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ORIGINAL PAPER

Evaluation of different lignocellulosic substrates for the production of cellulases and xylanases by the basidiomycete fungi *Bjerkandera adusta* and *Pycnoporus sanguineus*

Rosa Estela Quiroz-Castañeda · Nancy Pérez-Mejía · Claudia Martínez-Anaya · Lourdes Acosta-Urdapilleta · Jorge Folch-Mallol

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Abstract Agricultural waste products are potential resources for the production of a number of industrial compounds, including biofuels. Basidiomycete fungi display a battery of hydrolytic enzymes with prospective use in lignocellulosic biomass transformation, however little work has been done regarding the characterization of such activities. Growth in several lignocellulosic substrates (oak and cedar sawdust, rice husk, corn stubble, wheat straw and *Jatropha* seed husk) and the production of cellulases and xylanases by two basidiomycete fungi: *Bjerkandera adusta* and *Pycnoporus sanguineus* were analyzed. Growth for *P. sanguineus* was best in rice husk while corn stubble supported the highest growth rate for *B. adusta*. Among the substrates tested, cedar sawdust produced the highest cellulolytic activities in both fungal species, followed by oak sawdust and wheat straw. Xylanolytic activity was best in oak and cedar sawdust for both species. We found no correlation between growth and enzyme production. Zymogram analysis of xylanases and cellulases showed that growth in different substrates produced particular combinations of protein bands with hydrolytic activity.

Keywords Lignocellulose · Cellulases · Xylanases · Biofuels

Rosa Estela Quiroz-Castañeda, Nancy Pérez-Mejía are the authors contributed equally to this work.

R. E. Quiroz-Castañeda · C. Martínez-Anaya
Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001 Col. Chamilpa, 62209 Cuernavaca, Morelos, México

R. E. Quiroz-Castañeda · J. Folch-Mallol (✉)
Laboratorio de Biología Molecular de Hongos, Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001 Col. Chamilpa, 62209 Cuernavaca, Morelos, México
e-mail: jordi@uaem.mx

N. Pérez-Mejía
Facultad de Ciencias Biológicas, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001 Col. Chamilpa, 62209 Cuernavaca, Morelos, México

L. Acosta-Urdapilleta
Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001 Col. Chamilpa, 62209 Cuernavaca, Morelos, México

Introduction

Lignocellulose is the main component of biomass, comprising almost half of the photosynthetic material produced by plants and represents the most abundant renewable organic resource (Sanchez 2009). Lignocellulose consists of three main polymers: cellulose, hemicellulose and lignin, which are strongly intermeshed and chemically bonded by non-covalent forces and covalent cross-linkages (Perez et al. 2002). Cellulose is a glucose polymer with an organized crystalline and recalcitrant structure interspersed with regions of amorphous cellulose, a disorganized

structure susceptible to degradation by hydrolytic enzymes (Hendriks and Zeeman 2009). Hemicellulose is a complex polysaccharide comprised of various monomers such as pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and acids (ferulic acid, acetic acid); hemicellulose from hardwoods is rich in xylans, softwoods contain mainly mannans, and in grasses arabinoxylan is the main component (McMillan 1994; Olofsson et al. 2008). Lignin is an amorphous heteropolymer consisting of three different phenyl propane units (*p*-coumaryl, coniferyl and sinapyl alcohol), which produce respectively *p*-hydroxyphenyl, guaiacyl, and syringyl residues when incorporated into the lignin polymer. Lignin in hardwoods is composed mainly of guaiacyl and syringyl units, and in softwoods guaiacyl units predominate (Boerjan et al. 2003). Because sugar production from biomass is a key-limiting step in industrial processes, harsh physical–chemical pretreatments are used to loosen lignin and release fibrils of cellulose and monomers of the polysaccharide components (Wyman et al. 2005). Pretreatment also decreases the recalcitrance of crystalline cellulose through the rupture of hydrogen bonds, disorganizing its structure and making it more accessible to hydrolytic enzymes (Nguyen et al. 2000; Nagle et al. 2002; Soderstrom et al. 2002). The white-rot fungal group of Basidiomycetes is able to grow on lignocellulosic substrates and to exploit all wood components due the secretion of cellulases, xylanases and laccases (Perez et al. 2002; Martinez et al. 2005; Elisashvili and Kachlishvili 2009). Much attention has been focused on identifying these hydrolytic activities given their potential use in different industries such as paper biopulping, human and animal feedstock, textile and dye industries, bioremediation and the production of cellulosic bioethanol (Alborés et al. 2006). Recently, lignocellulolytic activities from white-rot fungi have been characterized, and some of them have shown robust cellulolytic activities at high temperatures or extreme pH values that match industrial conditions (Quiroz-Castañeda et al. 2009). Agro-industrial wastes generated from crop cultivation and food processing constitute a potential raw material source for value-added products such as biofuels (Elisashvili and Kachlishvili 2009). Wastes vary in their content of the three polymers and the composition of hemicellulose and lignin. Agricultural residues used in previous studies are wheat straw, corn stubble, rice husk or *Jatropha curcas* seed husk, and forest residues such as cedar and oak sawdust, softwood and hardwood (Saha 2003; Sommer et al. 2004). In this work, we analyzed the growth and the production of cellulolytic enzymes by two basidiomycete fungi *Bjerkandera adusta* and *Pycnoporus sanguineus* grown on lignocellulosic materials of different origin and composition and growth rates and levels of cellulolytic and xylanolytic activities were compared. Zymogram analyses permitted the detection of a number of bands with cellulolytic and hemicellulolytic proteins expressed by both fungi.

Methodology

Strains

Bjerkandera adusta UAMH 8258 was kindly provided by Dr. R. Vazquez-Duhalt and is well known for its elevated ligninase activity (Wang et al. 2003). *P. sanguineus* CEIBMD01 is a strain collected from a dead tree bark covered with crude oil in Veracruz, México, that is able to grow at moderately high temperatures (Dantan-Gonzalez et al. 2008).

Culture conditions

The fungi were grown and propagated on solid PDA medium at 28°C for 8 days. A passage was then performed with 1 cm^2 inoculum placed in a Petri dish containing a modified mineral base medium (Inglis et al. 2000) (7.8 mg/l $CuSO_4 \cdot 5H_2O$, 18 mg/l $FeSO_4 \cdot 7H_2O$, 500 mg/l $MgSO_4 \cdot 7H_2O$, 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 with phosphoric acid) to deprive the fungi of a carbon source (mycelia in this medium were very faint and failed to develop after 5 days at 28°C). For the experimental cultures, 1 cm^2 inocula were taken from the mineral base medium and placed on plates containing mineral base medium plus 2% powdered lignocellulosic materials as the carbon source (previously dried and pulverized in a coffee grinder (Braun) until a homogeneous powder was observed -with maximum and minimum particles sizes of 4 and 0.5 mm, respectively). We tested wheat straw (*Triticum aestivum*), rice husk (*Oryza sativa*), corn stubble (*Zea mays*), jatropha ground seed husk (*J. curcas*), and cedar (*Cedrus* spp.)

and oak (*Quercus* spp.) sawdust. The plates were incubated at 28°C for 6–15 days.

Growth rate calculation

Mycelial radial growth for each lignocellulosic material was measured daily with a vernier caliper, starting from the center of the disk to the periphery. Growth was followed, in triplicate, taking daily measurements for each substrate until mycelium reached the edge of the Petri dish (90 mm diameter). Mycelia diameter was plotted against time, and growth velocities ($\boldsymbol{\mu}$) were determined calculating the slope of the growth curve (cm/day).

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, it was then centrifuged at $2{,}504 \times g$ at 4°C for 30 min; the volume recovered varied between 3 and 4 ml. Supernatants were clarified by filtration through 0.45 µm filters (Millipore). For enzymatic activity measurements the following substrates were used: 2% carboxymethylcellulose (CMC, Sigma) and 2% 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 50°C for 45 min. Reducing sugars were determined using the 3,5-dinitrosalicylic acid (DNS) assay according to (Miller 1959) as described in Quiroz-Castañeda et al. (2009). 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 was added and absorbance was 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 plotted against time and the slope was calculated to determine the velocity of the reaction. Released reducing sugars versus time plots 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 (IU/mg protein), 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) standard curve.

Statistical calculations

Statistical analyses were performed with system (SAS) software 9.1 for Windows XP (SAS Institute Inc; Cary, NC, USA).

Zymograms

Total proteins secreted to the culture medium were precipitated with 80% acetone as described in Quiroz-Castañeda et al. (2009). 10% polyacrylamide gels containing 0.2% CMC were loaded with 5 or 10 µg of total protein previously mixed with a modified Laemmli loading buffer containing no SDS nor β-mercaptoethanol. Gels did not contain SDS, but SDS was added at 0.05% to the running buffer. For identification of CMCase activities after electrophoresis, gels were treated as described by Mateos et al. (1992); i.e., washed three times (40 min each) in PCA buffer (50 mM KH_2PO_4, 50 mM citric acid pH 5.2) in order to remove SDS. Gels were then stained with 0.1% Congo red for 30 min followed by one wash with 1 M NaCl and then submerged into 5% acetic acid for 10 min to turn Congo red to purple, thus improving visibility of the bands. For xylanase activity detection, 10% polyacrylamide gels containing 0.2% birchwood xylan were run. Gels were washed twice (30 min each) with 50 mM sodium acetate buffer pH 5.5 containing 25% isopropanol, followed by a single wash in 50 mM sodium acetate buffer pH 5.5 (Lee et al. 1993) and then incubated for 5 min at 60°C. Congo red staining of the gels was performed as mentioned above. The molecular weight of the bands was estimated against a protein marker (Invitrogen).

Results

Different substrates promote differential fungal growth rate

We were interested in determining how the fungi *B. adusta* and *P. sanguineus* grow on different

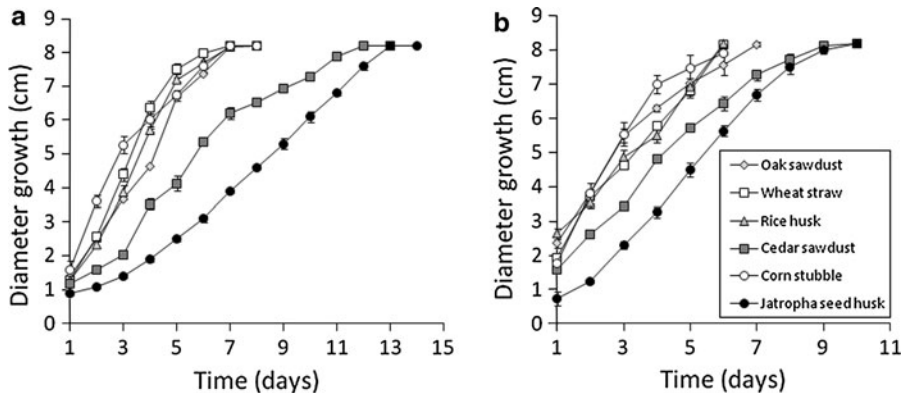

Fig. 1 Growth of fungi in Petri dishes with lignocellulosic substrate as carbon source. **a** *P. sanguineus*; **b** *B. adusta*

Table 1 Growth rate of *P. sanguineus* and *B. adusta* on different substrates

Substrate	*P. sanguineus* Growth rate (cm/day)[a]	*B. adusta* Growth rate (cm/day)[a]
Oak sawdust	1.06 ± 0.01	0.95 ± 0.01
Wheat straw	1.05 ± 0.02	1.19 ± 0.02
Rice husk	1.24 ± 0.05	1.10 ± 0.02
Cedar sawdust	0.85 ± 0.03	0.78 ± 0.01
Corn stubble	0.91 ± 0.03	1.23 ± 0.02
Jatropha seed husk	0.54 ± 0.01	0.92 ± 0.02

[a] Average and SD of three independent experiments

lignocellulosic materials as the only carbon source, by analyzing the velocity of growth on each substrate. Growth rate of *B. adusta* and *P. sanguineus* is presented in Fig. 1 and Table 1. Among the lignocellulosic substrates used in this study, rice husk medium permitted the fastest growth rate of *P. sanguineus* at 1.24 ± 0.05 cm/day. Mycelium in this substrate reached the edges of Petri dishes (90 mm Ø) in 7 days. On the other hand, *B. adusta* achieved its fastest growth rate on corn stubble, at a speed of 1.23 ± 0.02 cm/day, filling the Petri dish in 6 days. On the contrary, the substrate where *P. sanguineus* showed the slowest growth rate was jatropha seed husk medium (0.54 ± 0.01 cm/day) reaching the edge of the plate after 14 days. The growth rate of *B. adusta* was slowest in cedar sawdust (growing 0.78 ± 0.01 cm/day), filling the Petri dish after 10 days. All these data indicate differential utilization of the various materials by the two fungi.

Cellulolytic and xylanolytic activities are not related to mycelial growth

Mycelial growth indicates utilization of the substrate, thus we analyzed the cellulolytic and xylanolytic enzyme activities produced by the two fungi. Both, *B. adusta* and *P. sanguineus* culture supernatants from cedar sawdust medium showed the highest specific activity towards CMC, 1.40 and 2.73 IU/mg protein, respectively (Fig. 2); almost twofold the activity in *P. sanguineus* cultures in comparison with *B. adusta*. However, enzyme activities varied with respect to other substrates for the two species. The order of substrates for CMCase activity (UI/mg protein) for *P. sanguineus* was: cedar sawdust (2.73) > oak sawdust (0.72) > wheat straw (0.65) > corn stubble (0.26) > jatropha seed husk (0.25) > rice husk (0.24); with differences of 11.4 fold between the best inducer medium (cedar sawdust) and the worst inducer (rice husk). Whereas for *B. adusta* the order was: cedar sawdust (1.40) > wheat straw (0.96) > rice husk (0.87) > oak sawdust (0.79) > jatropha seed husk (0.58) > corn stubble (0.41). In this case the difference between substrates was less pronounced, with 3.5-fold difference between the best and the worst inducer media. *P. sanguineus* specific activities towards xylan (UI/mg protein) also presented variation among the substrates, with a 5.1-fold difference between the extremes, following the order: oak sawdust (0.31) > wheat straw (0.16) > cedar sawdust (0.13) > rice husk (0.07) = jatropha seed husk (0.07) > corn stubble (0.06). Finally, for *B. adusta* the levels of xylanase production where highest for oak sawdust (0.42) > cedar sawdust (0.40) > rice husk (0.24) > wheat

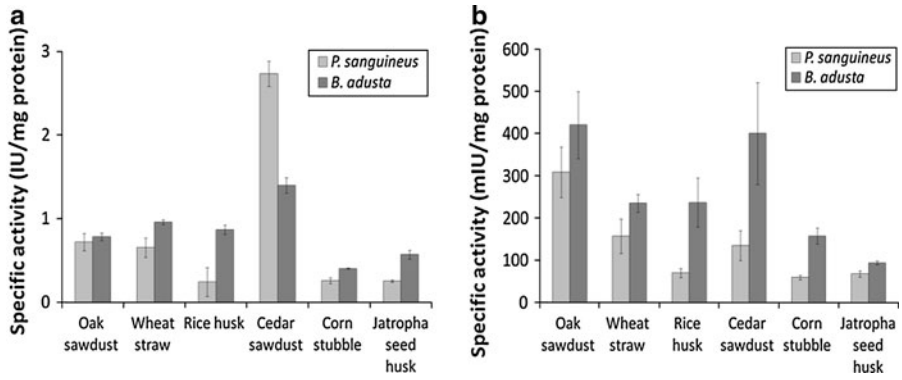

Fig. 2 Cellulolytic (**a**) and xylanolytic (**b**) activity towards different cellulosic substrates in supernatants of *P. sanguineus* and *B. adusta* from cultures from 8 and 6-day cultures, respectively. *Panel* **b** shows activities in mUI. Standard deviation of three independent experiments is shown

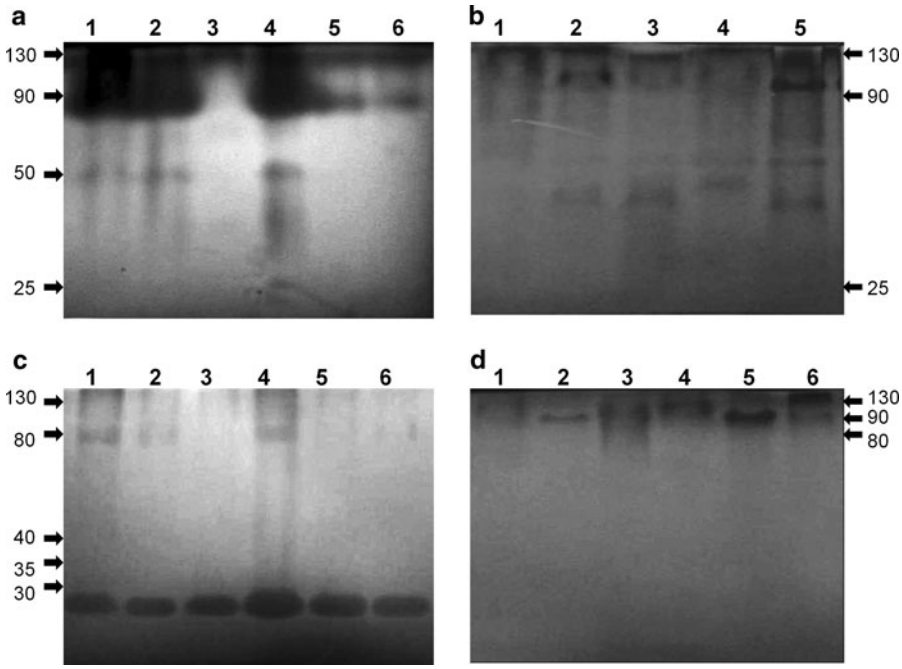

Fig. 3 Zymograms of acetone-precipitated CMCases from *P. sanguineus* (**a**) and *B.* adusta (**c**), and xylanases from *P. sanguineus* (**b**) and *B. adusta* (**d**) from culture supernatants. *Lane 1* oak sawdust; *2* wheat straw; *3* rice husk; *4* cedar sawdust; *5* corn stubble; and *6* jatropha seed husk. For details see "Methodology" section. *Arrows* indicate approximate MWs

straw (0.23) > corn stubble (0.16) > jatropha seed husk (0.09) (Fig. 2). *B. adusta* xylanolytic activity was 1.3 times greater than those of *P. sanguineus* in oak sawdust supernatant (Fig. 2). Interestingly, we did not find a direct correlation between the growth on the different substrates and the production of cellulolytic enzymes.

Different substrates produce diverse patterns of proteins with cellulolytic and xylanolytic activities

To characterize further the response of the fungi towards cellulose and xylan, zymogram analyses were performed. *P. sanguineus* showed a broad prominent CMCase activity region between 130 and 90 kDa approximately (Fig. 3a, lanes 1, 2 and 4) corresponding to growth on oak sawdust, wheat straw and cedar sawdust, respectively. In lanes 5 and 6, two discrete bands of 130 and 90 kDa were observed (corresponding to growth on corn stubble and jatropha seed husk, respectively). In addition, a band of approximately 50 kDa was also detected in supernatants of oak and cedar sawdust and wheat straw media (Fig. 3a, lanes 1, 2 and 4). In supernatants of rice husk medium, a band of 130 kDa was observed together with a faint band of approximately 70 kDa (Fig. 3a, lane 3). Cedar sawdust also induced a weak band of 20 kDa, which was not detected in any other substrate. The intensity of the bands of CMCase

activities correlated with the activities measured by the release of reducing sugars described above.

In the case of *B. adusta* CMCase activity, a major band of 25 kDa was detected in the supernatants of all the substrates tested (Fig. 3b, lanes 1–6). In addition, when grown in oak and cedar sawdust and wheat straw media, two bands of approximately 130 and 90 kDa were observed (Fig. 3b, lanes 1, 2 and 4), being more intense in preparations from cedar medium and less evident in wheat straw medium. When rice husk was used as a growing substrate, the 90 kDa band was not detected and the 130 kDa was very faint. From corn stubble and jatropha seed husk supernatants very low activity of these high molecular weight bands was detected (Fig. 3b, lanes 5 and 6). In this case a correlation with the release of reducing sugar measurements was found for cedar and wheat straw culture supernatants but not for rice and oak.

In the case of xylanolytic activity, *P. sanguineus* produced several bands for most substrates under the conditions analyzed. From oak medium, a broad band of activity ranging from 40 to 130 kDa was observed (Fig. 3c, lane 1). In contrast, wheat straw, rice, cedar and corn stubble produced similar band patterns of approximately 130, 90–80, 60, 38–40 and 28–32 kDa (Fig. 3c, lanes 2, 3, 4 and 5), being more active in supernatants from cedar sawdust. Surprisingly, no bands were detected in supernatants of *P. sanguineus* grown in jatropha seed husk (Fig. 3c, lane 6). We do not know if all these are variations of the same enzymes or, on the other hand, whether they are different proteins altogether. It is clear, however, that growth on specific substrates induces differential expression or modifications of xylanolytic proteins.

Finally, zymograms of *B. adusta* showed a less complex pattern of xylanolytic activities. Wheat straw, corn stubble and rice husk produced a 90 kDa band (Fig. 3d, lanes 2, 5 and 3). Oak and cedar sawdust induced a 110 kDa band, although activity seen in lane 1 is very low (Fig. 3d, lanes 1 and 4). Finally, in jatropha seed husk supernatants two bands of approximately 120 and 80 kDa are present (Fig. 3d, lane 6).

Discussion

Recently, we reported the characterization of cellulolytic activities of *P. sanguineus* and *B. adusta* grown on wheat straw (Quiroz-Castañeda et al. 2009). However, the analysis of cellulolytic activities of these fungi grown in other lignocellulosic substrates has not been reported. We found that mycelial growth rates varied among differing growing substrates, but that this growth variation does not appear to correlate with differing patterns of enzyme induction. Cedar sawdust was among the substrates that produced slowest growth rates for *P. sanguineus* and *B. adusta*; however the cellulolytic activity in this substrate was the highest for both fungi. Cellulolytic and xylanolytic activity levels varied among each substrate suggesting that the composition of these substrates induces different quantities and/or types of enzymes (see below). Cedar supernatants of both fungi had an elevated cellulolytic activity towards CMC, with *P. sanguineus* activity almost twofold higher than that of *B. adusta*. The xylanolytic activity towards birchwood xylan was 1.3 times higher in oak sawdust supernatant from *B. adusta* than from *P. sanguineus* growing in the same substrate. In fact, *B. adusta* presented an elevated xylanolytic activity in cedar sawdust supernatant as well. *B. adusta* seems to have a more efficient battery of hemicellulases than *P. sanguineus* since it presented higher levels of xylanolytic activity. Proteins with hydrolytic activity on CMC and birchwood xylan were detected in activity gels. The bands with CMCase activity identified in all culture supernatants from *B. adusta* and *P. sanguineus* showed molecular weights comparable to those reported for cellulases from *Trichoderma reesei* and *Phanerochaete chrysosporium*, ranging from approximately 25–50 kDa. Enzymes with almost double this size have also been reported in *Sclerotium rolfsii* and *Gloeophyllum sepiarium* (Sadana et al. 1984; Bhattacharjee et al. 1993; Lee et al. 1993).

We explored if the reported composition of the substrates could correlate with the different patterns of hydrolytic enzymes. For *P. sanguineus*, it seems that the content of cellulose in substrates is related to the intensity of the activity bands observed in zymograms of oak and cedar sawdust and wheat straw, in which the estimated cellulose content is 45–55, 45–50% (Baldrian and Valaskova 2008) and 37.5% (Sun and Cheng 2002), respectively. It seems that higher cellulose content could induce greater activity and more complex patterns of cellulase activity bands. Jatropha seed husk, with a cellulose content of only 13.52% (Department of Energy,

USA), induced less intense activity bands compared to the above mentioned substrates, while rice husk and corn stubble, with cellulose content of 35–37% (Saha 2003; Liang et al. 2010), induced intermediate CMCase activity.

P. sanguineus zymograms for xylanolytic activity also show different patterns for different substrate compositions. Hemicellulose from oak sawdust is mostly glucuronoxylans (24–40%) while in cedar sawdust is glucomannans (25–35%) (Department of Energy, USA), and a differing pattern of activity bands with variations in intensity was observed for these substrates. A hemicellulose content of approximately 25–28% (Betts et al. 1991) is found in wheat straw, rice husk, corn stubble and jatropha seeds husk. We found a correlation between the pattern of activity bands that shows only a slight variation in the number and molecular weight of bands, but not in the intensity, among these substrates.

Although cellulose content within the substrates analyzed is different, in *B. adusta* cellulolytic zymograms showed only slight variations in the intensity and number of high molecular weight cellulases induced. It is possible that, in this fungus, the small band (25 kDa) found in all cases is necessary for an efficient degradation regardless of the substrate composition. In xylanolytic zymograms, a significant difference in the number of bands was observed compared to those of *P. sanguineus.* Probably, the combination of hemicellulases induced in every substrate is exclusive to each fungus.

Another possibility for the differential expression patterns of activities, besides poly-carbohydrate composition, is that some of the substrates could still have more easily assimilable sugars. These would serve as a carbon source and possibly repress hydrolytic activities allowing nonetheless good growth rates (this could be the case for corn stubble for *P. sanguineus* or wheat straw for *B. adusta*).

Cellulases play a key role in industry in increasing the yield of fruit juices, beer filtration and oil extraction, improving the nutritive quality of animal feed, and enhancing the luster, smoothness, and overall quality of cellulosic garments (Liang et al. 2010). In this context, it is of particular interest to study the cellulolytic activity-containing band of 25 kDa found in the *B. adusta* supernatants since it was produced in all substrates, shows a very clear activity and its small size could make it suitable for heterologous expression. Experiments are under way to sequence the protein and to clone its corresponding gene. Xylan breakdown involves at least the action of endo-1,4-β-xylanases and β-xylosidases. Under the conditions tested, both fungi produced patterns of bands with xylanolytic activity of low and high molecular weight from 30 to 130 kDa. Xylanases and xylosidases of comparable low molecular size have been reported in other fungi (Collins et al. 2005). The results obtained in this work show that both species of basidiomycetes produce cellulolytic and xylanolytic activities, with sawdust being the best inducers for the production of these enzymes.

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