Selection and Characterization of a Native *Pycnoporus sanguineus* Strain as a Lignocellulolytic Extract Producer from Submerged Cultures of Various Agroindustrial Wastes

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A native strain of *Pycnoporus sanguineus* (CS2) was selected because of its lignocellulolytic potential on a color-based agar plate screening system; pectinolytic and amylolytic capabilities were also assessed. The effects of different lignocellulosic substrates under submerged cultures of selected fungi on the enzymatic production of cellulases, xylanases, amylases, and laccases were evaluated. Seven different treatments, in which different combinations of wheat straw (WS), wheat bran (WB), orange peels (OP), grapefruit peels (GP), and apple peels (AP) were used, were established. Controls of 2% (w/v) carboxymethyl-cellulose (CMC) and xylan were used in a rich medium for basidiomycetes (RMB). The highest titers were achieved using OP-based cultures, with large titers of CMCases (33.5 U), avicelases (15.7 U), ß-D-glucosidase (72.9U), and xylanases (18.3 U). The best levels of amylase and laccase activity were obtained in the RMB plus CMC (RMB-CMC) (7.2 U) and in the medium OF/AP/GP (6.4 U), respectively. The relative molecular sizes of cellulase, xylanase, and amylase were 66.2, 56.5, and 90.8 kDa, respectively. Laccase and amylase had maximum activities at 60 °C whereas cellulase and hemicellulase had maximum activities at 70 °C. The optimal pH for cellulases, xylanases, and amylases was 5.0 in every case, and more than 95% activity was observed at pH 6. These results reveal some efficient operating parameters for the application of these enzymatic extracts as adjuvants to improve animal nutrition.

Keywords: Amylases; Cellulases; Pycnoporus sanguineus CS2; Thermostability; Xylanases

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INTRODUCTION

Novel techniques for reutilizing waste byproducts from the food industry are rising in popularity along with the strengthening of the green movement around the world. The main objective of these bioprocesses is to obtain compounds with high industrial value such as enzymes, carbohydrates, amino acids, lipids, and many other biomolecules from organic sources (Castillo *et al*. 2009; Yang *et al.* 2011; Cunha *et al.* 2012). These biotechnological processes are sustainable because the enzymes and other compounds are produced cheaply due to the abundance of lignocellulose-rich wastes (Altaf *et al.* 2010).

Organisms like white rot fungi (WRF) yield simple sugar molecules by degrading the main components of wood's cell wall, including lignin, hemicellulose, and cellulose, depending on their extracellular enzymes (Henrissat *et al.* 1998; Steffen *et al.* 2007). Lignocellulolytic enzymes can be classified into two groups. The first group degrades cellulose and xylan and includes β-glucosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91), endo-1,4-β -glucanase (EC 3.2.1.4), and xylanases (EC 3.2.1.8). The second group includes the entire lignin oxidative complex, composed of laccase (EC 1.10.3.2), lignin peroxidase, LiP (EC 1.11.1.14), and manganese peroxidase, MnP (EC 1.11.1.13) (Rana 2011).

The potential of cellulases and other hydrolytic enzymes to convert cellulosic biomass has made the industrial application and design of new bioprocesses more popular in the last few years (Kuhad *et al.* 2011). Most research has focused on either solid state fermentation (SSF) or submerged (SF) fermentation, both of which have exhibited good enzyme production (García and Torres 2003; Sinegani *et al*. 2005; Altaf *et al*. 2010; Philippoussis *et al.* 2011). Enzyme production under agitated or solid state fermentation depends on the characteristics of the substrates used. Best conditions involve a balance between the cellulose, xylan, and lignin contents. The characteristics of the media are translated to enzyme production depending on the organism under study.

Research on lignocellulolytic enzymes has demonstrated their potential in the food, animal feed, brewing and wine, agriculture, pulp and paper, textile, bioremediation, medical, and cosmetic industries (Elisashvili *et al*. 2008; Mendonça *et al*. 2010; Kuhad *et al*. 2011). Studies have shown that WRF can produce lignocellulolytic enzymes when grown in food industry by-products and other lignocellulose-rich substrates such as oak leaf litter, tea leaves, reed grass, bean stalks, wheat and pea straws, apple peels, and banana peels (Sinegani *et al*. 2005; Valášková and Baldrian 2006; Levin *et al*. 2007; Eliasashvili *et al*. 2008; Philippoussis *et al*. 2011).

The aim of the present work was to evaluate the effects of different agroindustrial residues during submerged fermentation of *Pycnoporus sanguineus* CS2 on the production of lignocellulolytic enzymes, including cellulases, xylanases, laccases, and amylases. Furthermore, some biochemical and operative properties, including the relative molecular weights, optimal pH, and optimal temperature were characterized.

EXPERIMENTAL

Materials

Chemicals and plant residues

All of the chemicals used were reactive grade purchased from Sigma-Aldrich (St. Louis, MO), Becton, Dickenson and Co. (Le Pont de Claix, France), or Bio-Rad (Hercules, CA). Wheat straw, oat flakes, orange peels, grapefruit peels, and apple peels were collected on the campus of Marin and La Ascensión of Facultad de Agronomía-UANL and from the metropolitan area surrounding Monterrey, N. L., Mexico. All residues were washed and chopped into small pieces about 1.0 cm in size.

Fungal strains and inocula preparation

Pycnoporus sanguineus CS2, *P. sanguineus* CS20, *P. sanguineus* CS43, and *P. sanguineus* CS90 strains were obtained from carpophores collected in oak forests and scrubland around Monterrey, N. L., Mexico from different ecotypes. All strains belong to

the strain collection of vegetative mycelium of the Enzymology Laboratory of the Facultad de Ciencias Biológicas, UANL. Reference strains *Phanerochaete chrysosporium* ATCC 24785, *Trametes hirsuta* UAMH 8156, and *Trametes versicolor* UAMH 8272 were used for comparison. All strains were conserved with YMGA medium (10 g/L malt extract, 4 g/L glucose, 4 g/L yeast extract, and 15 g/L agar; Hernández-Luna *et al.* 2008), with periodic subcultures every three months. Inocula preparation consisted of 5 d of strain reactivation on YMGA medium. Agar cylinders (0.5-cm) cut from the periphery of the cultures were used to inoculate solid and liquid media.

Methods

Screening in solid medium assay

Identification of the main enzymatic activities involved in the modification of plant cell wall components was performed using the medium described by Sin *et al.* (2002). The medium was supplemented with 2% carboxymethyl (CM) cellulose (Sigma-Aldrich) to detect cellulases, with xylan (from birch wood, Sigma-Aldrich) to detect xylanases, with 0.5% pectin (from citrus peel, Sigma-Aldrich) to detect pectinases, 0.02% Poly R-478 dye for lignin modifying enzymes (LME), and 0.02% syringaldazine for laccases. Solid media were seeded with one 0.5-cm cylinder and incubated at 28 °C. Mycelium growth was recorded daily, Poly R-478 discoloration was measured for 30 d, and the syringaldazine reaction was registered until the fifth day of testing. To determine the hydrolytic activities, medium was revealed with iodine solution during the third day of growth (Sin *et al.* 2002). A numeric scale in which values from 0 to 3 were assigned was as follows: 3 corresponds to a reaction diameter greater than the mycelium growth, 2 corresponds to a reaction diameter similar to the growth diameter, 1 corresponds to a growth diameter larger than the reaction diameter, and 0 corresponds to no reaction or discoloration.

Enzyme production in submerged medium

The submerged fungal cultivation was carried out in a rotary shaker at 200 rpm and 28 °C in 500-mL flasks containing 200 mL of medium. As a positive control for cellulase and xylanase production, a rich medium for basidiomycetes (RMB) supplemented with CM-cellulose (RMB-CMC) and xylan (RMB-Xyl) at a concentration of 2% (w/v) was used. The average composition as reported by Pozdnyakova *et al.* (2006) was 10 g of glucose, 0.5 g of yeast extract, 10 g of peptone, 0.72 g of NH_4NO_3, 1.0 g of $MgSO_4$, 0.5g of KCl, and 1 mL of trace element solution (1 g/L $FeSO_4$, 2.8 g/L $ZnSO_4$, and 3.3 g/L $CaCl_2$) per 100 mL double-distilled water. Submerged fermentation of the residues was performed using 60-mM potassium phosphate buffer at pH 6. Concentrations and combinations of residues for each medium are shown in Table 1. All media were autoclaved twice for 60 min (121 °C, 15 psi) and allowed to cool to room temperature before each flask was inoculated with three 0.5-mm diameter agar plugs of mycelium taken from the periphery of a 5-d growth fungal colony in YMGA.

Table 1. Agroindustrial Residues Based Media: Abbreviations, Combinations, and Concentrations

Medium	Composition
RMB-CMC	Rich medium for basidiomycetes - Carboxymethyl cellulose, 2%
RMB-Xyl	Rich medium for basidiomycetes - Xylan from birch wood, 2%
WS	Wheat straw, 1.5%
WS/OP	Wheat straw, 1.5%; Orange peels, 7.5%
OP	Orange peels, 7.5%
OF/AP/OP	Oat flakes, 1.5%; Apple peels, 5%; Orange peels, 7.5%
OF/AP/GP	Oat flakes; 1.5%; Apple peels, 5%; Grapefruit peels, 7.5%
WB/AP/GP	Wheat bran, 1.5%; Apple peels, 5%; Grapefruit peels, 7.5%
WB	Wheat bran, 1.5%

Enzymatic assays

A 2-mL aliquot was taken from each flask every 2 d for enzymatic assays. Determinations of the cellulases, xylanases, amylases, laccases, lignin peroxidases (LiP), and manganese-dependent peroxidases (MnP) present in cultures were performed. Cellulase, xylanase, and amylase activities were assayed by measuring the concentration of reducing sugars released according to the method described by Miller (1959). Reaction mixtures consisted of 0.5 mL of a 50-mM sodium citrate buffer at pH 5.0, 0.3 mL of CM-cellulose, Avicel, Cellobiose, Starch or D-xylan as the substrate (1% final concentration) in 2 mL of sample. The reaction mixtures were incubated for 30 min at 50 °C. At the indicated times, samples of 0.1 mL of the reaction mixture were taken and boiled with 0.1 mL of dinitrosalicylic acid (DNS) for 5 min and submerged in an ice bath for 5 min. To perform photometric determination, 1 mL of double-distilled water was added, and samples were analyzed at 540 nm using a Shimadzu UV-Vis 1240 mini spectrophotometer (Japan). Standard curves for glucose, xylose, and maltose were used to determine the cellulase, xylanase, and amylase titers, respectively. All assays were performed in duplicate and enzyme units were expressed as the amount of enzyme that catalyze the liberation of 1 μmol of reducing sugar per minute. Laccase determinations were done according to the method reported by Abadulla *et al*. (2000), using a reaction with 2,6-dimethoxyphenol (2,6-DMP) as the substrate in a 200-mM sodium acetate buffer at pH 4.5; spectrophotometer determinations were performed at 468 nm. These assays were also used to determine the optimal pH and temperature. Laccase units were expressed as the amount of enzyme that oxidize 1 μmol of DMP per minute.

SDS-PAGE and zymograms

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 10% polyacrylamide gel according to the method detailed by Laemmli (1970) and stained with Coomassie blue. Cellulase activity was detected following the protocol of Blanco *et al.* (1998). After electrophoresis, the gels were soaked in 2.5% (v/v) Triton X-100 for 30 min and washed twice in 50-mM phosphate citrate buffer, pH 5.0) for 30 min at room temperature. The gels were overlaid onto a 1-mm-thick 1% agarose gel containing 0.2% substrate (CMC, xylan, or starch) and incubated at 50 °C to allow the reaction to proceed. After incubation, substrate-agarose gels were stained, depending on the substrate, with 0.1% (w/v) Congo red or iodine solution for 30 min and 1 min, respectively, and washed with 1-M NaCl until bands became visible. Congo red-stained gels were immersed in 5% (w/w) acetic acid to develop a dark blue background.

RESULTS AND DISCUSSION

Pycnoporus sanguineus is a worldwide distributed basidiomycete known for its high lignolytic potential. This potential has been associated with production of high-redox potential laccases as well as low-molecular weight mediators (Eggert *et al.* 1996; 1997). Sigoillot *et al.* (2002) reported that the lignocellulolytic and hemicellulolytic activities of *Pycnoporus cinnabarinus* are thermostable, and the potential of this fungus for use in different industrial and biotechnological processes has been explored, which takes advantage of agroindustrial residues for enzyme or bioethanol production (Quiroz-Castañeda *et al.* 2011). The lignolytic, cellulolytic, and hemicellulolytic potentials on solid media, of four native strains isolated from different ecotypes of Northeast Mexico were evaluated in this study. Taking into account the versatility of these fungi as producers of hydrolytic enzymes, the pectinolytic and amylolytic capabilities were also assessed.

Screening in Solid Plate Assay

From a collection of 120 native strains from different Northeast Mexico ecotypes, the lignocellulolytic, pectinolytic, and amylolytic potentials of four *Pycnoporus sanguineus* isolates were explored. Among them, a color-based agar plate system was established and the enzymatic capabilities of these autochthonous strains were compared to those of three well-studied international reference strains, as exemplified in Fig. 1. In general (Table 2), the four *Pycnoporus sanguineus* strains were good cellulase producers and exhibited better production rates than the reference strains *Phanerochaete chrysosporium* ATCC 24785, *Trametes hirsuta* UAMH 8156, and *Trametes versicolor* UAMH 8272. Strains *P. sanguineus* CS2 and *P. sanguineus* CS43 exhibited higher hydrolytic activity over pectin and starch than the rest of the strains evaluated. All strains had lower capability for xylanase production, but *P. sanguineus* CS2, *P. sanguineus* CS43, and *Trametes hirsuta* UAMH 8156 strains were the best producers.

P. sanguineus CS2 and *P. sanguineus* CS43 had the highest lignolytic activities. Both strains had entirely discolored the growth area on Poly R-478 plates starting on the fifth day of growth. The rest of the strains evaluated had moderate activity. The same behavior was observed in the medium supplemented with syringaldazine, in which the positive reaction diameter was larger than the growth diameter. In a similar study with 37 thermophilic fungal strains, isolated from different lignin-rich ecotypes (Younes *et al*. 2011), it was observed that isolates with the highest lignolytic potential had low hydrolytic potential and that the strains with the best hydrolytic activities did not produce lignolytic enzymes. These findings highlight the limitations of studied isolates in the simultaneous degradation of plant cell wall components. On the other hand, filamentous fungi have been reported to be the best cellulase producers. Under similar conditions of hydrolase activity detection to those in this work, the best filamentous strains exhibit hydrolysis areas as large as the growth area (Khokhar *et al.* 2012). The native basidiomycetes evaluated in the present study had hydrolysis areas twice as large as the growth area. In the case of pectinases, some studies of basidiomycetes (Pericin *et al.* 1992; Pericin *et al.*1997; Levin and Forchiassim 1998; Xavier-Santos *et al*. 2004) report *P. sanguineus* as the best polygalacturonase producer.

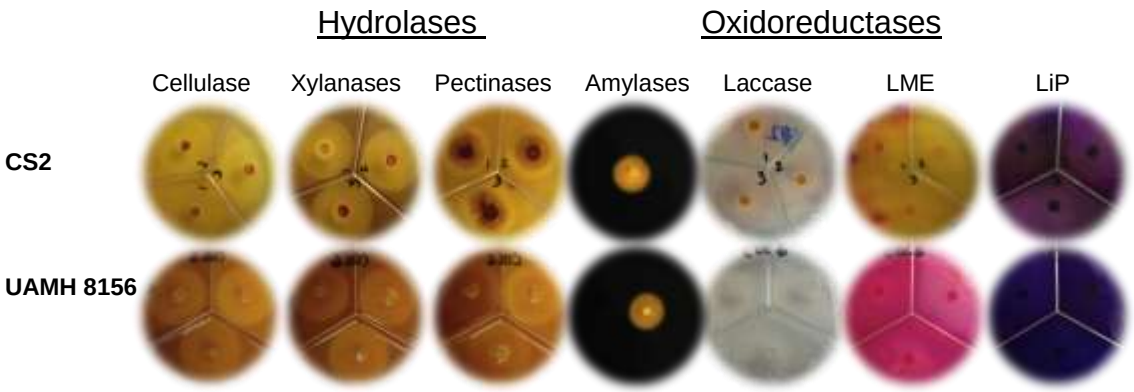

Fig.1. Color-Based System for Screening on Agar Plates. Figure shows a series of representative results for *Pycnoporus sanguineus* CS2, in comparison with *Trametes hirsuta* UAMH8156. Enzymatic potentials were estimated as described in methods.

Table 2. Lignocellulolytic Profiles of *Pycnoporus sanguineus* Native Strains and Reference Strains

Strain	Cellulase	Xylanase	Pectinase	Amylases	LME	Laccase
P. sanguineus CS2	3	2	3	3	3	3
P. sanguineus CS20	3	1	2	2	2	2
P. sanguineus CS43	3	2	3	3	3	3
P. sanguineus LE90	2	1	2	2	2	2
Ph. chrysosporium ATCC 24785	1	1	0	1	2	0
T. hirsuta UAMH 8156	2	2	2	2	0	1
T. versicolor UAMH 8272	1	1	2	2	1	2

3 = High activity, 2 = Mild activity, 1 = Weak activity, and 0 = No activity
LME, lignin modifying enzymes

Enzyme Production in Submerged Medium

Once the best lignocellulolytic enzyme producer, *Pycnoporus sanguineus* CS2 strain, for enzymatic production was selected, the submerged fermentation process was evaluated. A rich medium and agroindustrial residues based on a potassium phosphate buffer were used. Cellulase (CMCases, Avicelases, and β-D-glucosidase) as well as xylanase, amylase, and laccase production curves were determined. Under all tested conditions, β-D-glucosidase had the highest titers (43 to 73 U/flask) and amylase and laccase had lower titers. Amylase production ranged from 1 to 7 U/flask and laccase production ranged from 1 to 6 U/flask. The CMCases were more abundant than the Avicelases. This was not the case in WB and WB/AP/GP media in which CMCase titers were in the range of 20 to 30 U/flask. Avicelases exhibited a similar production pattern as CMCases with respect to the media used, but their titers were lower, ranging from 12 to 15 U/flask. All results are shown in Table 3. The highest enzymatic activities were observed when using WS/OP medium, except in the case of laccases. However, lignolytic activity could be inhibited by the polyphenols present in orange peels. This theory is supported by the results obtained under the same condition but when replacing orange peels with grapefruit peels, in which higher laccase titers (6.4 U/flask) were observed.

Amylases were present at higher levels in RMB-CMC and RMB-Xyl. The highest titers for all enzymatic activities were detected in WB media.

Table 3. Lignocellulolytic Enzyme Production of *Pycnoporus sanguineus* CS2

	Cellulases	Xylanases	β-D-glucosidases	Avicelases	Amylases	Laccases
	U/Flask*					
RBM-CMC	23.27	18.35	72.94	13.53	7.2	5.64
RBM-Xyl	21.75	17.83	67.86	15.34	6.48	3.67
WS	31.82	13.3	60.08	12.27	4.6	2.01
WS/OP	33.53	18.39	72.64	15.75	4.17	2.14
OP	31.82	13.3	60.08	12.27	4.6	1.16
OF/AP/OP	22.64	15.61	64.71	13.45	1.03	3.99
OF/AP/GP	29.27	18.15	43.64	16.6	5.31	6.44
WB/AP/GP	12.38	9.89	52.82	7.2	3.41	3.0
WB	3.6	4.02	44.94	0.16	0.76	2.0

* Liquid media on flask = 200 mL total volume

With regards to cellulase production *via* submerged fermentation, the greatest production was in WS/OP medium. In general, a variation of titers as a function of the culture media occurred. This was similar to the results obtained by Pukahuta *et al.* (2004) in their study of 19 strains of *Lentinus polychrous* cultivated in media supplemented with agroindustrial residues. They observed that, on different isolates of the same species, activity titers varied between 0.017 and 1.041 U/mL, and cellulases were not detected at all in five strains. These values are low compared to those reported by Elisashvili *et al.* (2009), a study in which *Pseudotremella gibbosa* IBB22 strain was selected as the best producer with 122 U/mL. In the case of the *Trametes* strains assayed, varied titers from 2 to 74 U/mL were observed. For *Pycnoporus* genus, it has been reported that cellulase production reached 20 U/mL from a *P. coccineus* IBB310 strain *via* the submerged fermentation of tangerine peels (Elisashvili *et al.* 2011). For *P. sanguineus* PF-2, 0.06 U/mL production *via* the submerged fermentation of milled corn cobs was reported (Falkoski *et al.* 2012). These results are very close to those of the present work.

Xylanases were produced at contents ranging from 4 to 18 U/flask, titers similar to those of exocellulases and lower than those of endocellulases. Production variability was once again a function of the media used. Even though these titers are lower than those reported for *P. coccineus* (17 to 32 U/mL), a similar behavior was observed using different residues in solid-state fermentation. In this case, banana peels were better natural inductors than leaf litter, wheat straw, or apple peels (Elisashvili *et al.* 2008). For *P. sanguineus* PF-2 under submerged fermentation in media supplemented with milled corncobs, the level detected was 10 U/mL (Falkoski *et al.* 2012), much higher than that in the present study for *P. sanguineus* CS2. The difference in these values may be due to media composition, culture conditions, or the origin of the strains.

With regard to ß-glucosidase production, both *P. sanguineus* PF-2 and *P. sanguineus* CS2 strains showed similar production levels at 0.20 and 0.36 U/mL, respectively.

In the present work, the amylase production curves of *P. sanguineus* CS2 were obtained under different culture conditions, where the greatest titles were detected in RMB-CMC and RMB-Xyl media (0.035 and 0.032 U/mL, respectively). Regarding

amylase production *via* submerged fermentation, *P. sanguineus* IBAMA-Brazil has been examined using starch or wheat bran as the carbon source. This is the first use of this fungus as a new amylase source reported by Siqueira *et al.* (1997). The greatest production of this strain was on the fourth day of culture, whereas for *P. sanguineus* CS2, the greatest production occurred on the fifth day.

Regarding laccase production from *P. sanguineus* CS2, maximum production of 7.5 U/mL in a Kirk medium modified with 350-μM cupric sulphate and 3% ethanol as inducers was reported (Salcedo-Martínez *et al.* 2013). Under the culture conditions used in this study, laccase titers were notably lower. This could be due to the presence of pigments and phenolic compounds in the lignin rich-substrates used, which could inhibit laccase activity while stimulating cellulase, hemicellulose, and pectinase activities (Elisashvili *et al.* 2009).

Molecular Weights, Optimal pH, and Temperature Estimation

The relative molecular sizes, optimal pH, and temperature for each enzymatic activity are shown in Table 4. Only one band of protein with laccase activity was detected on zymograms and SDS-PAGE gels copolymerized with CMC and xylan. For this band, a relative molecular size of 61 kDa was estimated. Amylase activity was associated with an activity band with a relative size of 91 kDa. Two protein bands with sizes of 66.2 and 56.5 kDa were detected for CMCase and xylanase activities, respectively. In the case of CMCase, the estimated weight for *P. sanguineus* CS2 was heavier than reported for *Pycnoporus sanguineus* CEIBMD01 (25 and 50 kD). However, native xylanase was smaller than reported for this strain with values between 80 and 90 kDa (Quiroz-Castañeda *et al.* 2009). With regard to size estimation for amylases, no reports of the molecular weight of *P. sanguineus* were found. Nevertheless, the enzyme from the strain used in the present work (90.8 kDa) was larger than those of reports for bacteria (20 to 30 kDa), fungi, and yeast (40 to 68 kDa) (Gupta *et al.* 2003).

Laccase activity was detected by component analysis with a relative molecular weight of 61 kDa, close to that reported for other laccases produced by other *Pycnoporus sanguineus* strains (Garcia *et al.* 2006; Acosta-Urdapilleta *et al.* 2010). Even though this is smaller than reported for this strain in another study (Salcedo-Martínez 2013), this may be due to adaptations of the techniques for the other enzymes of this work. All molecular weights reported here are relative.

The optimal pH for laccase production over 2,6-DMP was 3.5. The CMCases, xylanases, and amylases had an optimal pH of 5.0 with more than 95% of their activity conserved at pH 6.0. These results were similar to those reported for other *P. sanguineus* enzymes (Quiroz-Castañeda *et al.* 2009; Lomascolo *et al.* 2011).

The optimal temperature for laccase and amylase was 60 °C, whereas CMCase and xylanase had an optimal activity at 70 °C. In general, hydrolytic enzymes preserve more than 95% of their activity at 90 ºC (100% for cellulases, 99% for xylanases, and 98% amylases, as shown in Fig. 2). The optimal activity for CMCases and xylanases was higher than previously reported for other strains (Falkoski *et al.* 2012). The apparent increase in activity at 90 °C may be due to the increased physical accessibility of the remaining active enzymes in between branches of the polysaccharide structure that were somehow separated from one another at the elevated temperature. This phenomenon can be explained as a combination of two important factors, substrate solubility and enzyme thermostability.

Table 4. Molecular Weights, pH, and Temperature of *P. sanguineus* CS2 Extracellular Enzymes

Enzyme	MW (kDa)	pH	T (°C)
Cellulase	66.2	5.0	70
Xylanase	56.5	5.0	70
Amylase	90.8	5.0	70
Laccase	61.0	3.5 *	60

* Detected with 2, 6-DMP (2-mM)

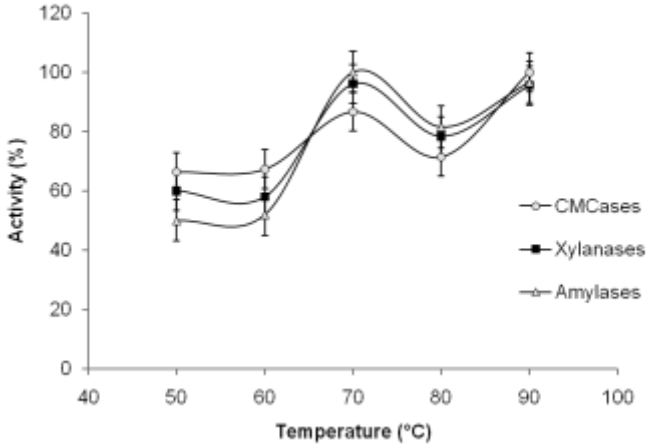

Fig. 2. Effects of temperature on enzyme activity. All assays were performed in triplicate and determined using the DNS method. Results showed a relative standard deviation lower than 5%.

All results allowed the strain *P. sanguineus* CS2 to be selected for the development of new adjuvants to improve animal nutrition for both ruminants and monogastric animals (Ribeiro *et al.* 2012). This can be further improved if it is considered that all degrading activities of the plant cell wall components are produced by one organism and, thanks to its plasticity, any enzyme production can be intensified by taking advantage of the agroindustrial residues generated in the region. This possibility will be explored in further studies.

CONCLUSIONS

1. *Pycnoporus sanguineus* CS2 is a thermostable cellulase, xylanase, and amylase producer that retains greater than 90% of its activity at 90 °C.
2. Because of its high lignocellulolytic, amylolytic, and pectinolytic potential, *Pycnoporus sanguineus* CS2 could be used for digestive adjuvant development in animal nutrition.
3. As a result of its metabolic plasticity and depending on the culture medium, it is possible to obtain high concentrations of all enzymes studied or to increase the production of specific enzymes. This would lend itself well to a broad range of applications in various industrial processes.

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REFERENCES CITED

Abadulla, E., Tzanov, T., Costa, S., Robra, K., and Gübitz, G. (2000). "Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsute*," *Appl. Environ. Microb.* 66(8), 3357-3362. DOI: 10.1128/AEM.66.8.3357-3362.2000

Acosta-Urdapilleta, L., Paz, G. A. A., Rodríguez, A., Adame, M., Salgado, D., Salgado, J., Montiel-Pena, M., Medrano-Vega, and Villarreal, E. C. V. (2010). "*Pycnoporus sanguineus* un hongo con potencial biotecnológico," in: *Hacia un Desarrollo Sostenible del Sistema de Producción-Consumo de los Hongos Comestibles y Medicinales en Latinoamérica: Avances y Perspectivas en el Siglo XXI*. D. Martínez-Carrera, N. Curvetto, M. Sobal, P. Morales, and V. M. Mora (eds.), pp. 188-220.

Altaf, S. A., Dahot, M. U., and Mangrio, S. M. (2010). "Production of xylanase enzymes by *Pleurotus eryngii* and *Flammulina velutipes* grown on different carbon sources under submerged fermentation," *World Appl. Sci. J.* 8 (Special Issue of Biotechnology & Genetic Engineering), 47-49.

Blanco, A., Díaz, P., Martínez, J., Vidal, T., Torres, A. L., and Pastor, F. I. (1998). "Cloning of a new endoglucanase gene from *Bacillus* sp. BP-23 and characterisation of the enzyme. Performance in paper manufacture from cereal straw," *Appl. Microbiol. Biotechnol*. 50(1), 48-54. DOI: 10.1007/s002530051255

Eggert, C., Temp, U., Dean, J. F., and Eriksson, K. E. L. (1996). "A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase," *FEBS Lett.* 391(1), 144-148. DOI: 10.1016/0014-5793(96)00719-3

Eggert, C., Temp, U., and Eriksson, K. E. L. (1997). "Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*," *FEBS Lett.* 407(1), 89-92. DOI: 10.1016/S0014-5793(97)00301-3

Elisashvili, V., Kachlishvili, E., and Penninckx, M. (2008). "Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes," *J. Ind. Microbiol. Biotechnol.* 35(11), 1531-1538. DOI: 10.1007/s10295-008-0454-2

Elisashvili, V., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Kahardziani, T., and Agathos, S. (2009). "Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the forest of Georgia," *World J. Microbiol. Biotechnol*. 25(1), 331-339. DOI: 10.1007/s11274-008-9897-x

Elisashvili, V., Torok, T., Kachlishvili, E., Khardziani, T., Metreveli, E., Kobakhidze, A., and Berikashvili, I. (2011). "Evaluation and regulation of the lignocellulolytic activity of novel white-rot basidiomycetes," *Global J. Biochem.* 1(2), 134-141.

Falkoski, D. L., Monteze, V. V., Almeida, M., Couto Alfenas, A., Colodette, J. L., and Tavares, S. (2012). "Characterization of cellulolytic extract from *Pycnoporus*

sanguineus PF-2 and its application in biomass saccharification," *Appl. Biochem. Biotechnol.* 166(6), 1586-1603. DOI: 10.1007/s12010-012-9565-3

García, A. M., and Torres, R. G. (2003). "Producción de enzimas lignolíticas por basidiomicetes mediante la técnica de fermentación en sustrato sólido," *J. Colom. de Biotech.* 5(1), 56-64.

Garcia, T. A., Santiago, M. F., and Ulhoa, C. J. (2006). "Properties of laccases produced by *Pycnoporus sanguineus* induced by 2,5-xylidine," *Biotechnology Letters* 28(9), 633-636. DOI: 10.1007/s1059-006-0026-3

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., and Chauhan, B. (2003). "Microbial α-amylases: A biotechnological perspective," *Process Biochem.* 38(1), 1599-1616 DOI: 10.1016/S0032-9592(03)00053-0

Henrissat, B., Teeri, T. T., and Warren, R. A. J. (1998). "A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants," *FEBS Letters* 425(2), 352-354. DOI: 10.1016/S0014-5793(98)00265-8

Hernández-Luna, C. E., Gutiérrez-Soto, G., and Salcedo-Martínez, S. M. (2008). "Screening for decolorizing Basidiomycetes in Mexico," *World J. Microbiol. Biotechnol*. 24(4), 465-473. DOI: 10.1007/s11274-007.9495-3

Khokhar, I., Haider, M. S., Mushtaq, S., and Mukhtar, I. (2012). "Isolation and screening of high cellulolytic filamentous fungi," *J. Appl. Sci. Environ. Manage*. 16(3), 223-226.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature* 227 No.5259, 680-685. DOI: 10.1038/227680a0

Levin, L., and Forchiassim, F. (1998). "Culture conditions for the production of pectinolytic enzymes of white-rot fungus *Trametes trogii* on the laboratory scale," *Acta Biotechnol.* 18(2), 157-166. DOI: 10.1002/abio.370180213

Levin, L., Villalba, L., Da Re, V., Forchiassin, F., and Papinutti, L. (2007). "Comparative studies of loblolly pine biodegradation and enzyme production by Argentinean white rot fungi focused on biopulping processes," *Process Biochem.* 42(6), 995-1002. DOI: 10.1016/j.procbio.2007.03.008

Lomascolo, A., Uzan-Boukhris, E., Herpoël-Gimbert, I., Sigoillot, J. C., and Lesage-Meessen, L. (2011). "Peculiarities of *Pycnoporus* species for applications in biotechnology," *Appl. Microb. Biotechnol.* 92(6), 1129-1149. DOI: 10.1007/s00253-011-3596-5

Mendonça, M. J., Castro, A., and Telles, H. C. (2010). "Industrial and biotechnological applications of ligninolytic enzymes of the Basidiomycota: A review," *Electronic Journal of Biotechnology* 13(6), 14-21. DOI: 10.2225/vol13-issue6-fulltext-2

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem*. 31(3), 426-428. DOI: 10.1021/ac60147a030

Pericin, D., Kovrosan, S., Banka, L., Antov, M., and Skrinjar, M. (1992). "Separation of the components of pectinolytic complex produced by *Polyporus squamosus* in submerged culture," *Biotechnol. Letters* 14(2), 127-130.

Pericin, D., Antov, M., Dimic, N., and Vujicic, B. (1997). "Rapid method for detection of low basal activity of exo-pectinase of *Polyporus squamosus*," *Biotechnol. Tech*. 11(11), 833-836. DOI: 10.1023/A:1018485510779

Philippoussis, A., Diamantopoulou, P., Papadopoulou, K., Lakhtar, H., Roussos, S., Parissopoulos, G., and Papanikolaou, S. (2011). "Biomass, laccase and endoglucanase production by *Lentinula edodes* during solid state fermentation of reed grass, vean

stalks and wheat straw residues," *World J. Microbiol. Biotechnol.* 27(2), 285-297. DOI: 10.1007/s11274-010-0458-8

Pozdnyakova, N. N., Turkovskaya, O. V., Yudina, E. N., and Rodakiewicz-Nowak, Y. (2006). "Yellow laccase from the fungus *Pleurotus ostreatus*: Purification and characterization," *J. Appl. Biochem. Microbiol*. 42(1), 56-61. DOI: 10.1134/S000368380601008X

Pukahuta, C., Suwanarit, P., Shinagawa, E., Hoshida, H., and Nishizawa, Y. (2004). "Combination of laccase, xylanase, and cellulase in lignocellulose degradation by white rot fungi, *Lentinus polychrous* Lév. and *L. squarrosulus* Mont.," *Kasetsart J (Nat. Sci.)* 38(1), 65-73.

Quiroz-Castañeda, R. E., Balcázar-López, E., Dantán-González, E., Martinez, A., Folch-Mallol, J., and Martínez-Anaya, C. (2009). "Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium," *Electron. J. Biotech.* 12(4), 1-8. DOI: 10.2225/vol12-issue4-fulltext-3

Quiroz-Castañeda, R. E., Martínez-Anaya, C., Cuervo-Soto, L. I., Segovia, L., and Folch-Mallol, J. L. (2011). "Loosenin, a novel protein with cellulose- disrupting activity from *Bjerkandera adusta*," *Microb. Cell Fact.* 10(8), 1-9. DOI: 10.1186/1475-2859-10-8

Ribeiro, L., Pinheiro, V., Outor-Monteiro, D., Mourão, J., Bezerra, R. M. F, Dias, A. A., Bennett, R. N., Marques, G., and Rodrigues, M. A. M. (2012). "Effects of dietary incorporation of untreated and white-rot fungi (*Ganoderma resinaceum* Boud) pre-treated olive leaves on growing rabbits," *Anim. Feed Sci. Tech.* 173(3-4), 244-251. DOI: 10.1016/j.anifeedsci.2012.01.014

Salcedo-Martínez, S. M, Gutiérrez-Soto, G., Rodríguez, C. F., Villarreal, T. J., Contreras, J. F., and Hernández-Luna, C. E. (2013). "Purification and partial characterization of a thermostable laccase from *Pycnoporus sanguineus* CS-2 with ability to oxidize high redox potential substrates and recalcitrant dyes," in: *App. Bioremediat. - Active and Passive Approaches*. Y. B. Patil and P. Rao (eds.), InTech, Rijeka, Croatia. DOI: 10.5772/56374

Sigoillot, C., Lomascolo, A., Record, E., Robert, J. L., Asther, M., and Sigoillot, J. L. (2002). "Lignocellulolytic and hemicellulolytic system of *Pycnoporus cinnabarinus*: Isolation and characterization of a cellobiose dehydrogenase and a new xylanase," *Enz. Microb. Tech.* 31(6), 876-883. DOI: 10.1016/S0141-0229(02)00208-9

Sin, M. K. W., Hyde, D. K., and Pointing, S. B. (2002). "Comparative enzyme production by fungi from diverse lignocellulosic substrates," *J. Microb.* 40(3), 241-244.

Siqueira, E. M., Mizuta, K., and Giglio, J. R. (1997). "*Pycnoporus sanguineus*: A novel source of [alpha]-amylase," *Mycol. Res.* 101(2), 188-190. DOI: 10.1017/S0953756296002547

Steffen, K. T., Cajthaml, T., Šnajdr, J., and Bladrian, P. (2007). "Differential degradation of oak (*Quercus petraea*) leaf litter by litter-decomposing Basidiomycetes," *Res. Microbiol.* 158(5), 447-455. DOI: 10.1016/j.resmic.2007.04.002

Valášková, V., and Baldrian, P. (2006). "Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* - Production of extracelular enzymes and characterization of the major cellulases," *Microbiology* 152(2), 3613-3622. DOI: 10.1099/mic.0.29149-0

Xavier-Santos, S., Carvalho, C. C., Bonfá, M., Silva, R., Capelari, M., and Gomes, E. (2004). "Screening for pectinolytic activity of wood-rotting Basidiomycetes and

characterization of enzymes," *Folia Microbiol*. 49(1), 46-52. DOI: 10.1007/BF02931645

Younes, B. S., Karray, F., and Sayadi, S. (2011). "Isolation of thermophilic fungal strains producing oxido-reductase and hydrolase enzymes from various Tunisian biotypes," *International Biodeterioration and Biodegradation*, 65(7), 1104-1109. DOI: 10.1016/j.ibiod.2011.08.0 03

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