F3. Alternatively, the improvement observed in the saccharification could be provided by endoglucanases in synergy with other crude supernatant proteins.

When evaluating the dynamic viscosity profile, the hydrolysates produced by the action of CT2Aa and CT2F3 had the same profile. At the same time, the use of CT2 resulted in samples with a slightly higher viscosity compared to the other two enzymatic cocktails, indicating that endoglucanases of *A. awamori* are involved in the viscosity reduction observed for the TrAa preparation (Figure 2). In parallel, CMCase enzymatic activity was used to infer endoglucanases' activity; curiously, CT2 had 1000-fold more CMCase activity than Aa. Considering the total volume added of each of these cocktails to the experiments, CT2 contributed with about 384 U of CMCase/g of biomass while Aa accounted for 9.7 U of CMCase/g of biomass. Altogether, these results indicate that the probable endoglucanases of *A. awamori* involved in the improvement of high-solids enzymatic hydrolysis have characteristics that provide an advantage over the endoglucanases present in CT2. However, further assays to purify, characterize, and compare these enzymes would be required.

4. Conclusion

Interestingly, our results suggested that different endoglucanases have different effects on liquefaction. The endoglucanases present in the *A. awamori* pool could promote faster liquefaction when compared to the Cellic® CTec 2 endoglucanases. These data suggest that *A. awamori* endoglucanases have a distinctive feature that improves the liquefaction during high-solids enzymatic hydrolysis. Future studies to characterize the endoglucanases identified in the F3 fraction of the *A. awamori* supernatant will contribute to elucidate the characteristics of these enzymes that boost the liquefaction of pretreated sugarcane bagasse in assays with a high content of solids.

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Tables

Table 1. Enzymes related to the enzymatic hydrolysis of cellulose and xylan identified in F1, F2, and F3 fraction of *A. awamori* supernatant.

Fraction	Access number	Protein	Estimated molecular mass (kDa)	Relative abundance (emPAI number)
F1	A0A401KS37	β-glucosidase	94	5.4
	A0A401L4C3	β-xylosidase	87	3.7
	A0A401KZ55	periplasmic β-glucosidase	88	0.8
	A0A401KLT2	probable β-glucosidase M	82	0.2
F2	A0A401KS37	β-glucosidase	94	1.0
F3	A0A401KJH0	endoglucanase A	25	18.3
	A0A401L2J5	β-xylosidase	34	16.3
	A0A401L2H3	endoglucanase E1	45	3.0
	A0A401L630	endo-1,4- β-xylanase	24	1.7
	A0A401KU32	endoglucanase-4	41	1.1
	A0A401L877	probable endo-β-1,4-glucanase D	36	1.0
	A0A401KS37	β-glucosidase	94	0.4
	A0A401L4C3	β-xylosidase	87	0.2

Table 2. Glucose concentration profile during 24 h of enzymatic hydrolysis and dynamic viscosity with a shear rate of 1 s^{-1} of hydrolysates obtained after 6 h of hydrolysis of 30% hydrothermally pretreated sugarcane bagasse (initial total solids - dry matter) using three enzymatic preparations: Cellic® CTec 2 (CT2); Cellic® CTec 2+Aa (CT2Aa) and Cellic® CTec 2+F3 (CT2F3).

Enzyme	Glucose concentration (g/L)				Viscosity (Pa.s)
	3 h	6 h	9 h	24 h	6 h
CT2	29.8†	38.9†	55.6†	82.2†	326
CT2Aa	41.2‡	50.6‡	68.2‡	96.3‡	179
CT2F3	37.9‡	47.4‡	61.6§	86.2†	143

†, ‡, §: Statistically different

Figure legends

Figure 1. Glucose concentration profile obtained during 24 h of enzymatic hydrolysis of 30% hydrothermally pretreated sugarcane bagasse (initial total solids - dry matter) using (▲) Cellic® CTec 2, (●) TrAa and (■) Celluclast + Novozyme 188.

Figure 2. Dynamic viscosity, using shear rates of 1-100 s^{-1} for 3 h (A), 6 h (B), 9 h (C), and 24 h (D) of the enzymatic hydrolysis of 30% (w/w) hydrothermally pretreated sugarcane bagasse. The figure depicts results for the use of the enzymes preparations (□) Celluclast + Novozyme 188; (Δ) Cellic® CTec 2 and (○) TrAa and for the control experiment carried out in the absence of enzymes (◊).

Figure 3. (A) Glucose concentration profile during 24 h of enzymatic hydrolysis of 30% hydrothermally pretreated sugarcane bagasse (initial total solids - dry matter) using four enzymatic preparations: (□) Celluclast + Novozyme 188; (■) Celluclast + Novozyme 188 + Aa; (Δ) Cellic® CTec 2 and (▲) Cellic® CTec 2 + Aa. Dynamic viscosity, using shear rates of 1-100 s^{-1} for 6 h (B), 9 h (C), and 24 h (D) of the enzymatic hydrolysis of 30% (w/w) hydrothermally pretreated sugarcane bagasse. The figure depicts results for the use of the enzymes preparations (□) Celluclast + Novozyme 188; (■) Celluclast + Novozyme 188 + Aa; (Δ) Cellic® CTec 2; (▲) Cellic® CTec 2 + Aa and for the control experiment carried out in the absence of enzymes (◊).

Figure 4. Protein and enzymes activity profile from the fractionation of *Aspergillus awamori* culture supernatant in Sephadex G-75. Absorbance at 280 nm (○); β-glucosidase activity (■); xylanase activity (♦) and endoglucanase activity (▲). Tubes corresponding to fractions F1, F2, F3, F4, and F5 were pooled and reserved for further analysis.