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RESEARCH ARTICLE

Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium

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Keywords: *B. adusta*, cellulases, *P. sanguineus*, wheat straw, xylanases.

Abbreviations: CMC: carboxymethylcellulose
DNS: dinitrosalycilic acid
PA: polyacrylamide

Cellulolytic properties of two white rot fungi, *Bjerkandera adusta* and *Pycnoporus sanguineus*, cultivated on wheat straw agar medium, were characterized and compared. Optimal growing parameters for maximum enzyme production for both fungi were wheat straw medium pH 5 and 28ºC. *B. adusta* showed, on the 6th day of culture, carboxymethylcellulose (CMC)ase activity levels 1.6 times higher than maximal *P. sanguineus* activity, achieved on the 8th day. *B. adusta* supernatants also displayed higher activity levels towards xylan (3.6-fold) compared to those of *P. sanguineus*. However, enzymes from *P. sanguineus* were more robust resisting one hour incubation at high temperatures (up to 80ºC), and exhibiting activity and stability in pH range from 2 to 8. Cellulolytic activities, with molecular masses ranging from 25 to 90 kDa, from the two species were detected in zymograms.

White rot fungi have the capacity to exploit all wood components due to the secretion of a variety of lignocellulolytic enzymes (Perez et al. 2002; Martínez et al. 2005). Much attention has been focused on finding such hydrolytic activities given their potential use in different industries such paper biopulping, human and animal feeding, and the production of cellulosic bioethanol

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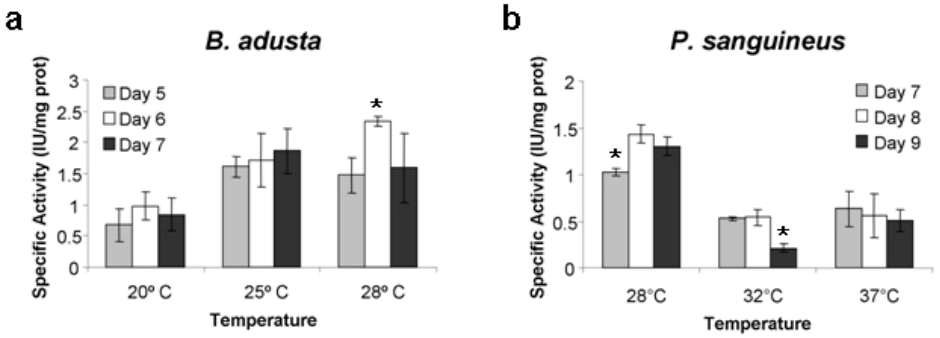

Figure 1. Influence of different growing temperatures at different times of culture on CMCase production by *B. adusta* (a) and *P. sanguineus* (b). Asterisks indicate statistically significant differences ($P < 0.05$, ANOVA) within each group of days analyzed.

(Alborés et al. 2006; Lee et al. 2007; Doi, 2008). Bioethanol is produced mainly by fermenting sucrose or glucose from sugarcane, sugar beets and corn. Given that not all countries overproduce these feedstocks, other carbohydrate sources are being explored (Kim and Dale, 2004; Bayer et al. 2007). Vegetal biomass is composed of three main polymers, lignin, cellulose and hemicelluloses. Cellulose is organized as glucose polymeric long lineal chains, arranged in different levels of fibrils that are associated by intramolecular interactions. Hemicelluloses, in contrast, are ramified polymers composed of different types of carbohydrates and phenolic compounds, among which xylan, glucan and arabinoglucan form the sugar backbone of the structure. Because sugar production from biomass is a key limiting step in industrial processes, harsh physical-chemical pretreatments (such as steam explosion, with or without diluted sulfuric acid) are used to loosen lignin and release fibrils of cellulose and monomers of hemicellulose components (Wyman et al. 2005). Pre-treatment also decreases the recalcitrance of crystalline cellulose by generating pores on its structure making it more accessible to hydrolytic enzymes (Nguyen et al. 2000; Nagle et al. 2002; Söderström et al. 2002). The holocellulose (cellulose and hemicellulose) can be then completely saccharified by a number of enzymes due to the synergism displayed by combinations of endo- and exo-glucanases and β-glucosidases (Nidetzky et al. 1994). Industrially-produced biomass sugars require large-scale cellulose degradation for fuel production. Traditionally, commercial cellulases preparations from *Trichoderma* have been used, and most studies regarding fungal cellulose degradation have been carried out in this genre and other ascomycetes (Gerhardt et al. 2007; Villena and Gutiérrez-Correa, 2007). However, contrary to ascomycetes, basidiomycetes are organisms specialized in wood degradation, and it is surprising that little work has been done regarding the characterization of basidiomycetes cellulolytic capacities. It is for this reason, important to establish the whole cellulolytic capabilities of wood-degrading fungi. Given that harsh pretreatments are used to obtain sugars from lignocellulose, it is important to screen for robust cellulolytic activities that can be efficient at high temperatures or extreme pH values that match industrial conditions.

In this work we analyzed the cellulolytic properties of two white rot fungi, *Bjerkandera adusta* and *Pycnoporus sanguineus*, originally isolated from two different geographical locations. *B. adusta* strain UAMH 8258, a basidiomycete well known for its elevated ligninase activity (Wang et al. 2003), and unable to grow at 37ºC, was isolated from temperate woods of the northern hemisphere. *P. sanguineus* CEIBMD01, a fungal strain able to grow at moderately high temperatures given its tropical origin (Dantán-González et al. 2008) was isolated from an oil-polluted environment in the south of the state of Veracruz (México).

In this work, culture conditions and levels of cellulolytic enzymes production by *B. adusta* and *P. sanguineus* grown in wheat straw medium were compared. Zymogram analyses permitted the detection of a number of bands with cellulolytic and hemicellulolytic activities expressed by both fungi.

MATERIALS AND METHODS

Strains

Bjerkandera adusta UAMH 8258 was kindly provided by Dr. R. Vazquez-Duhalt (Wang et al. 2003). *Pycnoporus sanguineus* CEIBMD01 was growing on a dead tree bark covered with petroleum spill when collected (Dantán-González et al. 2008).

Culture conditions

To ensure that the fungi only used wheat straw as the sole carbon source, they were firstly grown at 28ºC in pre-cultures of GMY medium (1% glucose, 0.35% malt extract, 0.25% yeast extract, 0.2% KH_2PO_4 and 0.05% $MgSO_4x7H_2O$, 1.5% agar, pH 4.5 adjusted with phosphoric acid) until mycelia had filled the Petri dish. A passage was

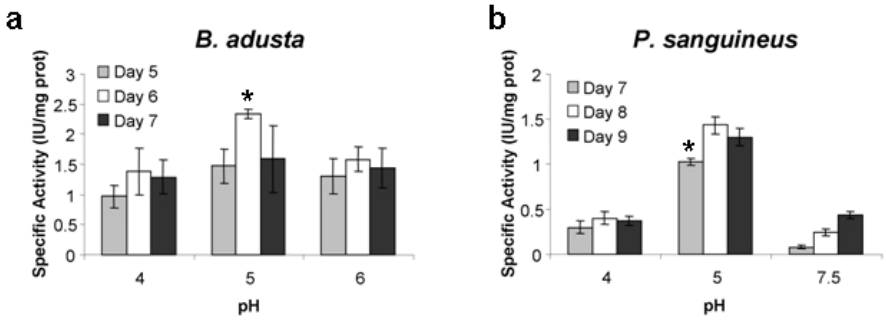

Figure 2. Influence of pH of the culture media at different times of culture on CMCase production by *B. adusta* (a) and *P. sanguineus* (b). Asterisks indicate statistically significant differences ($P < 0.05$, ANOVA) within each group of days analyzed.

then performed with 0.25 cm^2 inoculums placed in the center of another Petri dish containing a modified (Inglis et al. 2000) mineral base medium (7.8 mg/L $CuSO_4$ x $5H_2O$, 18 mg/L $FeSO_4$ x $7H_2O$, 500 mg/L $MgSO_4$ x $7H_2O$, 10 mg/L $ZnSO_4$, 50 mg/L KCl, 1 g/L K_2HPO_4 and 2 g/L NH_4NO_3, 1.5% agar; pH was adjusted to 5, or 4 and 6 -when required- with phosphoric acid) depleting and depriving the samples of a carbon source (mycelia in this medium were very faint and failed to develop after 5 days). Finally, for the experimental cultures, 0.25 cm^2 squares taken from the mineral base medium were placed in the center of a plate containing mineral base medium plus 2% powdered wheat straw as the sole carbon source. In this medium mycelia grew healthily to confluence. Wheat straw (*Triticum aestivum*) was pulverized in a coffee grinder (Braun) until a homogeneous powder was observed (with maximum and minimum particle sizes of 3 and 0.5 mm, respectively). Temperatures and periods of incubation varied according to each experiment (from 20 to 37ºC and from 1 to 12 days, respectively; see results).

Enzymatic assays and protein determination

Enzymatic activity and protein concentration were assayed from the supernatants obtained from the solid cultures as follows: agar media was cut into pieces for collection from the Petri dishes and placed in 50 ml centrifuge tubes, then centrifuged at 2,504 x *g* at 4ºC for 30 min; the volume recovered varied between 3 and 4 ml. Supernatants were clarified by filtration through 0.45 µm nitrocellulose filters (Pall). For enzymatic activity measurements the following substrates were used: 2% carboxymethylcellulose (CMC, Sigma), 2% microcrystalline cellulose (Avicel® PH-101, Fluka), 0.25% cellobiose (Sigma), and 2% oat spelt and birchwood xylans (Sigma), dissolved in 50 mM citrate buffer pH 5. Enzymatic reactions contained 200 µl of supernatant, 300 µl of 50 mM citrate buffer pH 5, plus 500 µl of each substrate solution. The reaction mixtures were incubated at 50ºC for 30 min. Reducing sugars were determined using the 3,5-dinitrosalycilic acid (DNS) assay according to Miller (1959). Briefly, 50 µl aliquots were taken every 5 min (after adding the supernatant to the reaction mixture) up to 45 min, then mixed with 50 µl of a DNS solution, boiled for 5 min and immediately cooled on ice for 5 min. Finally 500 µl of water were added and absorbance measured at 540 nm in a spectrophotometer (BioMate, ThermoSpectronic). Absorbance readings were compared to glucose or xylose standard curves ranging from 0.1 to 2 mg/ml; values were graphed against time, and the slope was calculated to determine the velocity of the reaction. Released reducing sugars vs. time were used to calculate enzymatic activities, considering 1 IU equivalent to 1 µmol of glucose or xylose released per min under the assayed conditions. For specific activity calculation, protein concentrations in mg/ml were determined by the Folin (Hycel de Mexico)-Lowry method (Lowry et al. 1951) with a bovine serum albumin (BSA) standar curve.

Optimal temperature and pH of CMCase activity

Enzymatic reactions were carried out as explained above at incubation temperatures between 30 and 90ºC. For optimal pH screening, a range from 3 up to 8 was tested with 50 mM sodium citrate buffer.

Thermal- and pH-stability of the cellulolytic enzymes

To test the heat stability of the cellulases in the crude supernatants, 200 µl of supernatant were incubated for 1 hr at different temperatures ranging from 30 to 90ºC. Treatments were stopped on ice and the remaining activity was measured at 50ºC and pH 5. For pH stability, 200 µl of supernatant were incubated in one volume of buffer solutions at pH values ranging from pH 2 up to 10 for 1 hr at room temperature. System buffers were McIlvaine (pH 2 and 3), sodium citrate (pH 4-8), and borate-HCl (pH 10). The remaining activity was measured at 50ºC and pH 5.

Statistical calculations

The general linear model (GLM) data analysis for this paper was generated using statistical analysis system (SAS) software 9.1 for Windows XP (SAS Institute Inc; Cary, NC, USA).

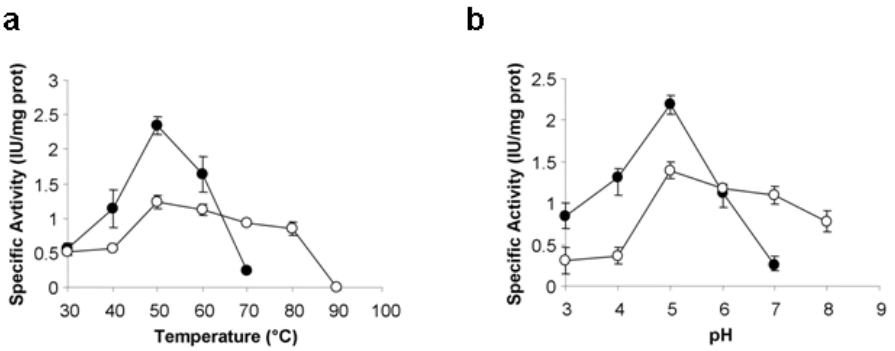

Figure 3. CMCase activity in culture supernatants at different reaction temperatures (a), and pH (b). Black circles, *B. adusta*; white circles, *P. sanguineus.*

Zymograms

Proteins in supernatants were precipitated at two different acetone concentrations: 20% and 80%. Pellets were dissolved in 100 µl (20%) and 2 ml (80%) of 50 mM citrate buffer. Protein concentration was quantified by the Folin-Lowry method, and 25 µg were run in 10% PAGE. For identification of CMCase activities, after electrophoresis, gels were treated as previously described (Mateos et al. 1992). Briefly, gels were washed three times (40 min each) in PCA buffer (50 mM KH_2PO_4, 50 mM citric acid pH 5.2). The polyacrylamide gel was laid on top of a 0.5% agarose gel containing 0.2% CMC, and incubated for 6 hrs at 30ºC in a humid chamber. Agarose gels were next stained with 0.1% Congo red for 30 min followed by one wash with 1 M NaCl. For xylanase activity detection, 10% SDS-polyacrylamide gels containing 0.2% birchwood xylan were run. Then the polyacrylamide (PA) gels were washed twice (30 min each) with 50 mM sodium acetate buffer pH 5.5 containing 25% isopropanol, and one wash without isopropanol (Lee et al. 1993), and then incubated for 5 min at 60ºC. Congo red staining of the PA gels was followed as mentioned above for agarose gels. Both, agarose and PA gels were submerged in 5% acetic acid for 10 min to convert the color of Congo red to purple, thus improving visibility of the bands. The molecular weight of the bands was estimated using a SDS-PAGE marker (Invitrogen).

RESULTS

Culture conditions for maximum CMCase activity production

Basidiomycetes fungi are excellent lignocellulolytic organisms. We were interested in establishing the optimal culture conditions for CMCase production of two species (*Bjerkandera adusta* and *Pycnoporus sanguineus*) isolated from very different geographical origins (cool forest the former and tropical the latter). Surprisingly, it was found that when the temperature of the growing cultures was varied, 28ºC was the optimum for CMCase activity production in both strains (Figure 1), even though *P. sanguineus* is able to grow better at more elevated temperatures (37ºC) (Dantán-González et al. 2008). *B. adusta* presented considerable levels of cellulase activity at 25ºC during the three days analyzed, reaching up to around 2 IU/mg (Figure 1a); on the oher hand, when *P. sanguineus* was grown at 32º or 37ºC the specific activity sharply decreased to less than half of that observed at 28ºC (Figure 1b). With respect to the pH influence of the culture medium, the highest cellulolytic activity from *B. adusta* was detected at pH 5 with no significant differences observed when grown at pH 4 and 6 (Figure 2a). Similarly, *P. sanguineus* preferred pH 5 for maximum CMCase activity levels, higher values of pH reduced the activity dramatically to less than one third, while at pH 6 the

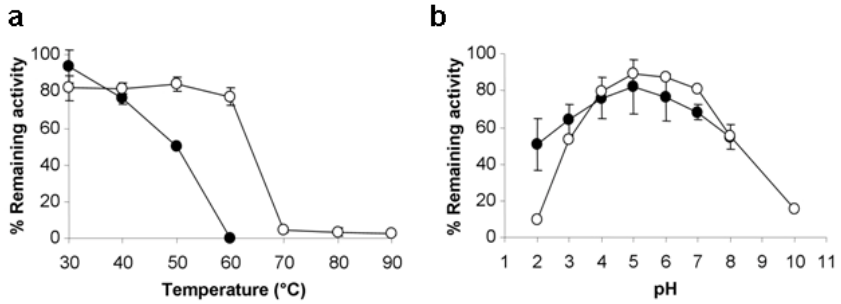

Figure 4. Stability of CMCase activity after incubation at different temperatures (a), and pH (b). Black circles, *B. adusta*; white circles, *P. sanguineus*.

activity was one half of that showed at pH 5 (Figure 2b). One difference between the species was the time at which CMCase activity was maximum. At 28ºC and pH 5 *B. adusta* exhibited its maximum CMCase activity on the 6th day, reaching 2.4 IU/mg, whereas the activity from *P. sanguineus* was highest on the 8th day showing 1.4 IU/mg (data not shown).

Cellulolytic activity characterization in culture supernatants

Enzymatic reaction conditions

Different properties of the enzymes contained in the supernatants from cultures of both species were analyzed. CMCase activity versus temperature of reaction profiles were obtained for each fungus. Despite both fungi showed maximal cellulolytic activities at 50ºC, *B. adusta* presented a peak with a significant decline both towards higher or lower temperatures. On the other hand, the enzymatic activity of the *P. sanguineus* supernatant maintained 40 to 85% of activity in the ranges of 30ºC to 40ºC and 60ºC to 80ºC (Figure 3a). Likewise, when the reaction was carried out at different pH, enzymes from both species were more active at pH 5 (Figure 3b); however, once again, those from *B. adusta* presented almost half the activity shown at pH 5 for pH below 4 and above 6. In contrast, the cellulolytic activity from *P. sanguineus* were elevated at more alkaline pH, showing more than 50% of the maximum activity at pH 8 (Figure 3b).

Enzymatic tolerance

Robustness of the cellulolytic enzymes is a key factor for industrial applications. The thermotolerance of the cellulases from the two fungi was analyzed. *B. adusta* supernatants showed 94% of remaining activity (2.2 IU/mg, compared to 2.4 IU/mg achieved at optimum conditions of pH and temperature) when incubated below 50ºC. However, this activity was rapidly lost when the temperature of incubation was increased (Figure 4a). At 40ºC only 76% of the original activity was retained, and at 50ºC half of the activity was lost. The enzymes that confer CMCase activity could not tolerate temperatures of 60ºC and above. In contrast, those from *P. sanguineus* resisted up to 60ºC showing approximately 80% of the original activity after 1 hr of incubation (Figure 4a). In this case CMCase activity was lost in 1 hr at temperatures above 70ºC. In addition, the effect of incubating the supernatants during 1 hr in buffers with pH ranging from very acidic to very alkaline was analyzed. In this case, while *B. adusta* enzymes retained above 50% of the CMCase activity at pH 2 and 8 (Figure 4b) *P. sanguineus* enzymes were almost inactive at pH 2, but retained 80% of activity at pH 7, and showed more than 50% residual activity at pH 3 and 8. However, activity at pH 10 was reduced by 84% (Figure 4b).

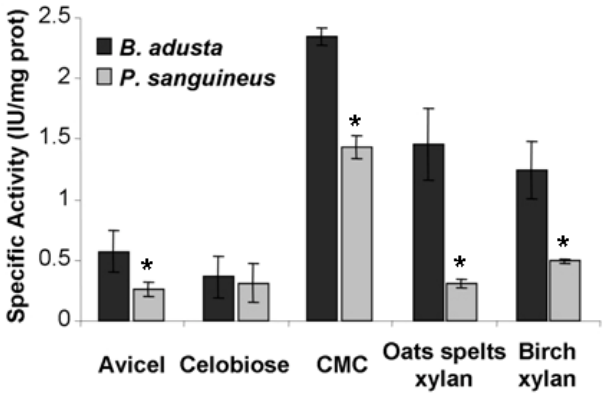

Figure 5. Cellulolytic activities towards different cellulosic substrates in supernatants of *B. adusta* (gray bars), and *P. sanguineus* (black bars) from 6- and 8-day cultures, respectively, after growth in wheat straw media at 28ºC and pH 5. Asterisk indicates statistically significant differences ($P < 0.05$, ANOVA) between the fungi.

Substrate specificities

To evaluate the enzymes specificities, different substrates were tested in the enzymatic reactions. Exo-glucanase activity was evident in both species by the use of microcrystalline cellulose (Avicel), with *B. adusta* showing higher enzymatic activity (0.6 IU/mg) than *P. sanguineus* (0.3 IU/mg; Figure 5). Cellobiose was hydrolyzed by β-glucosidases practically to the same extent by the two fungi (Figure 5). CMC is formed by amorphous cellulose, a substrate for endo-glucanases. Liberated cello-oligosaccharides from CMC by endo-glucanases then become substrate for β-glucosidases increasing the concentration of reducing sugars. Therefore, it was not surprise to find the most elevated values for both fungi when CMC was used as the reaction substrate (2.4 [*B. adusta*] and 1.43 IU/mg [*P. sanguineus*]; Figure 5). Xylanase activity towards xylan of two different sources (oat spelt and birchwood) was produced by the two fungi. *B. adusta* xylanases were more active than those from *P. sanguineus* by 5 and 2.5 times for both substrates respectively.

Zymograms

Protein bands with cellulolytic and xylanolytic activities were identified using gel electrophoresis and Congo red staining. Five possible cellulase bands were observed from acetone-precipitated supernatants of *B. adusta* (Figure 6a). The band of largest molecular weight was approximately of 90 kDa, whereas the other four bands were around 25, 35, 40 and 50 kDa (Figure 6a). On the contrary, *P.sanguineus* showed only two prominent CMCase activity bands of approximately 25 and 50 kDa when precipitated with 80% acetone (Figure 6a). The 50 kDa band was the only CMCase activity present following precipitation at 20% acetone (Figure 6a), which could be a useful characteristic for downstream processing. In the case of xylanases, *B.*

adusta produced a unique activity band which precipitated free of other contaminant proteins at 20% acetone (Figure 6b, and data not shown). Similarly, *P. sanguineus* produced only one xylanase activity band under the conditions analyzed. Both xylanases bands were in the range of approximately 80 to 90 kDa.

DISCUSSION

The cost of the cellulolytic enzymes is one of the factors determining the economics of a biocatalytic process and it can be reduced finding optimum conditions for their production (Lynd et al. 2002). According to this, it is important to determine the hydrolytic capabilities of wood-degrading organisms to achieve better digestion of cellulosic materials for industrial purposes. In the present work, *Bjerkandera adusta* and *Pycnoporus sanguineus*, growing on a natural cellulosic substrate (wheat straw), produced a number of cellulolytic activities with different characteristics. Previous reports exist on the pretreatment of cellulosic substrates with white-rot fungi, achieving high-quality levels of saccharification of the holocellulose (Taniguchi et al. 2005; Lee et al. 2007). At identical optimal culture conditions (28ºC and pH 5), maximal activities achieved by *B. adusta* were reached earlier (6 days of incubation vs. 8 in *P. sanguineus*), and at higher levels. Still, the requirements of culture conditions for both strains were the same. This was surprising given that *P. sanguineus* was isolated from a tropical location and grows better at high temperatures, indicating that the optimal growing temperature does not necessarily correlate with the optimal condition for producing cellulases. Nonetheless, one advantage of *P. sanguineus* (possibly due its increased thermotolerance) is the broad range of temperatures at which its enzymes achieve elevated activities (from 50 to 80ºC), whereas *B. adusta* activity showed a peak at 50ºC (Figure 3). An additional difference between fungi was the tolerance of *P. sanguineus* enzymes to incubation at temperatures up to 60ºC (Figure 4). This coincides with the notion that enzymes from thermophilic fungus present higher optimal temperature which could be related to longer "shelf-lives" for industrial applications (Maheshwari et al. 2000). Besides, thermostable cellulolytic enzymes also have great potential to be used in industrial processes such as food processing, textiles and bioconversion (Bhat and Bhat, 1997; Murray et al. 2004). One favorable attribute of *B. adusta* enzymes is their resistance to 1 hr incubation from pH 2 to 8 (Figure 4b). This is an important property that could allow degradation of previously acid- or alkali-treated material without the need to neutralize it. Since biological processes are more environmentally friendly compared to chemical pretreatments, these features make of these two species good candidates for biological pretreatment of wheat straw, using their enzymes either individually or in combination.

This is the first report of the characterization of the cellulolytic properties of any of the species herein presented. However, a recent report (Rodrigues et al. 2008), focused on animal feeding, describes the use of fungal extracts to improve wheat straw digestibility, in which a strain of *B. adusta* was included. In it, volumetric CMCase and Avicelase activities where determined from liquid cultures, with values of approximately 0.14 and 0.1 IU/ml, respectively, which correspond to 8.9- and 3.3-fold less than those found in this work (1.25 and 0.33 IU/ml, respectively). The differences could be due to the method used to grow the fungus (see below). Nevertheless, in accordance with their findings, CMCase activity was also higher than the activity towards crystalline cellulose. Due to the variety of cellulosic media used to cultivate fungi, and to the different reported expression of cellulase activities, it is difficult to compare the efficacy of the enzymes analyzed in this work with others already described. In a report from (Nozaki et al. 2007), the enzymatic activities of the white rot basidiomycete *Trametes hirsuta* were measured using wheat bran (among other substrates) in solid media. They found that *T. hirsuta* enzymes were ten times more active in solid media than in liquid ones. Similarly, we observed higher activities (3-fold higher for *B. adusta* and 1.8-fold higher for *P. sanguineus*) in agar than in liquid cultures (data not shown). However, the activities achieved by the white rot fungi strains tested herein were 12- (*B. adusta*) and 7- (*P. sanguineus*) fold higher than the values for *T. hirsuta* cultured under related conditions (Nozaki et al. 2007). The elevated activities presented in this work may be the result of the synergism of different types of enzymes produced by the evaluated fungi. Endo- and exo-glucanases, as well as -glucosidases and xylanases were expressed in the supernatants of both fungi, as revealed by the different substrates used in the reaction mixtures. It is important to note the existence of enzymes with elevated action on microcrystalline cellulose, in particular from *B. adusta* (0.6 IU/mg vs. 0.26 IU/mg of *P. sanguineus*). It is also worth noting the high activity of *B. adusta* xylanases compared to *P. sanguineus*, particularly for applications such as in the paper and animal feeding industries (Polizeli et al. 2005; Alborés et al. 2006). Finally, the proteins responsible for the hydrolysis of CMC and two

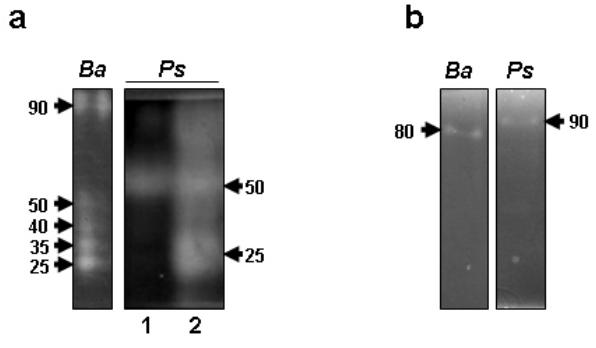

Figure 6. Zymograms of acetone-precipitated CMCases (a) and xylanases (b), from culture supernatants. *Ba*, *B. adusta*. *Ps*, *P. sanguineus*. Lane 1, 20%-acetone faction; lane 2, 80%-acetone fraction. For details see Materials and Methods. Arrows indicate approximate MWs.

types of xylan were detected by zymography. The CMCase activity bands identified in both strains showed molecular weights comparable to those reported for endo-glucanases from *Trichoderma reesei* and *Phanerochaete chrysosporium*, ranging from approximately 25 to 50 kDa. Endoglucanases play a key role in increasing the yield of fruit juices, beer filtration, and oil extraction, improving the nutritive quality of bakery products and animal feed, and enhancing the brightness, smoothness, and over all quality of cellulosic garments (Parry et al. 2002). Hence, there is a need for a wide range of enzymes with varying pH and temperature optima, stability, and substrate specifications. According to its size the high molecular weight band in supernatants from *B. adusta* could correspond to a β-glucosidase (Perez et al. 2002). In addition, under the conditions tested, both species expressed only one band of similar molecular weight (approximately 80 to 90 kDa) with xylanase activity. Xylanases have been clustered into different glycosyl hydrolases families (Collins et al. 2005). Given the MW encountered for xylanases from both fungi, it is tentatively suggested that they may belong to family 10, with a characteristic low isoelectric point (IP) (Wong et al. 1988). Purification of these proteins is currently being performed to confirm their features, and to establish their individual contribution to the total activity of the supernatants.

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