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Full Length Research Paper

Identification and characterization of cellulases produced by *Acremonium strictum* isolated from Brazilian biome

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Abstract

Explorations of biodiversity in the search for new biocatalysts by selecting microorganisms from nature represents a method for discovering new enzymes which may permit the development of bio-catalysis on an industrial scale. In face this, the objective of the present study was to identify and characterize cellulases produced by *Acremonium strictum* AAJ6 isolated from samples collected from different Brazilian biomes. The microorganism was cultivated in medium containing microcrystalline cellulose (Avicel), at temperature of 30°C and 150 rpm agitation for 240 h. After induction of enzymes, four specific activities were evaluated: endoglucanase (CMCase), total activity (filter paper activity), cellobiase and β-glucosidase. The optimal temperature and pH of the enzymes were determined using the central composite rotational design (CCRD). For the identification of cellulases produced by *Acremonium strictum*, the purified protein was subjected to trypsin digestion and analyzed by liquid chromatography coupled with mass spectrometry (LC-MS/MS). The peptides identified in the mass spectrometry were searched against the CAZy database and two potential cellulolytic enzymes were identified: endoglucanase Cel74a and β-glucosidase. This work shows not only studies of enzymatic characterization, but also the importance of the biodiversity exploitation for the identification of new microorganisms and new enzymes with potential application biotechnological.

Keywords: Brazilian biome, *Acremonium strictum,* cellulases, mass spectrometry.

INTRODUCTION

Cellulases are enzymes that form a complex capable of acting on cellulosic materials, promoting its hydrolysis. These biocatalysts are highly specific enzymes that act in synergy to release sugars of which glucose is of great interest to industry because it can be easily converted in a variety of bio-products, such as ethanol (Castro and Pereira 2010). The set of enzymes involved in degradation of cellulose is referred to as the cellulase complex. Most studies on the cellulase complex refer to microbial enzymes from fungi and bacteria, due to their potential to convert insoluble cellulosic material into glucose (Zhang et al., 2006).

In recent years the interest in production of cellulases has increased due to several potential applications, such as the production of bioenergy and biofuels, and application in textile and paper industries (Zhou et al., 2008; Soccol et al., 2010). The growing concerns about the shortage of fossil fuels, the emission of green house gases and air pollution by incomplete combustion of fossil fuel have also resulted in an increasing focus on production of bioethanol from lignocellulosics feed stocks and the possibility of using cellulases to perform enzymatic hydrolysis of the lignocellulosic materials (Soccol et al., 2010). However, in the bioethanol

production process, the cost of the enzymes used for hydrolysis must be reduced and efficiency of these enzymes improved in order to make the process economically viable (Zhou et al., 2008; Soccol et al., 2010).

Many studies have been published seeking new microorganisms to produce cellulolytic enzyme with higher specific activity and efficiency. Several strategies are available for improving the production and efficiency of cellulases, including optimization of the fermentation process, genetic modifications and mutagenesis. However, at present, the task of finding a good producer of cellulases still arouses the interest of researchers (Kitagawa et al., 2011; Sorensen et al., 2011). There is great interest in finding microorganism species that are not yet cataloged as interesting producers of inputs to industry in general, as well as optimizing production processes of these inputs from known microorganisms (Hernalsteens 2006; Hernalsteens and Maugeri 2007).

Brazil possesses the greatest biodiversity on the planet and home to seven biomes, forty-nine already classified as ecoregions, and an incalculable number of ecosystems. Due to the large Brazilian biodiversity, the species of fauna and flora may never be completely known, and the number of species not yet identified may reach the order of tens of millions. It is estimated that less than 5% of microorganisms existing on earth have been identified. In this context it is essential to implement programs capable of better utilizing Brazilian biodiversity (Hernalsteens 2006). Considering this scenario, the goal of the present work was to identify and characterize cellulases produced by *Acremonium strictum* isolated from samples collected from different Brazilian biomes with potential application biotechnological.

MATERIALS AND METHODS

Microorganism

Hernalsteens and Maugeri (2007) sampled flowers, fruit and soil from tropical Brazilian biomes, including: the Atlantic Rainforest (stretches along the Brazilian coast); the Cerrado (tropical savanna eco-region); the Pantanal (the world's largest wetland) and the Amazon Forest (complex biome due to the great diversity of vegetation present, considered the *lungs of the world*), aiming to isolate new microorganins and new enzymes. The microorganism studied in this work was *Acremonium strictum* AAJ6 that was isolated from Brazilian Biome (Cerrado), and selected as a potential producer of cellulases based on previous screening (Goldbeck et al., 2012). This microorganism belongs to the bank of cultures in the Laboratory of Bioprocess Engineering (LEB) - FEA/UNICAMP.

Fermentation

The inoculum was cultivated in agar slants (GYMP medium: 2.0% glucose, 0.5% yeast extract, 1.0% malt extract, 0.2% monobasic sodium phosphate, 2.0% agar and pH 5.5), at 30°C for 96 h. Fermentations were performed in shake flasks of 500 mL at 30°C, 150 rpm and monitored for 240 h. The culture medium consisted of: Avicel (microcrystalline cellulose) 20.0 g/L, yeast extract 0.60 g/L, KH_2PO_4 7.0 g/L, K_2HPO_4 2.0 g/L, $MgSO_4.7H_2O$ 0.15 g/L, $(NH_4)_2SO_4$ 1.0 g/L, $FeSO_4.7H_2O$ 0.01 g/L and KCl 0.50 g/L (Peixoto, 2006). The pH was adjusted to pH 5.5 with HCl. After 240 h, the fermentation broth was centrifuged in a RC26 PLUS refrigerated centrifuge (DuPont Instruments-Sorvall, Newtown, CT, USA) at 4°C and 18,200 x *g* for 10 minutes, and the supernatant (crude enzymatic extract) submitted to assays for determination of enzymatic activities.

Essays of Enzymatic Activities

Endoglucanase activity*:* A carboxymethylcellulose (CMC, 1%) solution was prepared in 0.2 M sodium acetate buffer (pH 4.2). One mL of the CMC solution was incubated with 1 mL of the crude enzymatic extract at 50°C for 10 min (Ogawa et al., 1982) and the amount of reducing sugar was measured by the 3,5-dinitrosalicylic (DNS) reagent method according to Miller (1959).

Filter paper activity: Total cellulase activity was determined by the filter paper assay procedure (Mandels and Sternberg, 1976). The assay system had a total volume of 2 mL, consisting of 1 mL of crude enzymatic extract and 1 mL of 0.2 M sodium acetate buffer (pH 4.2) and 50 mg of Whatman filter paper N° 1, incubated for 60 min at 50 °C. The amount of reducing sugar was measured by the 3, 5-dinitrosalicylic (DNS) reagent method, according to Miller (1959).

Cellobiase activity: Cellobiase activity was measured using a reaction mixture containing a cellobiose solution (0.02 M) in 0.2 M acetate buffer (pH 5.2). One mL of the cellobiose solution was mixed with 1 mL of crude enzymatic extract and incubated at 50 °C for 30 min. The amount of reducing sugar was then measured using a commercial enzymatic kit (Laborlab, Guarulhos, Brazil) containing glucose oxidase (Henry et al., 1974).

β-glucosidase activity: To determine the β-glucosidase activity, the method described by Afolabi (1997) utilized a substrate solution containing 1 mg/mL of pNPG (p-Nitrophenyl β-D-glucopyranoside) in 0.2 M acetate buffer, pH 5.2. A volume of 0.1 mL of crude enzymatic extract was added to 1.9 mL of substrate and incubated for 30 min at 50 °C. Subsequently, 0.5 mL of 2% sodium carbonate were added and quantification was performed in a spectrophotometer at 405 nm.

One unit (U) of endoglucanase, filter paper, and cellobiase activity is defined as the amount of the enzyme that released 1 μmol of glucose per minute from the substrate, at the three experimental conditions described above. For β-glucosidase activity, one unit (U) is defined as the amount of the enzyme that releases 1 μmol of *p*-Nitrophenol per minute from the *p*-Nitrophenyl β-D-glucopyranoside, at the experimental conditions.

The protein concentration was determined according to the methodology proposed by Lowry et al. (1951) using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard. The readings were taken in a spectrophotometer at 750 nm.

Precipitation with Ethanol

Precipitation of enzymes using ethanol was performed according to the methodology described in Santos (2002). Anhydrous alcohol with 99.3% (v/v) at -20 °C was slowly added to the crude enzyme extract until reaching the concentration of 70% (v/v) under gentle stirring using a Hanna magnetic stirrer, model HI 190 M, (Hanna Instruments, Eibar, Spain) at 2 °C, with the aid of jacketed reactors and Tecnal TE® 184 water bath to minimize enzyme denaturation. After the addition of ethanol, the solution was immediately centrifuged at 18,200 x *g* for 15 min from 4 °C in a RC26 PLUS refrigerated centrifuge (DuPont Instruments-Sorvall, Newtown, CT, USA). The precipitate containing the enzyme was resuspended in 0.2 M acetate buffer, pH 5.2, and analysis of enzyme activities (Endoglucanase, Filter Paper and Cellobiase) as well as protein concentration analysis was performed as described above.

Precipitation with Acetone

Precipitation of enzymes using acetone was performed as was conducted with ethanol described above. However, the acetone used had 99.5% (v/v) and was added until reaching a saturation of 60% (v/v), according to Mawadza et al. (2000).

Concentration by Nanofiltration

The enzymatic extract was concentrated by nanofiltration in a cylindrical cell under stirring, using a NP010 Microdyn Nadir membrane (Microdyn-Nadir, Wiesbaden, Germany) with average diameter of 1.4857 nm, nominal cut-off molecular weight of 1 kDa and permeability of 6.47×10^{11} m/Pa.s. Initially the membrane was conditioned with water at a pressure of 3000 kPa and a temperature of 2 °C. After conditioning of the membrane, 100 mL of the enzymatic extract were filtered using a pressure of 2000 kPa and temperature of 2 °C until approximately 90 mL were collected. Tests were later performed to determine the enzymatic activities (endoglucanase, filter paper and cellobiase), the enzyme concentration and the recovery percentage.

Characterization: Temperature and pH Profile

The enzymes, previously precipitated (after determining the best recovery method), were characterized regarding their pH and temperature profile. Optimal temperature and pH for enzymatic activities were determined using a central composite rotational design (Rodrigues and Iemma, 2005). Four CCRD (central composite rotational design) were performed with two independent variables (temperature and pH) for the enzymatic activity studies. Enzymatic activities were determined in function of temperature and pH using the methodologies described previously. The software Statistic 6.0 was used to analyze the results. The temperature range studied varied from 43°C to 57°C and the pH varied from 4.7 to 5.7.

Purification by Fast Protein Liquid Chromatography

After defining the best recovery method (precipitation and/or concentration) and characterizing the enzymes with regards to their pH and temperature profiles, the enzymes present in the precipitated enzymatic extract were purified by ion exchange chromatography using fast protein liquid chromatography (FPLC, Pharmacia Biotech, Piscataway, NJ, USA). Two resins were tested: Streamline-DEAE and Q-Sepharose (Pharmacia Biotech, Piscataway, NJ, USA) both mounted with bed volume of 10 mL. Several tests were performed and the following operating conditions were established: injection of 10 mL of enzyme (60% acetone-precipitated and resuspended in 0.2 M acetate buffer, pH 5.2), a flow gradient from 0 to 1 M NaCl in 0.05 M phosphate buffer, pH 7, and 2 mL fractions were collected.

Electrophoresis (SDS-PAGE)

Electrophoresis (SDS-PAGE) was used to separate the proteins and to estimate their molecular weight. This process was performed in 12% (w/v) polyacrylamide gel according the protocol proposed by Laemmli (1970). Gel was stained by silver nitrate. Samples were denatured in sample buffer at 99 °C for 5 minutes. Mixture of proteins of high molecular weight (HMW electrophoresis standard,

Sigma-Aldrich, St. Louis, MO, USA) was used as molecular weight standard.

Identification of Peptides by Mass Spectrometry

About 500 µL of eluted fractions from chromatography (FPLC) were precipitated with 10% trichloroacetic acid and resuspended into 100 µL of 0.050 M ammonium bicarbonate. Proteins were then digested with a trypsin solution 20 ng/µL in 0.001 M calcium chloride and incubated for 16 h at 37 °C. The reaction was stopped by adding 0.1% formic acid and aliquots were stored at -20 °C. For the identification of peptides, samples were analyzed by liquid chromatography coupled with mass spectrometry (LC-MS/MS, Applied Biosystems, Foster City, CA, USA) at a flow rate of 0.6 µL/min. The gradient was 2% to 90% acetonitrile in 0.1% formic acid over 60 minutes. The spectra were acquired using the software MassLynx v.4.1 (Waters, Milford, MA, USA) and the raw data files were converted to a peak list format (mgf), without summing the scans, by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science Ltd.). These were then searched against the CAZy Database using Mascot v.2.3.01 engine (Matrix Science Ltd.), with carbamidomethylation as the fixed modification, oxidation of methionine as a variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursors and fragment ions. Only peptides with a significance threshold of $p<0.05$ (Mascot-based score) were considered as a product of peptide cleavage (Franco Cairo, 2011).

RESULTS AND DISCUSSION

Recovery: Precipitation and Concentration of Enzymes

The microorganism, *Acremonium strictum* AAJ6, was subjected to fermentation in culture medium containing commercial cellulose (Avicel) to induce the production of the cellulase enzyme complex. After 240 h, the fermentation broth was centrifuged to remove the biomass and was subjected to studies of precipitation and concentration of enzymes present in the crude enzymatic extract. For recovery of enzymes present in the crude enzymatic extract several methodologies were tested, including ethanol precipitation (saturation of 70%), acetone precipitation (saturation of 60%) and concentration by nanofiltration with membrane cut-off of 1 kDa (NP010). The results obtained using these different methodologies were tested with regards to percentage of enzyme recovery and purification factor, as shown in Table 1.

Analyzing Table 1, it is verified that the methodology employing acetone (60% saturation) for the precipitation of enzymes presented the best results of enzyme recovery, registering 80.67% recovery of endoglucanases (CMCase), 65% of FPase and 25% of cellobiase. The calculated purification factor was the highest (8.8) recorded for endoglucanase activity and also presented the highest recovery percentage (80.67%). These values recorded in acetone precipitation were quite satisfactory and superior too many reported in the literature. Mawadza et al. (2000) used a methodology quite similar, employing acetone (55% saturation) for precipitation of cellulases from *Bacillus sp*. For the particular strain of *Bacillus* CH43, a 59% recovery was obtained but with a purification factor of only 0.6. For the strain named HR68, the average recovery was 64.5% and purification factor 2.5.

Kavian et al. (1999) studied different precipitation methods for cellulases produced by *Lumbricus rubellus* and *Trichoderma viride*, utilizing precipitation with acetone and ammonium acetate. However, when employing acetone precipitation for the enzyme extract, purification factors of 2.24 and 4.75 were registered for *Lumbricus rubellus* and *Trichoderma viride*, respectively. These values were lower than those recorded for enzymes produced by the microorganisms in the present study (*Acremonium strictum*) where 60% acetone was used for precipitation of endoglucanases (purification factor was up to 8.8). Thus, the precipitation process with acetone showed to be efficient and can be employee for recovery of cellulases produced by *Acremonium strictum.*

Precipitation with ammonium sulfate was also tested in different saturations (70, 80 and 90%), but in no condition studied was it possible to verify the precipitation of enzymes (data not shown). This method is very frequently cited in literature for precipitation of cellulases (Kavian et al., 1999; Sanwal 1999; Ye et al., 2001) however for the enzymes recovered in the present study it was not satisfactory. The enzyme precipitation methodology employing ethanol is also widely used in the recovery of several enzymes; however, in the present study, it showed the worst results of recovery for the enzymes, registering average recoveries of 12.25% for CMCase, 11.67% for FPase and only 8% for cellobiase (Table 1). Among the two organic solvents tested for precipitation of cellulases from *Acremonium strictum*, results with acetone prevailed over those obtained using ethanol.

In the nanofiltration process, by not using solvent it was expected to obtain satisfactory values of enzyme concentration and recovery, but this was not verified. As can be seen in Table 1, in this process a recovery percentage of 35% was obtained for CMCase, 23.33% for FPase and 20% for Cellobiase. These values are greater than those registered for precipitation with

Table 1. Comparison of results between the different methods employed for precipitation and recovery of enzymes from *Acremonium strictum*

	*Total Activity (U)	*Specific Activity (U/mg)	Factor of Purification	Percent Recover (%)
Crude Enzymatic Extract				
CMCase	4.00	0.139	1	100
FPase	0.60	0.021	1	100
Cellobiase	0.20	0.007	1	100
Precipitation: 70% Alcohol				
CMCase	0.49	0.225	1.62	12.25
FPase	0.07	0.032	1.54	11.67
Cellobiase	0.02	0.007	1.06	8.00
Crude Enzymatic Extract				
CMCase	3.00	0.119	1	100
FPase	0.40	0.016	1	100
Cellobiase	0.20	0.008	1	100
Precipitation: 60% Acetone				
CMCase	2.42	1.057	8.88	80.67
FPase	0.26	0.114	7.15	65.00
Cellobiase	0.05	0.022	2.75	25.00
Crude Enzymatic Extract				
CMCase	2.00	0.078	1	100
FPase	0.60	0.023	1	100
Cellobiase	0.10	0.004	1	100
Nanofiltration: NP010 (1 kDa)				
CMCase	0.70	0.203	2.61	35.00
FPase	0.14	0.041	1.74	23.33
Cellobiase	0.02	0.006	1.49	20.00

*Analyzes were performed in duplicate; standard deviations < 5%

ethanol, however lower than those obtained for precipitation with acetone.

Characterization Enzymatic: Temperature and pH Profile

After determining the best recovery method, the enzymes were characterized with regards to their pH and temperature profiles. The optimal temperature and pH of the enzymes were determined using a central composite rotational design, as can be visualized in the Table 2 which presents the matrix of the CCRD (central composite rotational design), consisting of the tests, independents variables and their responses (enzymatic activities). To analyze the effects of the central composite rotational design and verify that there was no significant difference at the 5% significance level ($p<0.05$), Pareto charts were constructed (Figure 1). The temperature and pH studied in the CCRD did not influence the endoglucanase activity at 5% significance, as can be observed in Figure 1a, where the effects of the parameters were lower than the “critical t” (2.57). The critical t-value provides the cut-off probability for observation of a t-distribution at the significance level of 5% ($p<0.05$). Thus, it can be verified for the pH range of 4.7 to 5.7 and temperature range of 43 to 57°C, the values obtained for CMCase activity of *Acremonium strictum* do not statistically differ ($p<0.05$).

Observing Figure 1b, the linear pH and quadratic pH presented significant effects at the significance level of 5% for determining filter paper activity. The interaction of temperature and pH was not significant ($p<0.05$), however when analyzing the results in Table 2 it is observed that enzyme activity at the axial points had an influence on the interaction of temperature and pH, not discarding this effect for the calculation of regression coefficients.

For cellobiase activity, 3 parameters were considered significant ($p<0.05$) for determining the regression coefficients, which are: temperature, linear pH and the interaction between pH and temperature (Figure 1c). The

Table 2. Matrix of the CCRD (central composite rotational design) to determine the optimal temperature and pH of enzymes studied

Tests	pH	Temperature (°C)	*CMCase (U/mg)	*FPase (U/mg)	*Cellobiase (U/mg)	*β-glucosidase (U/mg)
1	(-1) 4.9	(-1) 45	0.614	0.258	0.285	0.567
2	(+1) 5.5	(-1) 45	0.853	0.220	0.276	0.549
3	(-1) 4.9	(+1) 55	0.548	0.272	0.505	0.729
4	(+1) 5.5	(+1) 55	0.698	0.052	0.368	0.650
5	(-1.41) 4.7	(0) 50	0.455	0.314	0.342	0.644
6	(+1.41) 5.7	(0) 50	0.638	0.056	0.295	0.688
7	(0) 5.2	(-1.41) 43	0.398	0.038	0.239	0.585
8	(0) 5.2	(+1.41) 57	0.445	0.019	0.549	0.641
9	(0) 5.2	(0) 50	0.516	0.066	0.365	0.644
10	(0) 5.2	(0) 50	0.578	0.060	0.332	0.671
11	(0) 5.2	(0) 50	0.534	0.056	0.356	0.632

*Analyzes were performed in duplicate; standard deviations < 5%

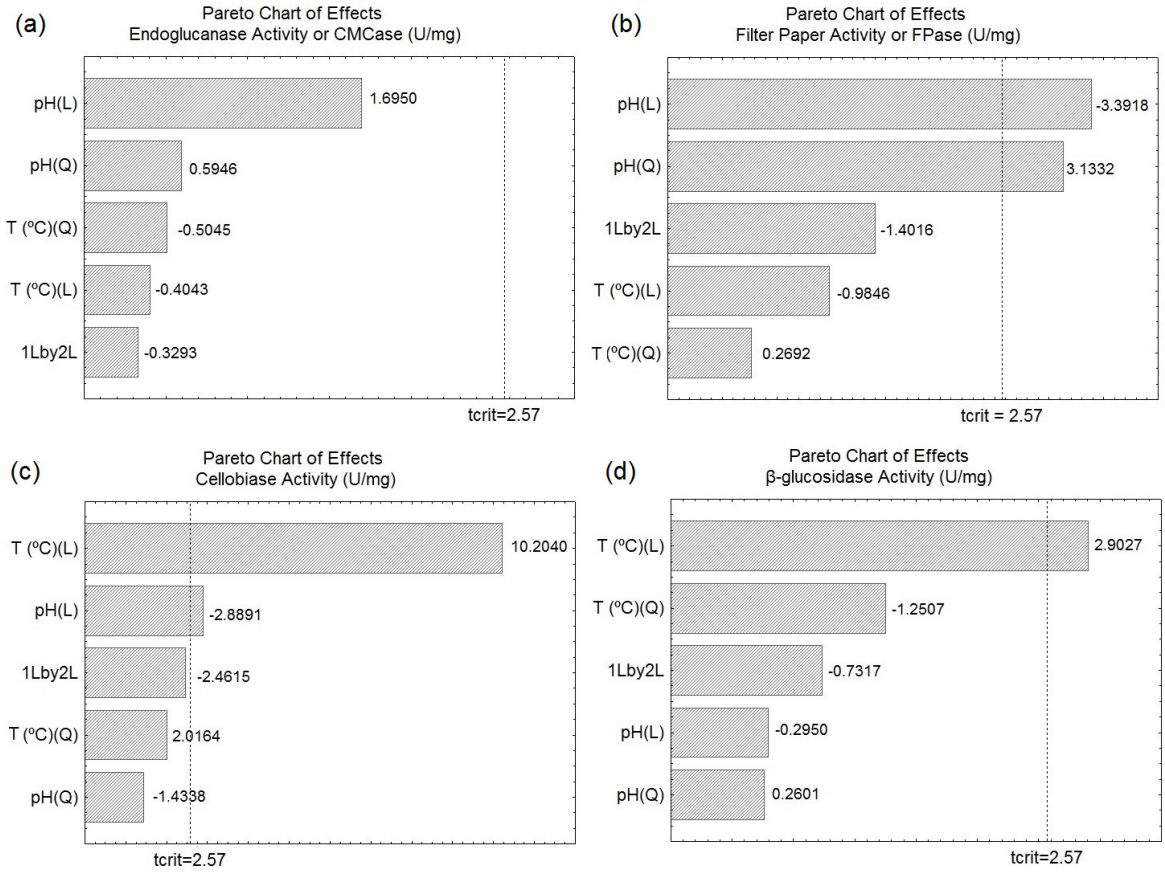

Figure 1. Pareto charts of the effects of pH and temperature, liner (L) and quadratic (Q), on enzymatic activity by *Acremonium strictum*: (a) Endoglucanase activity or CMCase; (b) Filter paper activity or FPase; (c) Cellobiase activity and (d) β-glucosidase activity

parameters of quadratic pH and temperature were ignored. Figure 1d presents the Pareto chart for analysis

Table 3. Analysis of variance (ANOVA) of the enzymatic activities what showed significant differences ($p<0.05$) at determination of pH and temperature profiles

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_{cal}/F_{tab}	R^2
Filter Paper Activity					
Regression	0.099817	3	0.033272	2.102	79.67
Residual	0.025471	7	0.003639		
Total	0.125288	10			
Cellobiase Activity					
Regression	0.080128	3	0.026709	4.687	89.73
Residual	0.009169	7	0.00131		
Total	0.089297	10			
β-glucosidase Activity					
Regression	0.012637	1	0.012637	1.677	52.52
Residual	0.013235	9	0.001471		
Total	0.027873	10			

F_{cal}=F calculated; F_{tab}=F tabulated; p-value < 0.00001

of the effects on β-glucosidase activity, registering significant effects at the level of 5% significance for only the linear temperature parameter.

To determine the regression coefficients, only significant effects ($p<0.05$) were considered. In order to check the validity of the regression coefficients and mathematical models, an analysis of variance (ANOVA) was performed, as shown in Table 3.

It can be observed that the value of F calculated for the regression, considering all enzymatic activities, was higher than the value of F tabulated, and the percentage of variation (R^2) explained for the 3 models was greater than 79.67, 89.73 and 52.52%, respectively. The first two models showed a very good coefficient of variation, and it can thus be probable that the values predicted by the model fit to the experimental values, validating the models. However, the coefficient of variation for the third model (β-glucosidase activity), due to the very low value (52.52%), cannot be considered valid because it does not fit well to the predicted model.

The validated models, with the coded variables, represent enzymatic activity (U/mg) as a function of temperature and pH for the enzymes from *Acremonium strictum* that are represented by the following equations, respectively:

Filter paper activity = 0.068 – 0.078 pH + 0.083 pH^2 – 0.046 pH x Temperature (Eq. 1)

Cellobiase activity = 0.356 – 0.027 pH + 0.094 Temperature – 0.032 pH x Temperature (Eq. 2)

Having defined the models, contour curves (Figure 2) were constructed in order to obtain the best temperature and pH conditions for the enzymatic reactions. From these curves it can be observed that the optimal temperature and pH varies with the enzyme under consideration. In the present study the pH and temperature ranges were not excessively large, pH (4.7 to 5.7) and temperature (43 to 57°C), precisely the range that literature considers ideal (Chi et al., 2009; Castro and Pereira, 2010) in order to verify even small variations can cause large differences to the process. According to Chi et al. (2009) the optimal pH of purified cellulases from fungi is in the range of pH 5.0 to pH 6.0 and optimal temperature from 40 to 60°C.

Filter paper activity of cellulases produced by *Acremonium strictum* showed an optimum temperature range of approximately 50 to 57°C and pH of 4.7, as can be visualized in Figure 2a, where the highest enzymatic activity is in the darker region of the graph corresponding to values near 0.4 U/mg. To validate this result, a temperature of 55°C and pH of 4.7 were tested, obtaining an average FPase activity of 0.386 U/mg. Almeida (2009) studied cellulases and hemicellulases from species of *Acremonium* endophytes and detected the greatest endoglucanase activity from *Acremoniun sp. EA0810* at pH 5.0 and 70°C, while for FPase activity, optimal pH and temperature were pH 6.0 and temperature 55°C.

A similar behavior to that obtained for filter paper activity was observed for the cellobiase activity, whose optimum conditions were in the temperature range of 54 to 57°C and pH range of 4.7 to 5.2, as verified in Figure 2b. Assays in triplicate of cellobiase activity at a temperature of 55°C and pH of 4.7 were also performed to validate the results, observing an average activity of 0.562 U/mg. These results are similar to the values found by Quin et al. (2008) who studied the purification and characterization of endoglucanases of *Trichoderma*

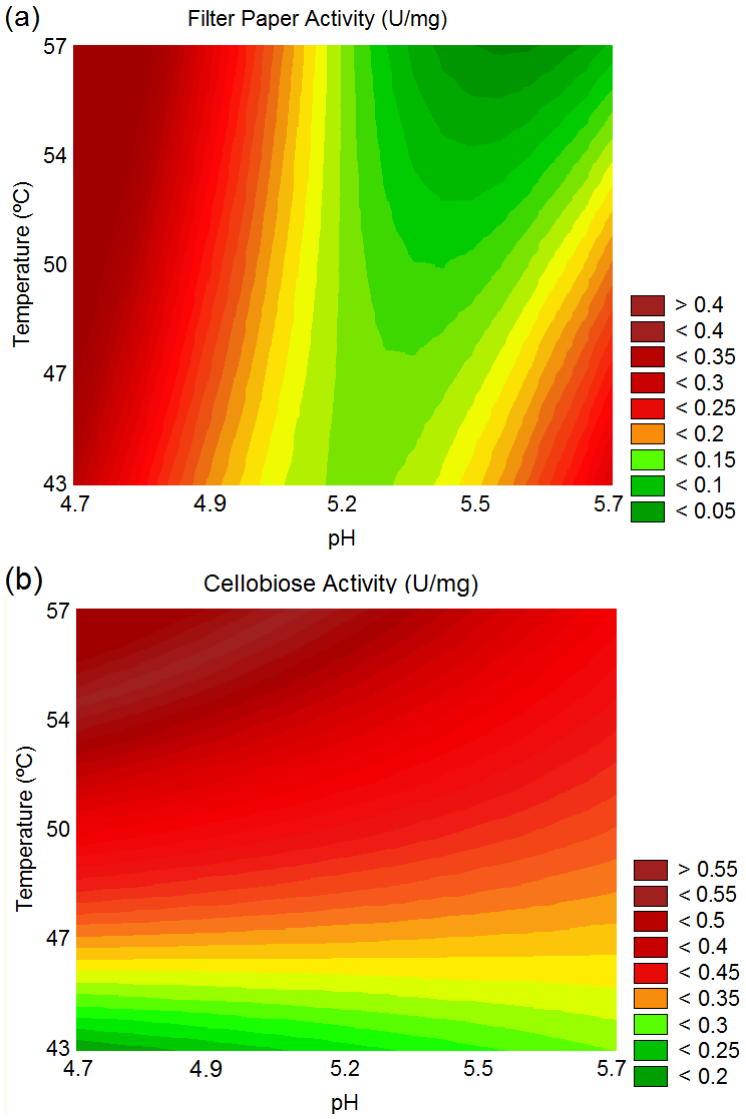

Figure 2. Contour curves generated in the central composite rotational design (CCRD) to determination of enzymatic activity as a function of pH and temperature: (a) Filter paper activity and (b) Cellobiase activity; p-value < 0.00001

reesei and obtained an optimum pH range of 4.6 to 5.0 and optimum temperature of 50℃. Regarding β-glucosidase produced by *Acremonium strictum*, the model was not considered valid and therefore it was not possible to analyze the contour curve, however, when analyzing the results obtained in the Pareto chart (Figure 1d), and the results of Table 2, it is understood that the only parameter that directly influence β-glucosidase activity was temperature. Higher temperatures are required for greater enzyme efficiency, from 55 to 57℃, while the pH range studied did not affect β-glucosidase activity at the significance level of 5%.

As can be observed in Figures 2a and 2b, the contour curves for both cellulolytic enzymes presented optimum temperatures which were at the maximum of the temperature range studied in the experimental design. Regarding pH, there is a predominance of more acid pH levels. This directs further studies for characterizing these enzymes which should utilize higher temperatures and more acidic pH levels. This characteristic of cellulases presenting activity at high temperatures may be of interest to the textile and detergent industries because it

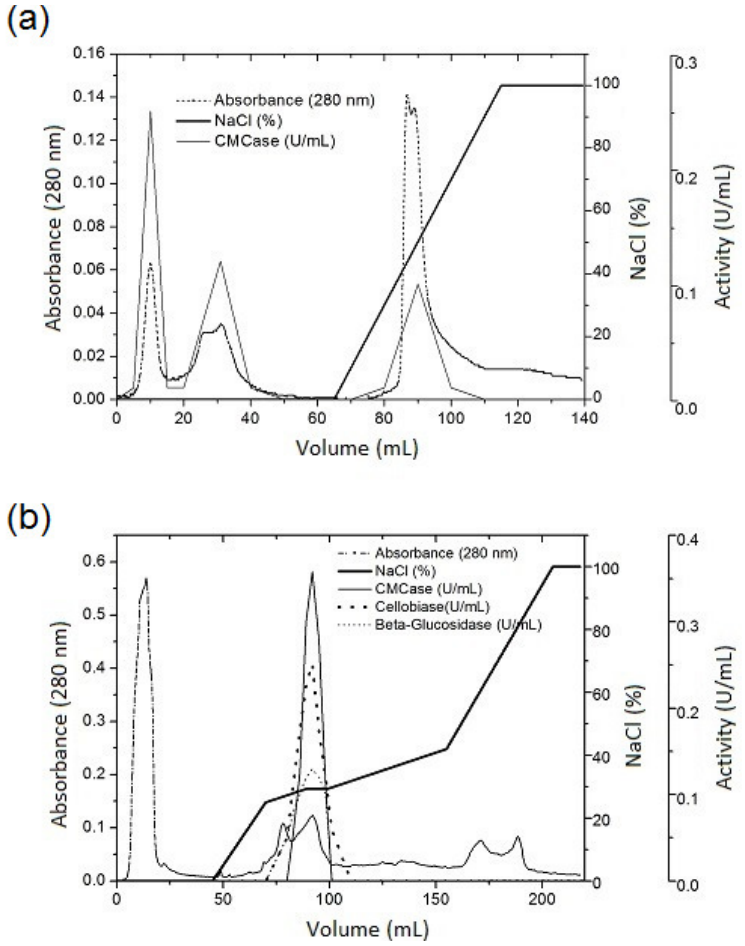

Figure 3. Chromatogram obtained by fast protein liquid chromatography (FPLC) for purification of enzymes present in the enzymatic extract from *Acremonium strictum*: (a) Streamline-DEAE resin and (b) Q-Sepharose resin

can be easily inactivated at room temperature after utilization (Chi et al., 2009). In general, cellulases produced by filamentous fungi have optimal pH values in the acidic range (3.6 to 5.0), and optimum temperature around 50°C (Castro and Pereira 2010).

Purification and Identification of Enzymes

Celluloltytic microorganisms produce complex enzyme systems which present considerable fractionation problems. Ion-exchange chromatographic methods have been used to separate and purify them. In this work, the enzymes present in the previously precipitated enzymatic extract were purified by ion exchange chromatography using fast protein liquid chromatography (FPLC), and two resins were tested: Streamline-DEAE and Q-Sepharose. Figure 3a and 3b exhibit the chromatograms obtained by FPLC during purification of enzymes present in the enzymatic extract (precipitated with acetone 60%) using the Streamline-DEAE and Q-Sepharose resins, respectively.

Analyzing the chromatogram of Figure 3a we can observe five recorded absorbance peaks, of which three peaks were recorded in the washing step. Following the initiation of NaCl gradient, in the saturation range of 40-50%, 2 more peaks were detected. Subsequently the fractions were analyzed for the activities of endoglucanase (CMCase) as shown in Figure 3a, where the major peaks of activity were recorded in the washing step, demonstrating that most of the enzymes did not adhere to the resin under study (Streamline-DEAE) and were eluted during the washing step. The resolution of the chromatogram during implementation of the NaCl

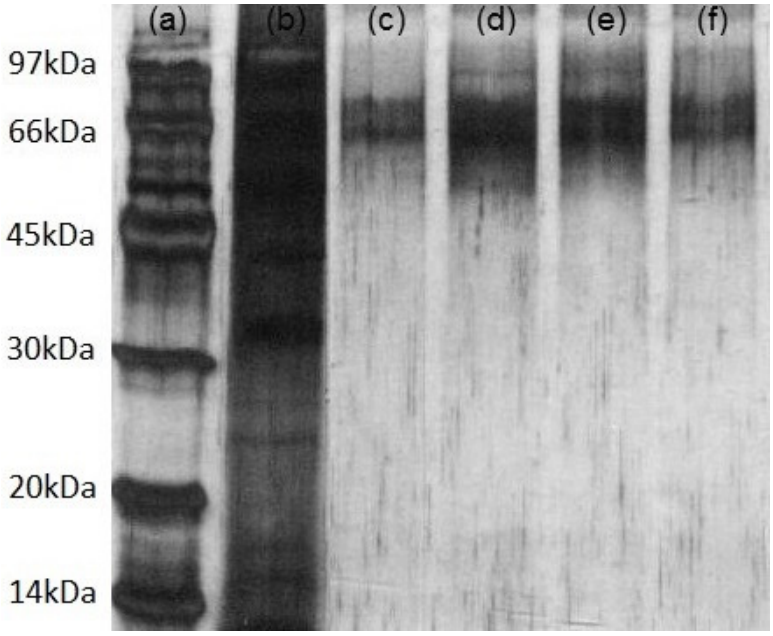

Figure 4. Electrophoresis gel (stained by silver nitrate) of cellulases from *Acremonium strictum.* *Bands (a) to (f) represents: (a) standard; (b) crude enzymatic extract; (c to f) fractions collected from the chromatograph (FPLC) when using the Q-Sepharose resin, where maximum enzymatic activity was recorded

gradient was also not satisfactory, since two peaks were partially overlapped. This demonstrates the low efficiency of Streamline DEAE resin for purification of cellulases present in the enzymatic extract produced by *Acremonium strictum*.

The second resin tested was Q-Sepharose. This resin presented good results in the initial tests; however several assays were performed to generate a satisfactory degree of purity. As can be observe in Figure 3b, in the fractions collected by FPLC the three enzymatic activities were analyzed (endoglucanase, cellobiase and β-glucosidase) where all presented peaks in activity with 29% NaCl saturation, with no significant losses during washing which indicates the adherence of the enzyme to the resin employed (Q-Sepharose). Quin et al. (2008) who studied the purification of endoglucanases of *Trichoderma reesei* using ion-exchange chromatography (CM-Sepharose resin), obtained a separation profile very similar to that recorded in the chromatogram of the present study, as can be seen in Figure 3b, presenting a single peak of CMCase activity and retaining 80% of the original CMCase activity.

Considering Figure 3b, not only was CMCase activity determined, but it was also possible to determine the activity of β-glucosidase and cellobiase. With analysis of fractions collected by chromatography, it was possible to observe that the enzymes showed maximum activity in a single absorbance peak. Other tests were performed to extend the period of the salt gradient, however the same pattern was always observed. Thus, we can note that the enzymes analyzed presented a very similar molecular weight. To better visualize these results and estimate the molecular weight of enzymes, gel electrophoresis SDS-PAGE was carried out, as can be visualized in Figure 4.

Analyzing Figure 4, one can see that the fractions collected by chromatography, although quite thick, appeared in only a single band, different from what occurred with the crude enzymatic extract which was very concentrated. This caused the dark color of the gel, but even so it is possible to observe the presence of many bands. However, it can be concluded that the ion-exchange chromatography was a very efficient method for the purification of proteins present in the enzymatic extract produced by *Acremonium strictum.*

From the electrophoresis gel used to separate the enzymes according to molecular weight, it is possible to determine the approximate molecular weight of the cellulase complex present in the extract produced by *Acremonium*, which was approximately 70 kDa. Almeida (2009) performed the zymogram analysis of the crude enzyme from *Acremoniun sp. EA0810* and detected endoglucanases of approximately 61.6 kDa and

Table 4. Peptides identified using a database containing all non-redundant proteins derived from the CAZy database

Peptide	Protein	Nunber
K.LVYTIAK.S	β-D-glucoside glucohydrolase (*Hyprocrea jecorina*)	AAA18473
	β-D-glucoside glucohydrolase (*Trichoderma viride*)	AAQ76093
	β-D-glucoside glucohydrolase I (*Trichoderma viride*)	ACS93768
	β-glucosidase (*Trichoderma sp. SSL*)	ACH92574
K.HYILNEQELNR.E	β-D-glucoside glucohydrolase (*Hyprocrea jecorina*)	AAA18473
	β-D-glucoside glucohydrolase (*Trichoderma viride*)	AAQ76093
	β-D-glucoside glucohydrolase I (*Trichoderma viride*)	ACS93768
R.TDIGGLYR.L	endoglucanase Cel74 (*Hypocrea jecorina*)	AAP57752
	hypothetical protein (*Podospora anserina*)	CAP66717
	hypothetical protein (*Magnaporthe grisea*)	EAA48924
R.HFDANGIEPR.F	β-glucosidase (*Schizophyllum commune*)	AA33925
R.LESYNYPGR.Y	putative glycosylhydrolase (*Streptomyces scabei*)	CGB68787
R.TLLESVESR.L	putative protein glucan (*Diaprepes abbreviatus*)	AAV68692
R.ALFGLMWAFPGR.K	1,4-alpha-glucan branching enzyme (*Azoarcus sp. BH72*)	CAL94413
R.KGDTDIFR.T	glycosise hydrolase family 57 (*Denitrovibrio acetiphilus)*	ADD67236
R.EAEFTLPEK.L	oligo-1,6-glucosidase (*Paenibacillus sp.)*	AAQ91295

xylanases of about 27.8 kDa. Zhou et al. (2008) studied the identification and purification of the main components of cellulases from a mutant strain of *Trichoderma viride* and found a wide variation range of molecular weights (21 kDa to 110 kDa) and the pI (isoelectric point) ranged from 4.2 to 6.2. Among the enzymes identified, the Cel7a (CBH I) presented weight molecular of 67 kDa and pI 4.2. The variations in molecular weight and pI are closely linked to the family of which the enzymes belong for example; β-glucosidase typically has a higher molecular weight than endo- or exoglucanase.

The enzymes purified were subjected to trypsin digestion and analyzed by liquid chromatography coupled with mass spectrometry (LC-MS/MS) for the identification of peptides present in the samples. The spectra were analyzed by Mascot Ions-Search Software (Matrix Science, London, GB, UK) for protein identification using a database containing all non-redundant proteins derived from the CAZy website, a specialized database (up to 67,000 protein sequences) that describes the families of structurally related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify or create glycosidic bonds (Cantarel et al., 2009). After spectra acquisition, using a stringent cut-off (cut-off >32; significant at $p<0.05$) allowed the explicit assignment of 9 unique peptides as glycoside hydrolases and hypothetical proteins from microorganisms next the phylogeny (Table 4).

For identification of peptides present in the samples a confidence criterion of two or more unique peptides of different peptide sequences was used (Marcotte 2007). Two potential cellulolytic enzymes were identified using the CAZy database: endoglucanase Cel74 and β-glucosidase. The sequence of peptide R.TDIGGLYR.L (Table 4) presented the confidence criterion for identification of peptides and was reported in the literature as a member peptide of endoglucanase Cel74 from *Hypocrea jecorina*. Since the sequences K.LVYTIAK.S and K.HYILNEQELNR.E (Table 4) are reported in the literature as member peptides of β-glucosidase (~78 kDa) found in the microorganisms *Hyprocrea jecorina* and *Trichoderma viride.* They also displayed the same confidence criterion mentioned above. Chhabra and Kelly (2002) studied the biochemical characterization of *Thermotoga maritima* endoglucanase Cel74, and reported that Cel74 is encoded by an open reading frame of 2124 bp corresponding to a polypeptide of 79 kDa with a signal peptide at the amino-terminus.

Franco Cairo et al. (2011) performed the metaproteomic analysis from the whole body of the lower termite *C. gestroi*. The proteomic approach identified a large number of polypeptides in the *C. gestroi* digestome. Potential cellulolytic enzymes were identified using the same criterion used in our study, such as the match to probable β-glucosidase (family GH3) from *Dictyoglomus turgidum*, two GH7 protein matches, one derived from *Pleurotus sp*. and another from the uncultivated symbiotic protist *Hodotermopsis sjoestedti.* And GH9 matches from *Reticulitermes flavipes* and *C. formosanus,* which are endogenous cellulases from termites.

CONCLUSION

Our findings show that the method utilizing 60% acetone for enzyme precipitation was the one which presented the best recovery percentages, registering 80.67% for endoglucanases (CMCase), 65% for filter paper activity (FPase) and 25% for cellobiase. Regarding characterization, the results showed that to endoglucanase activity, the temperature and pH had no significant difference at the significance level of 5%, among the range of values studied. For activity of FPase and cellobiase, optimum temperature was 55°C and optimum pH was 4.7. While that β-glucosidase enzyme, only temperature significantly influenced enzyme activity, favoring higher temperatures (55 to 57 °C). The molecular weight of the cellulase enzyme complex produced by *Acremonium strictum* was approximately 70 kDa. The peptides identified in the mass spectrometry were searched against the CAZy database and two potential cellulolytic enzymes were identified: endoglucanase Cel74a and β-glucosidase. This work shows not only studies of enzymatic characterization, but also the importance of the biodiversity exploitation for the identification of new microorganisms and new enzymes with potential application biotechnological.

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