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Cellulose-degrading enzyme production by *Clonostachys byssicola*: Partial purification and characterization of an endoglucanase

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Abstract

Agro-industrial wastes offer potential as renewable carbon sources for microbial growth and industrial enzyme production, with potential applications in textile and biofuel industries, among others. The filamentous fungus *Clonostachys byssicola* was cultivated for seven days in liquid medium containing 1% (w/v) soybean hulls as unique carbon source. Hemicellulase, cellulase and pectinase activities were observed in the crude extract after seven days growth. A CMCase, denominated Cb-Cel, was partially purified using ultrafiltration and chromatographic methods. With a molecular weight of 48 kDa, the enzyme was most active at 70°C and pH 5.0, and thermostable at 40 and 50°C. Cb-Cel showed K_m and V_{max} values of 15.81 ± 1.65 mg/mL and 0.59 ± 0.03 IU/mL, respectively. Phenolic compounds (tannic, 4-hydroxybenzoic, ferulic, ρ-coumaric, cinnamic acids and vanillin) had no inhibitory or deactivator effects on enzymatic activity when tested on the concentrated fraction or Cb-Cel. Enzymatic hydrolysis of CMC and filter paper hydrolysis resulted in the release of glucose, cellopentaose and cellohexaose. Enzymatic hydrolysis of soybean hulls released mostly cellohexaose, cellopentase, mannose and xylobiose. In comparison to hydrolysis of sugarcane bagasse, the hydrolysis of soybean hulls yielded higher amounts of reducing sugars, suggesting that the enzymes secreted during growth of *C. byssicola* are more active on this substrate as carbon source.

Keywords - Cellulase - Enzymatic Hydrolysis – Phenolic Compounds - Soybean Hulls

Introduction

Concern over the depletion of fossil fuel resources and climate change attributed to anthropogenic carbon dioxide emissions has led to considerable global interest in the exploitation of renewable and carbon-neutral energy sources (Doherty et al. 2011). Among available energy sources, non-food lignocellulosic residues such as sugarcane bagasse, corn cob, soybean hulls, oat hulls and sorghum straws offer considerable potential as renewable carbon-sources.

Soybean hull, a by-product in processing, is an external layer of the grain that constitutes approximately 8% of the whole seed (Gnanasambandan & Proctor 1999). Processed soybean is the world's largest source of animal protein feed and the second largest source of vegetable oil (USDA 2015). Considering the scale of global production, residues from this crop are expressive in volume,

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and offer potential as growth substrates for lignocellulolytic microorganisms and subsequent production of industrial enzymes.

Lignocellulosic biomass is highly recalcitrant to enzymatic degradation, which hinders enzyme access to the biomass. For this reason, the application of a biomass-disrupting pretreatment is often employed to increase enzyme access. Many of the pretreatments employed are performed under conditions that result in the formation of phenolic compounds from the degradation of lignin, which can have inhibitory effects on enzymes and/or fermentative organisms (Ximenes et al. 2010, Li et al. 2014). Efficient enzyme hydrolysis and reduced enzyme loading therefore depends upon a reduction of inhibition or deactivation effects on enzymes (Duarte et al. 2012).

The biotechnological application of fungal enzymes for agricultural waste degradation adds value to raw materials in an environmentally friendly manner (Siqueira 2010). Microbial cellulases are employed in the food, pulp and paper, feed and textile industries, as well as in the formulation of detergents and washing powders (Bhat 2000). In addition, these enzymes can be applied in the hydrolysis of lignocellulosic biomass to bioethanol, contributing towards sustainable development. Enzymatic hydrolysis of lignocellulosic biomass is considered as the most efficient and least polluting method for glucose production (Singhania et al. 2015). Endoglucanases act randomly on soluble and insoluble cellulose chains, releasing oligomers that will be attacked by cellobiohydrolases and liberating cellobiose. Cellobiose is subsequently converted to glucose by β-glucosidase (Moreira et al. 2011), with glucose then converted to ethanol by fermenting microorganisms. Considering that enzymatic hydrolysis followed by fermentation is the principal approach employed for second generation ethanol production from lignocellulosic biomass (Olofsson et al. 2008), there is increasing interest in the characterization of efficient enzymes for use in such applications.

The fungus *Clonostachys byssicola*, as well as its teleomorph *Bionectria byssicola*, is commonly found in neotropical regions. Although this fungus is known for its potential as a biological control agent against plant pathogenic fungi such as *Moniliophthora roreri* and *Rosellinia* spp. (Evans et al. 2003, García et al. 2003, Hoopen et al. 2003, Krauss et al. 2006, Alvindia & Natsuaki 2008), there are no reports focusing on its ability to produce lignocellulose-degrading enzymes, including cellulase. In this study, we employed soybean hull lignocellulosic biomass as a carbon source for the submerged cultivation of C. *byssicola* and production of cellulolytic enzymes. To the best of our knowledge, this is the first report on the production and characterization of cellulases in *C. byssicola*.

Materials and methods

Molecular characterization

The fungal isolate was identified through molecular characterization. For molecular identification, genomic DNA was extracted using a phenol-chloroform-based protocol. The ITS region of the rRNA gene was amplified using the forward primers ITS1 (5' - TCCGTAGGTGAACCTGCGG – 3') or ITS5 (GGAAGTAAAAGTCGTAACAAGG) and the reverse primer ITS4 (5TCCTCCGCTTATTGATATGC 3 (White et al. 1990). The β-tubulin gene was amplified using the forward primer Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and reverse primer Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC 3') (Glass & Donaldson. 1995). Each PCR reaction contained 10 ng of genomic DNA, 0.4 μM of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.0 U Taq DNA polymerase and 1× IB Taq polymerase buffer (Phoneutria, Belo Horizonte, MG, Brazil). Thermocycling was conducted with the following program: denaturation at 95°C for 4 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min, plus a final elongation period at 72°C for 5 min. All PCR products were purified using ExoSAP-IT® (USB, Cleveland, Ohio, USA) and forward and reverse- sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). PCR products were run on an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA,

USA). The sequences were then compared with the sequences in the NCBI nucleotide database using the BLAST algorithm (Altschul et al. 1997).

Chemicals

All substrates were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soybean hulls and sugar cane bagasse were obtained from a local source. Sephacryl S100, Q-Sepharose and DEAE-Sepharose columns were acquired from GE Healthcare Life Sciences, (Piscataway, NJ. USA). CarboPac PA-100 columns were purchased from Dionex Co. (Sunnyvale, CA, USA). Saccharides were purchased from Megazyme Co. (Wicklow, Ireland).

Residue pretreatment

Soybean hulls and sugarcane bagasse were thoroughly washed with tap water and autoclaved at 121°C for 2 h. Material was subsequently dried at 60°C for 48 h and ground to form a homogeneous blend. The fine powder obtained was used as carbon source for fungal growth and substrate for analysis of enzymatic hydrolysis.

Organism and enzyme production

C. byssicola was obtained from the fungal culture collection at the Enzymology Laboratory, University of Brasília, Brazil (under genetic heritage number 010237/2015-1 for authorization to access and ship sample components). Following maintenance and growth on PDA medium (10% potato broth, 1% dextrose and 2% agar), spore concentrations were determined under microscopy using a Neubauer chamber. Spore concentrations were adjusted with sterile saline solution (0.9%) and 3 mL aliquots of spore suspensions (10^7 spores/mL) inoculated into Erlenmeyer flasks containing 300 mL of liquid medium (0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% $(NH_4)_2SO_4$, and 0.06% yeast extract) at pH 7.0 with 1.0% (w/v) soybean hulls as carbon source. The fungus was grown for 7 days at 28°C under agitation at 120 rpm. The medium was then filtered through filter paper and the resulting filtrate, hereafter called crude extract, used as source of enzymes.

Enzyme assays

Endoglucanase (CMCase), mannanase, pectinase and xylanase activities were determined by mixing 5 µL of enzyme sample with 10 µL of substrate (4% (w/v) carboxymethylcellulose (CMC), 1% galactomannan, 1% pectin and 1% oat spelt xylan, respectively) at 50°C for 30 min (Salles et al. 2000, with modifications). The substrates were diluted in 100 mM sodium acetate buffer (pH 5.0). FPase activity was determined through incubation of 150 µL of enzyme with a 1 x 6 cm strip of filter paper (Whatman No. 1) at 50°C for 1 h (Mandels et al. 1976). The amount of reducing sugar released was measured using the DNS method (Miller 1959). Activity was expressed as µmol of reducing sugars formed per min per mL (IU/mL). Glucose, mannose, galacturonic acid and xylose were used as standards. Protein concentration was determined by the Bradford assay (1976) using bovine serum albumin as ~~a~~ standard. Glucose content was measured by the glucose oxidase method (Trinder 1969).

Partial purification of CMCase

Partial purification steps were carried out at room temperature. Crude extract of *C. byssicola* was concentrated 10 times by ultrafiltration using an Amicon System (Amicon Inc., Beverly, MA. USA) fitted with a 10 kDa (PM 10) cut-off-point membrane. Aliquots (10 mL) of the concentrated samples were fractionated by gel filtration chromatography on a Sephacryl S100 (3.0 x 68.2 cm) column, pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0 and 0.15 M NaCl. Fractions of 5 mL were collected at a flow rate of 20 mL/h. Fractions 48 – 52 with higher CMCase activity were pooled and subjected to ion-exchange chromatography on a HiTrap Q Sepharose Fast Flow (1 mL) column coupled to a purification Äkta system (GE Healthcare). The column was previously equilibrated with 50 mM sodium acetate buffer, pH 5.0, followed by a linear gradient of NaCl (0 - 1 M). Fractions (5 and 6) of 1 mL were collected at a flow rate of 1 mL/min. Fractions with CMCase

activity were pooled and applied onto a HiTrap DEAE Sepharose Fast Flow column under the same conditions as described above. Fraction number 5 was collected and named Cb-Cel. All samples were stored at 4°C.

Electrophoresis and zymography

Samples were precipitated with 10% TCA (trichloroacetic acid) and washed three times with cold 100% acetone. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) using a 12% (w/v) gel, subjected to 25 mA for 50 min. The gel was stained for proteins using silver nitrate according to the method of Blum et al. (1987). Identification of CMCase activity was carried out by zymography, in which the substrate CMC was copolymerized in the polyacrylamide gel (12% w/v) in a final concentration of 0.4% (v/v). The gel was incubated at 60°C for 90 min and stained with Congo red (0.1% w/v) as described by Bischoff et al. (1998).

Determination of hydrolysis products

Reactions containing enzymatic fractions (concentrated and Cb-Cel) and substrates (soybean hulls, sugarcane bagasse, avicel, mannan and xylan (1%) CMC (1% and 4%) and filter paper were incubated at 40ºC under agitation at 120 rpm. Soybean hulls and sugarcane bagasse were incubated for 240 hours (aliquots were harvested initially every 24 h), while avicel, mannan, xylan and CMC were incubated during 48 hours (aliquots were harvested every 12 h). Aliquots of 125 µL were withdrawn at intervals for quantification of total reducing sugars using the DNS method, for glucose quantification by the glucose oxidase method (Trinder 1969) and for analysis of hydrolysis products by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) (Ryan et al. 2003). In this process, aliquots (100 µL) of hydrolysate samples were analyzed by HPAEC-PAD on a Dionex ICS 3000 Ion Chromatography DC System (Dionex Co., California, USA) using a CarboPac PA-100 column and guard column at 20°C, with an operating flow rate of 0.25 mL/min. Samples were eluted for 80 min in 100 mM sodium hydroxide solution (Sigma-Aldrich) and a gradient of 0-500 mM sodium acetate (Merck) with 100 mM NaOH. The reducing sugars were identified and quantified on an ED40 electrochemical detector (Dionex Co.), and the data analyzed by Chromeleon 6.8 SR8 (Dionex Co.). Oligossaccharide solutions with a degree of polymerization from 2 to 6 units (G2, cellobiose; G3; cellotriose; G4, cellotetraose; G5, cellopentaose; G6, cellohexaose; M2, mannose; M3, manotriose; M4, manotetraose; M5, manopentaose; M6, manohexaose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose) (Megazyme Co.) and glucose (G1), mannose (M1) and xylose (X1) (Sigma-Aldrich) were used as standards for analysis of the reaction products.

Enzymatic characterization

The effect of temperature on CMCase activity was determined by performing the standard assay at a temperature range of 30-80°C (Δ = 10°C) and pH 5.0. The effect of pH on cellulase activity was determined at 50°C over a pH range of 3.0 to 9.0, using the following buffers (50 mM): sodium acetate (pH 3.0–6.0), sodium phosphate (pH 6.0–7.5) and Tris–HCl (pH 7.5–9.0). The evaluation of CMCase thermostability was performed by pre-incubating the enzyme samples at 40, 50 and 60°C. At various time periods, aliquots were taken and the residual activity measured under standard conditions.

The effect of phenolic compounds (vanillin, tannic acid, 4-hydroxybenzoic acid, ferulic acid, ρ-coumaric acid and cinnamic acid, at 1 mg/mL) on CMCase activity was assessed by pre-incubating enzyme samples at 25°C in the presence of these compounds. Incubation was carried out for 7 days, and aliquots were taken for measurement of residual CMCase activity by the standard protocol. Appropriate controls were included for all experiments, and the average values were reported as relative activity (%).

The effect of metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Na^{+}, Zn^{2+}) and EDTA (ethylenediaminetetraacetic acid), at concentrations of 1 mM and 10 mM, on CMCase activity was evaluated by pre-incubating the enzyme samples at 25°C for 20 min in the presence of these compounds. The residual activity was determined using standard protocols.

For kinetics experiments, CMC concentration ranged from 0.5 to 26.7 mg/mL. K_m and V_{max} values were estimated from the Michaelis-Mentem equation with the non-linear regression data analysis program Enzifitter (Leatherbarrow, 1999). All experiments were carried out in triplicate, with a standard deviation less than 10 %.

Statistical Analysis

Statistical analyses were performed with the Statistica 8.0 software. Data were submitted to factorial ANOVA and Fisher post hoc tests with significance level of $p < 0.05$.

Results

Partial purification of CMCase

The crude extract obtained following 7 days growth was assayed for CMCase (0.904 IU/mL), pectinase (0.845 IU/mL), mannanase (0.814 IU/mL), xylanase (0.744 IU/mL) and FPase activities (0.268 IU/mL). Due to its higher activity, CMCase was chosen as the main target of this research. Crude extract was then subjected to ultrafiltration through a PM-10 ultrafiltration membrane. After this procedure, CMCase activity was only detected in the concentrated fraction, which in turn was subjected to successive chromatographic methods. In the concentrated fraction, a beta-glucosidase activity of 2.37 IU/mL was detected, while pectinase, mannanase and xylanase activities were 2.08, 1.37 and 1.26 IU/mL, respectively.

The concentrated fraction (CF) was fractionated by gel filtration chromatography on Sephacryl S100 (Figure 1a). The elution profile showed a single CMCase peak (1.06 IU/mL) which was further fractionated by anion-exchange chromatography on a Q Sepharose Fast Flow. The elution profile of the anion exchange chromatography showed two peaks with endoglucanase activity, with the second (0.70 IU/mL) chosen for purification due to its lower protein peak (Figure 1b). The second peak was pooled and loaded onto a DEAE Sepharose Fast Flow chromatography column (Figure 1c). Two peaks containing cellulase activity were observed, with the fractions of the second peak pooled and denominated Cb-Cel for further enzyme characterization. All collected fractions were stored at 4°C.

The degree of purity obtained by all purification steps was evaluated by SDS-PAGE and zymography (Figure 2). As demonstrated in the zymogram, Cb-Cel and an enzyme preparation from the Q Sepharose Fast Flow showed only one band of CMCase activity. Under denaturing conditions, the gel showed a protein band of approximately 48 kDa for the Cb-Cel preparation. The results of the partial purification of CMCase are summarized in Table 1. The successive purification of Cb-Cel resulted in a seven-fold increase in purity and 19.5% recovery. Following the final purification step, Cb-Cel showed activities of CMCase (0.424 IU/mL), xylanase (0.250 IU)/ml), mannanase (0.145 IU/ml) and pectinase (0.108 IU/mL).

Enzymatic hydrolysis

Concentrated and Cb-Cel samples were incubated with cellulose, hemicellulose and lignocellulosic substrates. With the hydrolysis of filter paper by the concentrated fraction, an increase was observed in the amounts of reducing sugars released during the 48 hours of incubation. Moreover, an increase in reducing sugar release was also observed after the hydrolysis of CMC 4% by Cb-Cel (Figure 3).

Given their high recalcitrance, hydrolysis of soybean hulls and sugarcane bagasse was performed over a 10 day period. Sugar concentrations gradually increased during the 10 day hydrolysis, indicating that hydrolysis time period was an important factor for enzyme action. The amount of sugar released following soybean hull degradation was markedly higher than observed

following sugarcane bagasse hydrolysis, mostly by action of enzymes from the concentrated fraction. After 10 days of hydrolysis, 5.2 mg/mL of reducing sugars were released from the hydrolysis of soybean hulls and 0.8 mg/mL from sugarcane bagasse (Figure 4).

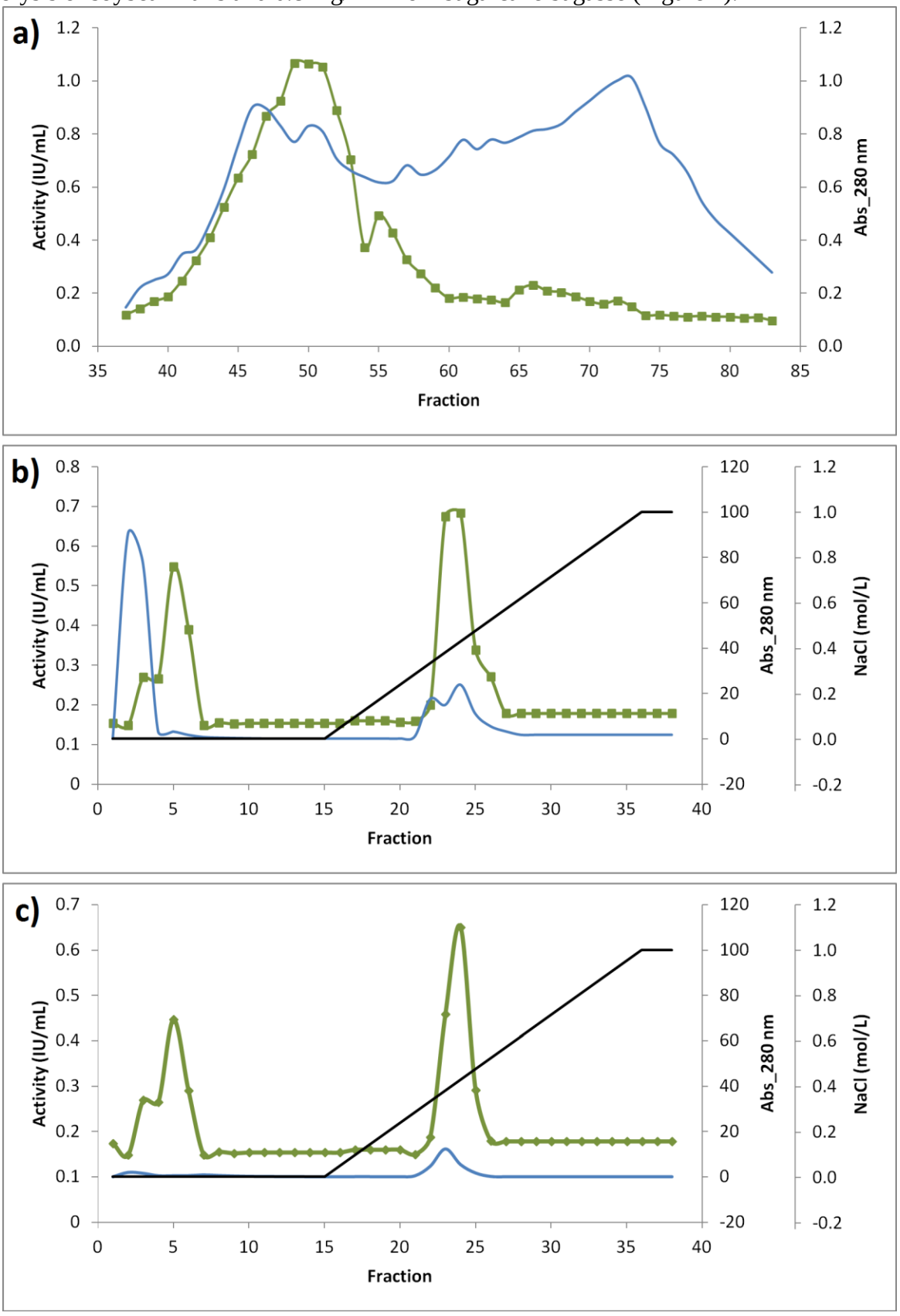

Fig. 1 Purification of Cb-Cel. **a)** Gel filtration chromatography on Sephacryl S100 column. **b)** Ion-exchange chromatography on HiTrap Q Sepharose Fast Flow column. **c)** Ion-exchange

chromatography on HiTrap DEAE Sepharose Fast Flow column. CMCase activity (IU/mL) (■), Protein (Abs_280 nm) (▬) and NaCl gradient (mol/L) (▬).

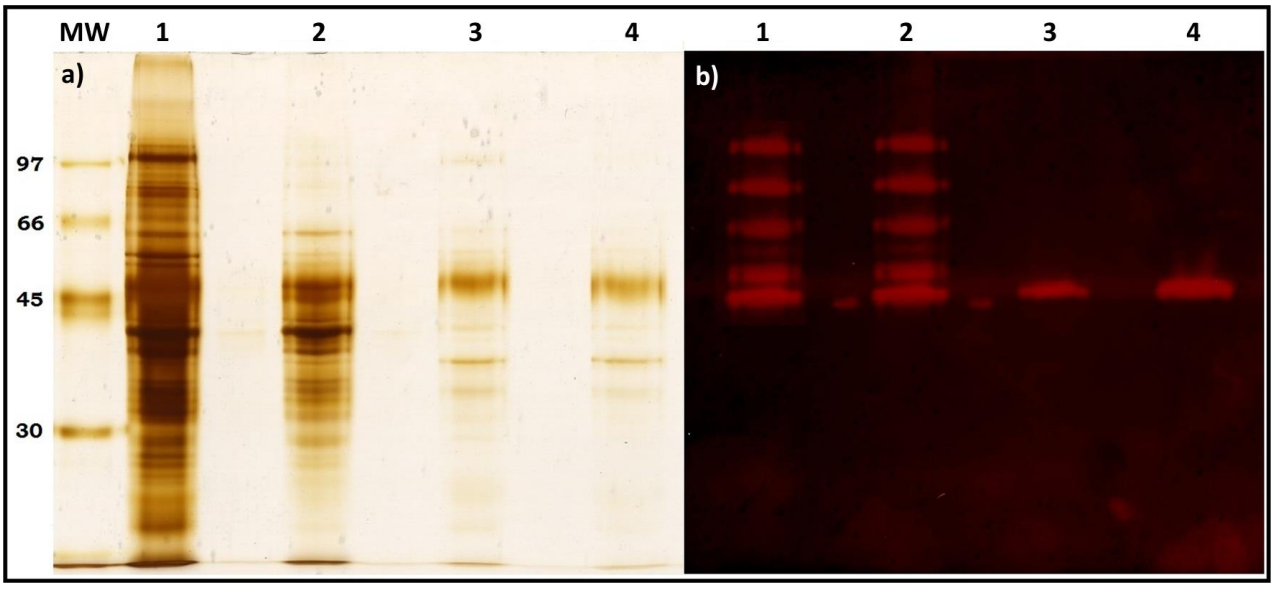

Fig. 2 SDS-PAGE (12%) of the successive purification steps of Cb-Cel. The gel was stained with **a)** silver nitrate or **b)** 0.1% congo red. MW (molecular weight marker): phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). Line 1, concentrated fraction (PM 10 kDa); line 2, fractions pooled from S100 chromatography; line 3, fractions pooled from Q-FF chromatography; line 4, fractions pooled from DEAE-FF chromatography (Cb-Cel).

Table 1 Summary of partial purification of Cb-Cel.

Purification Step	Total Protein (mg)	Total Activity (IU)	Specific Activity (IU/mg)	Purification Fold	Yield (%)
Crude Extract	51.6	258.0	5.0	1.0	100.0
Concentrated Fraction	11.0	45.0	4.1	0.8	17.4
Gel filtration on Sephacryl S100	14.0	122.4	8.7	1.7	47.4
Ion-exchange on Q-FF	3.8	69.6	18.3	3.7	27.0
Ion-exchange on DEAE-FF	1.4	50.4	36.0	7.2	19.5

Moreover, the amount of glucose gradually increased during saccharification of CMC, filter paper and lignocellulosic substrates. After 48 hours of hydrolysis of filter paper and CMC 4% by the concentrated fraction, 3.96 and 1.50 mg/mL of glucose were released, respectively. In the case of hydrolysis of soybean hulls by the concentrated fraction, 2.36 mg/mL of glucose was released after 240 hours of hydrolysis. Cb-Cel released lower amounts of glucose in all hydrolysis evaluations (data not shown).

The products of hydrolysis of substrates by the concentrated and Cb-Cel were identified by HPAEC-PAD, with results summarized in Table 2. Glucose was released from the hydrolysis of 1 and 4% CMC, filter paper and soybean hulls. All manooligosaccharides were observed following hydrolysis of mannose. A xylooligomers range of X1-X5 was obtained after xylan hydrolysis.

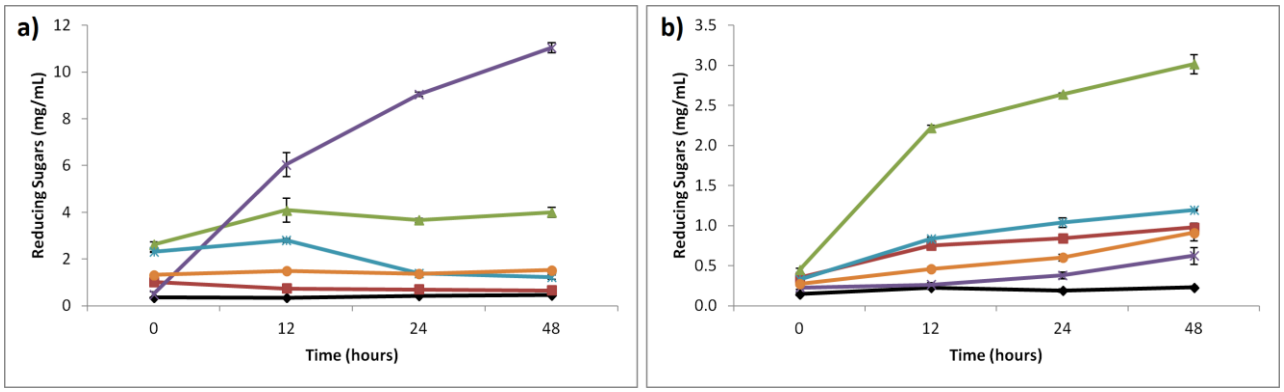

Fig. 3 DNS method-based quantification of total reducing sugars following hydrolysis of 1% avicel (♦), 1% (■) and 4% (▲) carboxymethylcellulose (CMC), filter paper (×), 1% mannan (**x**) and 1% xylan (●) by **a)** concentrated fraction and **b)** Cb-Cel

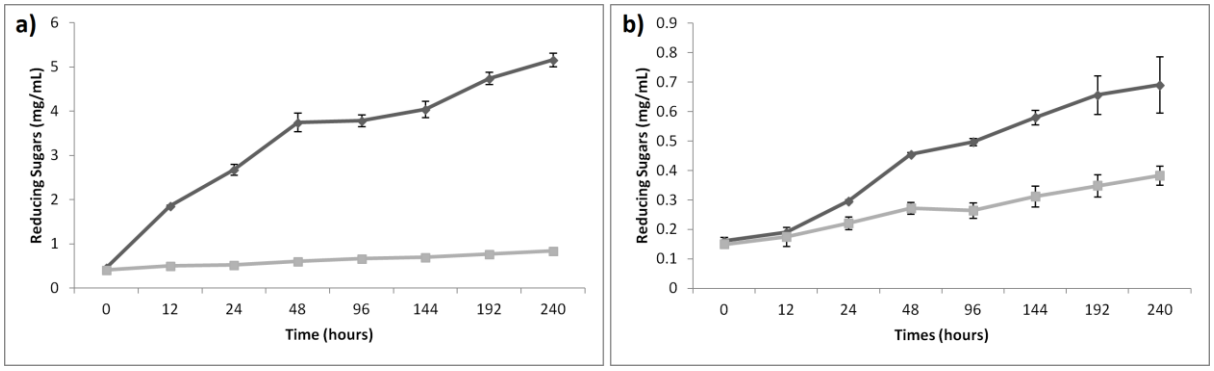

Fig. 4 DNS method-based quantification of total reducing sugars following hydrolysis of 1% soybean hulls (♦) and 1% sugarcane bagasse (■) by **a)** concentrated fraction and **b)** Cb-Cel

Table 2 HPAEC-PAD-based identification of the hydrolysis products released by concentrated fraction and Cb-Cel.

	Products of hydrolysis	
Substrate	Concentrated Fraction	Cb-Cel
CMC 1%	G1, G5	G1, G2
CMC 4%	G1, G5, G6	G1
Filter Paper	G1, G5	G1
Mannan 1%	M1, M2, M3, M4, M5	M1, M2, M3, M4, M5, M6
Xylan 1%	X1, X2, X3, X4, X5	X2, X3
Soybean Hulls 1%	G1, G4, G5, G6, M2, M4, X2	G1, G5, M1, X2, X5
Sugarcane Bagasse 1%	G6, M1, X1, X4	G6, X2

G1: glucose, G2: cellobiose, G5: cellopentaose, G6: cellohexaose, M1: mannose, M2: mannobiose, M3: mannotriose, M4: mannotetraose, M5: mannopentaose, M6: :mannohexaose, X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose.

The enzymatic hydrolysis of soybean hulls by the concentrated fraction yielded predominantly cellohexaose and cellopentaose, which were released between 12h and 48h, a period during which a low amount of glucose was also released (Figure 5). Moreover, mannotetraose (during the interval of 12 and 48h) and mannobiose (during the interval of 96 and 240h) were released in lower amounts (< 0.2 µg/mL) (data not shown). Following the hydrolysis by Cb-Cel, xylobiose and mannose were the main reducing sugars detected between 12 and 48h. Comparatively, smaller quantities of saccharides were obtained after hydrolysis of soybean hulls by Cb-Cel.

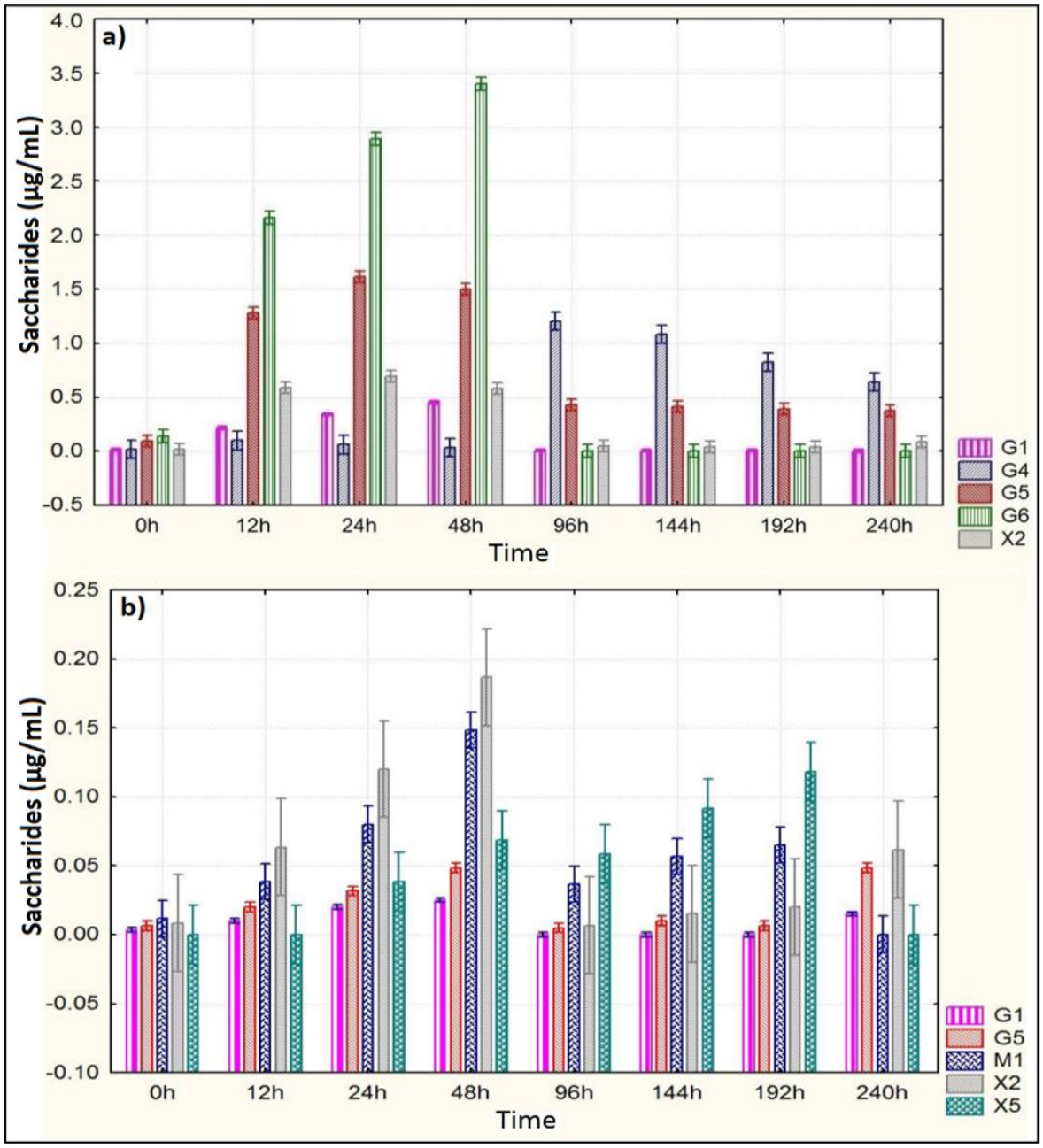

Fig. 5 Identification and quantification of the main saccharides released from 1% soybean hulls following hydrolysis over time by enzymes from **a)** concentrated fraction and **b)** Cb-Cel

As presented previously, the quantification of total reducing sugars by the DNS method showed that enzymatic hydrolysis of sugarcane bagasse released lower amounts of reducing sugars compared to hydrolysis of soybean hulls. This result was corroborated by the identification and quantification of hydrolysis products by HPAEC-PAD. In this case, the degradation of sugarcane

bagasse released only cellohexaose, mannose, xylose and xylotetraose (concentrated fraction), with cellohexaose and xylobiose (Cb-Cel) in low quantities (< 0.3 µg/mL) (data not shown).

Enzymatic characterization

Effect of temperature, pH and thermostability on CMCase activity

The effect of temperature and pH on CMCase activity from the concentrated fraction and Cb-Cel is shown in figure 6. The highest activity was obtained at 60 and 70°C, and pH 4.5 and 5.0, for the concentrated fraction and Cb-Cel, respectively. Cb-Cel was active over a broad range of temperatures and pH, retaining at least 60% of its activity between 40 and 80°C and pH 4.0 to 7.5. The concentrated fraction retained at least 68% of its activity at temperatures from 30 to 70°C, with this enzymatic fraction also active over a wide range of pH values, retaining at least 60% of relative activity between pH 3.5 to 9.0.

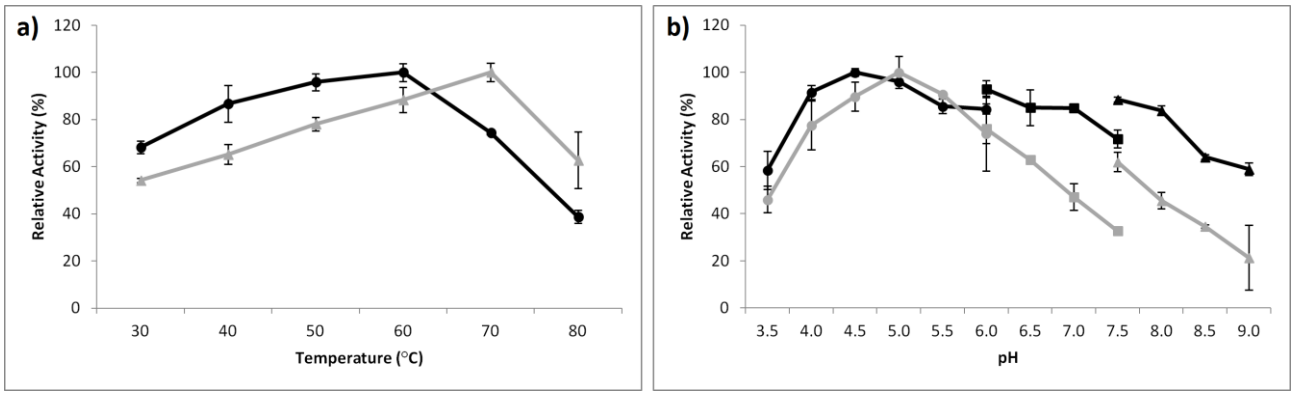

Fig. 6 Effect of temperature (**a**) and pH (**b**) on CMCase activity from concentrated fraction (black line) and Cb-Cel (gray line). Buffers: sodium acetate (pH 3.5-6.0), sodium phosphate (pH 6.0-7.5) and Tris-HCl (pH 7.5-9.0).

Cb-cel showed elevated thermostability at 40 and 50°C. After 30 days incubation, 97% of its initial activity was maintained at 40°C. At 50°C, this enzyme also maintained 67% of its relative activity for 25 days (Figure 7). At 60°C, the Cb-Cel half-life was approximately 3 hours.

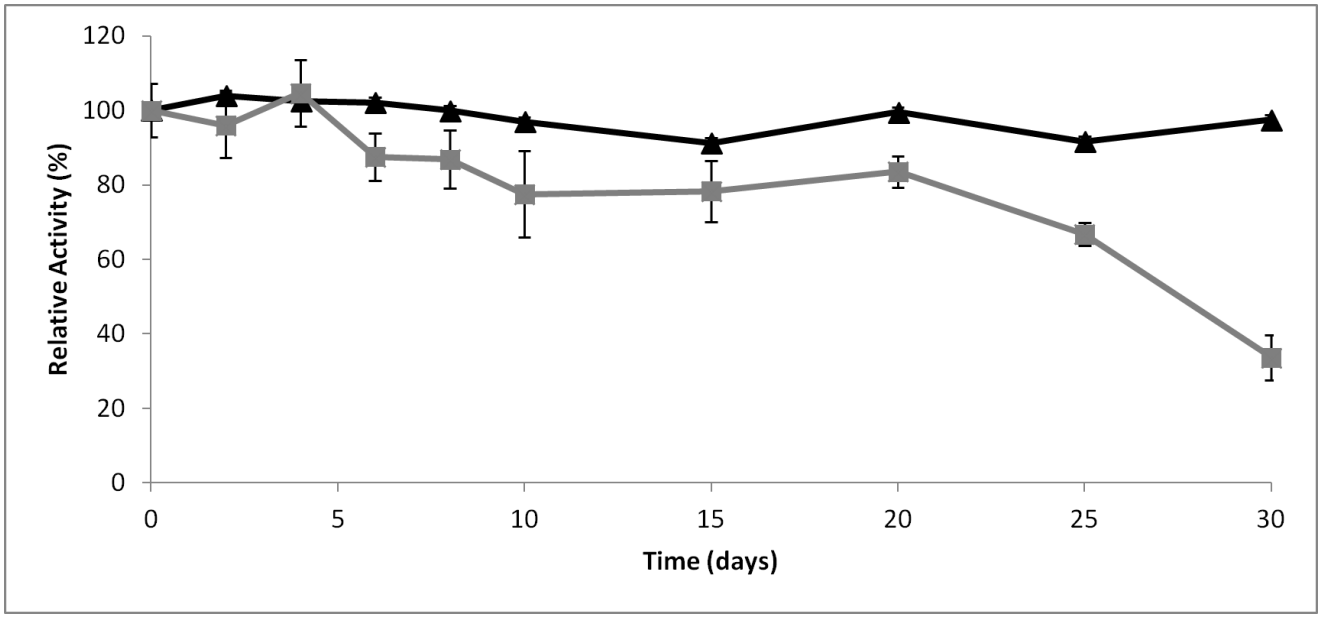

Fig. 7 Thermostability of Cb-Cel at 40°C (▲) and 50°C (■).

Effect of phenolic compounds on CMCase activity

The inhibitory and deactivator effects of oligomeric (tannic acid) and monomeric phenolic compounds (vanillin, 4-hydroxybenzoic, ferulic, ρ-coumaric and cinnamic acids) on the concentrated fraction and Cb-Cel activities were evaluated. According to Ximenes et al. (2011), inhibition occurs immediately after the incubation of the enzyme with the phenols, while deactivation is related to the incubation time. Figure 8 shows the effect of such compounds with the concentrated fraction and Cb-Cel. The concentration of phenolic compounds at 1 mg/mL did not interfere with the DNS assay, in contrast to higher concentrations (5 and 10 mg/mL) of such compounds which were found to interfere with the assay (data not shown). Overall, Cb-Cel and the concentrated fraction were resistant to phenolic compounds. CMCase activity of Cb-Cel showed an increase of 25% and 18% at zero time after incubation with ferulic and tannic acids, respectively.

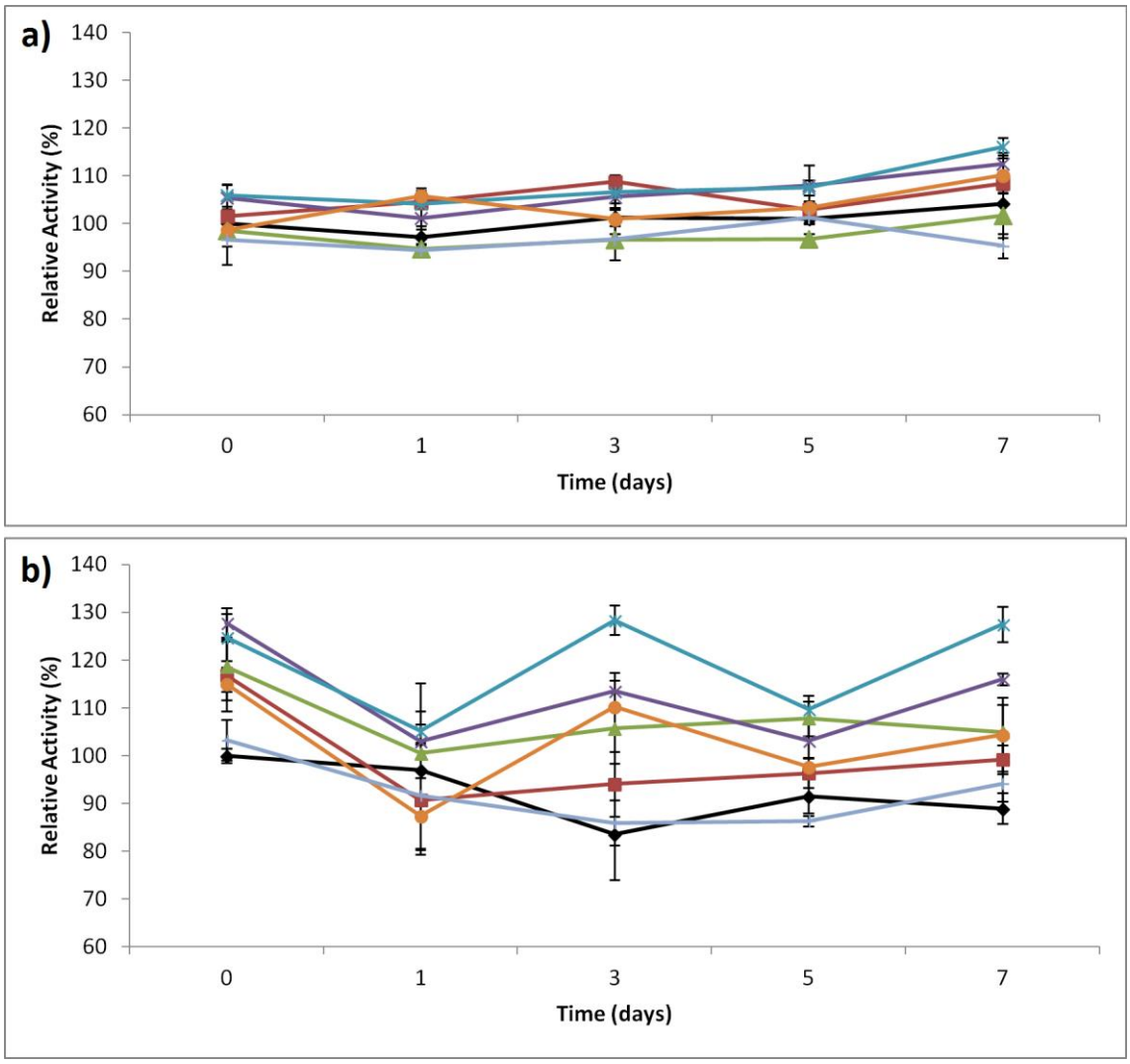

Fig. 8 Inhibitory and deactivator effects of phenols (1 mg/mL) on CMCase activity from **a)** concentrated fraction and **b)** Cb-Cel. Control (♦), vanillin (■), tannic acid (▲), 4-hydroxybenzoic acid (×), ferulic acid (ɤ), ρ-coumaric acid (●) and cinnamic acid (+).

Effect of metal ions and EDTA on CMCase activity and determination of kinetic parameters

The effect of metal ions and EDTA on Cb-Cel activity at concentrations of 1 and 10 mM was evaluated (Table 3). Fe^{3+} and Fe^{2+} at 10 mM resulted in a severe inhibition (more than 90%) of

Cb-Cel activity. $CuSO_4$ and $ZnSO_4$ (10 mM) decreased the enzyme activity by approximately 70% and 40%, respectively. By contrast, Co^{2+} at 10 mM had an activator effect on CMCase activity from the concentrated fraction and Cb-Cel of 16.4 and 24.6%, respectively. Moreover, the chelating agent EDTA showed no inhibitory or activation effect on CMCase activity from the samples evaluated. The rate dependence of the endoglucanase reaction on CMC followed Michaelis–Menten. Non-linear regression data analysis of Cb-Cel acting on CMC showed Km and Vmax values of 15.81 ± 1.65 mg/mL and 0.59 ± 0.03 IU/mL, respectively.

Table 3 Effect of metal ions and EDTA at 1 mM and 10 mM on CMCase activity from the concentrated fraction (CF) and Cb-Cel

	Relative Activity (%)			
	1 mM		10 mM	
	CF	Cb-Cel	CF	Cb-Cel
Control	100.0 ± 1.08	100.0 ± 0.10	100.0 ± 3.13	100.0 ± 3.32
$CaCl_2$	96.7 ± 2.75	96.8 ± 1.42	107.5 ± 4.66	90.1 ± 2.94
$CoCl_2$	114.4 ± 0.64	116.4 ± 2.11	116.4 ± 4.71	124.6 ± 2.94
$CuCl_2$	94.6 ± 4.95	66.0 ± 5.08	90.6 ± 3.34	86.8 ± 4.54
$CuSO_4$	87.9 ± 0.86	67.0 ± 1.47	88.9 ± 2.49	30.1 ± 4.98
$FeCl_3$	104.9 ± 5.25	47.6 ± 1.62	90.5 ± 2.37	0.0 ± 0.00
$FeSO_4$	102.4 ± 0.82	75.6 ± 1.05	94.8 ± 2.91	6.1 ± 7.23
KCl	100.0 ± 1.24	95.0 ± 1.79	91.1 ± 2.01	97.1 ± 6.19
$MgCl_2$	121.7 ± 0.82	98.0 ± 6.49	98.3 ± 0.36	107.8 ± 1.78
$MgSO_4$	96.6 ± 1.55	96.0 ± 4.87	98.5 ± 1.32	98.9 ± 2.74
NaCl	106.3 ± 1.14	96.2 ± 3.64	106.7 ± 3.88	90.4 ± 3.39
$ZnCl_2$	107.9 ± 0.40	99.7 ± 2.20	107.6 ± 0.53	63.1 ± 2.36
$ZnSO_4$	102.7 ± 2.14	72.9 ± 3.86	106.1 ± 0.96	58.9 ± 5.79
EDTA	98.5 ± 0.66	102.6 ± 3.48	99.7 ± 2.66	102.3 ± 2.84

Discussion

Agro-industrial wastes represent an important alternative source of carbon for microbial growth and subsequent production of industrial enzymes. For example, cellulases are known to be synthesized by microorganisms during growth on wheat bran as cellulosic material (Rawat & Tewari 2012). Within this context, a variety of lignocellulosic substrates were evaluated as carbon sources for *C. byssicola* growth and cellulase production. In this study, *C. byssicola* secreted multiple cellulases, as demonstrated by zymogram analysis, wherein five bands with CMCase activity were observed. As with *C. byssicola*, many other microorganisms, such as *Trichoderma reesei* (Miettinen-Oinonen 2004), *Myceliophthora* sp. (Badhan et al. 2007) and *Aspergillus fumigatus* (Lima et al. 2009), are also known to produce cellulolytic enzymes in multiple isoforms, which have distinct biochemical properties, molecular weight and substrate specificity. This multiplicity of isoforms is an interesting feature, given that many enzymes are known to act synergistically to hydrolyze substrates more efficiently.

Analysis of the hydrolysis of all the substrates evaluated showed greater amounts of reducing sugars released by the concentrated fraction when compared to Cb-Cel. Taking into account that CMCase activity was not detected in the ultrafiltrate fraction, during purification of Cb-Cel at least five CMCase activities were present. Since these enzymes act synergistically for the complete hydrolysis of cellulose, the yield and fold values were probably underestimated.

As shown previously, soybean hull hydrolysis by the concentrated fraction and Cb-Cel released markedly higher amounts of reducing sugar than sugarcane bagasse degradation. Given that the fungus *C. byssicola* was grown in medium supplemented with soybean hulls as carbon source, it is likely that the enzymes secreted during microbial growth are more active on this substrate. A hypothesis for this fact is that differences in the structure of the cell wall of soybean hulls and sugarcane bagasse leads the fungus to release enzymes that are more suitable to the deconstruction of the cell wall of this lignocellulosic biomass employed for its growth. Juhász et

al. (2005) also observed that the enzymatic hydrolysis of willow and corn stover was more efficient when enzymes were produced by *T. reeseei* following grown in liquid medium containing the respective substrate as carbon source.

Identification and quantification by HPAEC-PAD showed that hydrolysis products of CMC and filter paper by secreted enzymes from *C. byssicola* were mainly glucose, cellopentaose and cellohexaose. The ability to hydrolyze CMC suggests that the semi-purified enzymes obtained in this study are endoglucanases, since cellobiohydrolases are not able to degrade this substrate due to the carboxymethyl side groups, which prevent the cellulose chain from entering the narrow tunnel leading to the active site of the cellobiohydrolases (Jørgensen et al. 2003). In addition, HPLC analysis showed a major liberation of cellooligomers with greater polymerization degree instead of glucose, corroborating the classification of these enzymes as endoglucanases. Given that endoglucanases hydrolyze glycosidic bonds at different positions of the cellulose chain, hydrolysis products vary considerably. For example, enzymatic hydrolysis of CMC by a purified CMCase from *Daldinia eschscholzii* generated cellobiose, cellotriose, cellotetraose and lower amounts of glucose (Karnchanatat et al. 2008). Furthermore, Liu et al. (2011) performed enzymatic hydrolysis of cellooligosacharides (G2, G3, G4 and G5) and CMC with two purified CMCases from *Aspergillus fumigatus*, named Egl2 and Egl3. They observed that Egl2 hydrolyzed cellotetraose and cellopentaose, while Egl3 hydrolyzed cellotriose, cellotetraose and cellopentaose. In CMC degradation, Egl2 released cellobiose, cellotriose and cellotetraose, while Egl3 generated glucose and cellobiose.

With regard to the characterization of Cb-Cel, activity was observed over a wide temperature and pH range, indicating suitability for application in industrial processes. CMCases commonly present optimum temperature around 60 – 70°C. Previous studies have reported highest activity at 60°C, such as EGa from *Penicillium occitanis* (Chaabouni et al. 2005) and EG3 from *Aspergillus fumigatus* (Liu et al. 2011). Similarly, numerous studies have described CMCases more active at 70°C, for example in *Penicillium purpurogenum* KJS506 (Lee et al. 2010), *Thermoascus aurantiacus* (Parry et al. 2002, Dave et al. 2014), *Daldinia eschscholzii* (Karnchanatat et al. 2008) and *Penicillium brasilianum* (Krogh et al. 2009). Optimum activity at pH 5.0 was reported for CMCases from *Thermomonospora* sp. (George et al. 2001), *Streptomyces drozdowiczii* (Lima et al. 2005), *Aspergillus aculeatus* (Naika et al. 2007), *Penicillium purpurogenum* (Lee et al. 2010) and *Aspergillus fumigatus* (Liu et al. 2011).

Our results indicated an elevated thermostability of Cb-Cel at 40 and 50°C. Cb-Cel presented higher thermostability than several other endoglucanases, such as an endoglucanase from *Aspergillus terreus* that lost 50% of its activity in less than 6 hours of incubation at 50°C (Narra et al. 2014). Cb-cel was also more thermostable than the recombinant endoglucanases ApCel5A (from *Aureobasidium pullulans*), GtCel12A (from *Gloeophyllum trabeum*) and StCel5A (from the thermophilic fungus *Sporotrichum thermophile*), which all presented a half-life of 2 hours at 45°C, 50°C and 55°C, respectively (Tambor et al. 2012). Lee et al. (2010) reported that CMCase from *Penicillium purpurogenum* showed a half-life of 72h, 48h, 7h and 2h at 40, 50, 60 and 70°C, respectively. Since Cb-Cel was partially purified, it is likely there are stabilizing factors enhancing its thermostability. Thus, Cb-Cel shows potential for future biotechnological applications.

Phenolic compounds, which result from the degradation of lignin, may be inhibitory to enzymes and/or fermentative organisms. As such, the employment of enzymes resistant to phenols is crucial for efficient biomass hydrolysis (Duarte el al. 2012). Cb-Cel was resistant to inhibition and deactivation by the evaluated phenolic compounds. Our results showed that tannic acid (1 mg/mL) increased Cb-Cel activity at the beginning of the assay (time zero). On the other hand, there are numerous reports in the literature reporting inhibitory effects of phenols on cellulase activities. For instance, Ximenes et al. (2011) investigated inhibitory and deactivator effects of tannic acid on cellulases from *Trichoderma reesei*. Tannic acid at 2 mg/mL inhibited CMCase and FPase activities by approximately 25% and 60%, respectively. After 24h incubation, tannic acid at 5 mg/mL presented a deactivator effect and decreased CMCase and FPase activities by 80% and 60%, respectively. Other studies have reported that vanillin, syringaldehyde, trans-cinnamic acid

and hydroxybenzoic acid inhibit cellulose hydrolysis in wet cake by endo- and exo-cellulases (Ximenes et al. 2010). The effects of phenolic compounds are observed not only in terms of enzymatic activity but also in the production of enzymes by microorganisms. Vohra et al. (1980) observed that vanillin at 0.5 mg/mL inhibited the production of CMCase, avicelase and β-glucosidase from *T. reesei* by 78%, 100% and 100%, respectively, and also inhibited fungal growth by 94%. Ferulic acid inhibited the production of the same enzymes by 75%, 86% and 100%, respectively, but had little effect on microbial growth. Berlin et al. (2006) demonstrated that seven cellulase preparations were inhibited by a dissolved lignin preparation. Similarly, phenolic derived from pretreated sugarcane bagasse deactivated cellulolytic enzymes (Michelin et al. 2015). By contrast, there are also reports of increased hemicellulase activities in the presence of phenolic compounds, such as observed with a commercial xylanase preparation, with activity enhanced by 50% after the addition of vanillic acid at low concentrations (0.05%), and hydrolysis of xylan improved by the addition of vanillin and guaiacol (Kaya et al. 2000). Similarly, another xylanase, named XylT2, showed an improvement in its activity by approximately 50% in the presence of vanillin, ferulic acid and 4-hydroxybenzoic acid (Moreira et al. 2013). Phenolic compounds can have a wide variety of effects on enzymes activity, increasing enzymatic activity under certain conditions. As such, enzyme activities and the presence of lignin degradation products are not likely to be correlated in a simple manner (Kaya et al. 2000). In this way, as Cb-cel displayed resistance to several phenolic compounds, it shows potential for use in industrial processes.

The inhibition or activation of cellulases by selected chemicals is a useful approach for the study of active site structure and its mechanism of action. Among the divalent metal ions, most often Cu^{2+} and Fe^{2+} are found to exert strong inhibition on cellulolytic enzymes, such as cellobiohydrolases, endoglucanases and β-glucosidases (Tejirian & Xu 2010). The data reported here is corroborated by studies performed by Li et al. (2006) and Karnchanatat et al. (2008), wherein endoglucanase activity decreased in the presence of both Cu^{2+} and Fe^{2+}. Elvan et al. (2010) and Xiang et al. (2014) also observed inhibitory effects of Cu^{2+} on CMCase. The inhibitory effect of these ions can be ascribed to a detrimental interaction with cellulases, which can cause conformational changes or replacement of native metal cofactors (Tejirian & Xu 2010). Moreover, the chelating agent EDTA showed no inhibitory effect on CMCase activity from *C. byssicola*, indicating that the activity of this enzyme does not depend on the presence of metal ions. In comparison with Km value of an endoglucanase from *Penicillium funiculosum* (Castro et al. 2010), Cb-Cel showed lower affinity for CMC.

In conclusion, both the concentrated fraction and Cb-Cel from *C. byssicola* were able to hydrolyze multiples substrates, releasing both monomers and oligomers. Greater amounts of reducing sugars were detected from the hydrolysis of soybean hulls than from sugarcane bagasse. This is probably a result of the fact that soybean hulls were used as initial carbon source for fungal growth, with the release of enzymes more suitable to the degradation of this substrate. Cb-Cel showed activity over a broad range of pHs and temperatures, with elevated thermostability. Additionally, Cb-Cel displayed resistance to several phenolic compounds which is of relevance for industrial biofuel production, given that such compounds are formed during lignocellulosic biomass pre-treatment.

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