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Short Communication

Characterization of laccase isoforms produced by *Pleurotus ostreatus* in solid state fermentation of sugarcane bagasse

Susan Grace Karp [a,c], Vincenza Faraco [b,e], Antonella Amore [b], Leila Birolo [b,e], Chiara Giangrande [b], Vanete Thomaz Soccol [a,d], Ashok Pandey [f], Carlos Ricardo Soccol [a,*]

[a] Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná, Coronel Francisco H. dos Santos Avenue, 210, Zip Code 81531-990 Curitiba, Brazil
[b] Department of Organic Chemistry and Biochemistry, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Naples, Italy
[c] National Service of Industrial Apprenticeship (SENAI-CIC), Curitiba, Brazil
[d] Industrial Biotechnology Program, Positivo University, Curitiba, Brazil
[e] School of Biotechnological Sciences, University of Naples "Federico II", Italy
[f] Biotechnology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum, India

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ABSTRACT

Laccases are oxidative enzymes linked to biological degradation of lignin. The aim of this work was to evaluate the effect of inducers and different concentrations of nitrogen on production level of total laccase activity and pattern of laccase isoforms, produced in solid state fermentation of sugarcane bagasse by a selected strain of *Pleurotus ostreatus*. The addition of yeast extract 5 g/L, copper sulfate 150 μM and ferulic acid 2 mM provided highest enzymatic activity (167 U/g) and zymograms indicated the presence of six laccase isoforms (POXA1b, POXA3, POXC and three other isoforms). Results of protein identification by mass spectrometry confirmed the presence of POXC and POXA3 as the main isoenzymes, and also identified a glyoxal oxidase and three galactose oxidases. The fact that the isoenzyme POXA1b was not identified in the analyzed samples can be possibly explained by its sensitivity to protease degradation.

1. Introduction

Sugarcane bagasse is an important agro-residue generated in high amount (186 million tons/year) by the sugar and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane, and is composed of 19–24% lignin, 27–32% hemicellulose, 32–44% cellulose and 4.5–9% ashes (Soccol et al., 2011). Lignin is a recalcitrant aromatic polymer that can be oxidized by laccases, manganese peroxidases and lignin peroxidases. These enzymes catalyze one-electron oxidation of lignin units, producing aromatic radicals (Giardina et al., 2000). Laccases (E.C. 1.10.3.2, *p*-diphenol:dioxygen oxidoreductases) are blue multicopper oxidases able to oxidize a variety of phenolic compounds, with concomitant reduction of molecular oxygen to water. These enzymes are secreted in multiple isoforms depending on the fungal species and the environmental conditions, and this variety is related to the diversity of their roles: lignin degradation/synthesis, fruiting bodies development, pigment production, cell detoxification (Piscitelli et al., 2011). The biochemical diversity of laccase isoenzymes appears to be due to the multiplicity of laccase genes; however, regulation of their expression can be substantially diverse between fungal species (Palmieri et al., 2003).

Pleurotus ostreatus belongs to a class of white-rot fungi that produces laccases, manganese peroxidases but not lignin peroxidases (Giardina et al., 2000). The sequencing and annotation of the *P. ostreatus* PC 15 genome version 2.0 (Joint Genome Institute, 2011) indicates the presence of at least 12 genes of multicopper oxidases. Some of the corresponding enzymes have been purified and characterized, and these include POXA1b (Giardina et al., 1999), POXA1w and POXA2 (Palmieri et al., 1997), POXA3 (Palmieri et al., 2003) and POXC, previously named POX2 (Giardina et al., 1996) where POX means phenol oxidase. Other isoenzymes whose sequences have been determined are POX1 (Giardina et al., 1995), POX3 and POX4 (Pezzella et al., 2009). POXC is the most abundantly produced under all growth conditions examined according to Giardina et al. (1999). The objective of these studies was to evaluate the effect of known inducers of laccase expression (copper sulfate and ferulic acid) and two levels of organic nitrogen concentration in the form of yeast extract, on the production level of total laccase activity and laccase isoforms – identified by zymograms and mass spectrometry – produced in solid state fermentation, utilizing the sugarcane bagasse as substrate and a selected strain of *P. ostreatus* (coded Pl 22 Em).

* Corresponding author. Tel.: +55 41 33613191.
E-mail address: soccol@ufpr.br (C.R. Soccol).

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2. Methods

2.1. Solid state fermentation

Erlenmeyer flasks of 125 mL containing 1 g of sugarcane bagasse were autoclaved and humidified with a saline solution (sterilized by filtration, 15 mL/g bagasse) presenting the following constant composition: $MgSO_4{\cdot}7H_2O$ (0.3 g/L), $FeSO_4{\cdot}7H_2O$ (0.005 g/L), $MnSO_4{\cdot}H_2O$ (0.00156 g/L), $ZnSO_4{\cdot}7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), $CoCl_2$ (0.002 g/L), KH_2PO_4 (1.5 g/L), pH 5.5, with some differences regarding the following variables: yeast extract (2 or 5 g/L, containing 10.5% w/w total nitrogen, sterilized by autoclaving at 121 °C, 1 atm for 15 min); $CuSO_4$ (0 or 150 μM, sterilized by filtration), ferulic acid (2 mM, sterilized by filtration and added after 48 h of fermentation). These concentrations were chosen on the basis of optimization experiments for laccase production by *P. ostreatus* (data not shown). The strains *P. ostreatus* 22 Em (Pl 22 Em – selected for presenting the highest laccase production in sugarcane bagasse among 45 strains of basidiomycetes) and *P. ostreatus* ATCC MYA-2306 (Pl ATCC – standard dikaryotic strain for laccase production in liquid culture) were reactivated in PDA dishes. After 7 days of growth, 4 disks of 7 mm diameter were transferred to Erlenmeyer flasks of 250 mL containing 50 mL of Czapek liquid medium and the antibiotic streptomycin sulfate (0.1 g/L), added to reduce the risk of bacterial contamination. After 5 days of growth at 28 °C and 125 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a homogenizer (IKA T-10 basic Ultra-Turrax) and resuspended in the residual medium to a lower final volume (10% of the initial volume). 0.2 mL of the homogenized mycelium was transferred to the fermentation flasks, which were manually homogenized and incubated at 28 °C for different times.

2.2. Extraction of the enzymes

Enzymes produced by solid fermentation were extracted by solid–liquid extraction using sodium phosphate buffer as solvent ($NaH_2PO_4{\cdot}H_2O$, 50 mM, pH 7.0) containing 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), at the proportion of 1:10 (w/w) (Iandolo et al., 2011). The mixture was homogenized in vortex for 1 min and centrifuged for 7500 rpm, 4 °C, 45 min. The supernatant was separated and submitted to analyses.

2.3. Liquid fermentation

Liquid fermentation was conducted in PDY medium (potato dextrose 24 g/L and yeast extract 5 g/L) containing Cu^{2+} 150 μM, added after autoclaving, in Erlenmeyer flasks of 500 mL filled with 250 mL (final volume). The strains Pl 22 Em and Pl ATCC were reactivated in PDA dishes and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Erlenmeyer flasks of 250 mL containing 50 mL of Czapek liquid medium and the antibiotic streptomycin sulfate (0.1 g/L). After 5 days of growth at 28 °C and 125 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a homogenizer (IKA T-10 basic Ultra-Turrax) and resuspended in the residual medium to a lower final volume (10% of the initial volume). 2.5 mL of the homogenized mycelium were transferred to the fermentation flasks, which were incubated at 28 °C and 125 rpm.

2.4. Laccase activity and protein concentration assays

The enzymatic activity of laccases was assayed by the oxidation of ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid). The reaction mixture contained 100 μL of ABTS 20 mM (in sodium citrate buffer 0.1 M, pH 3.0), sample (usually 20–50 μL) and sodium citrate buffer ($C_6H_8O_7{\cdot}H_2O$ 0.1 M, pH 3.0) up to 1 mL. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 $M^{-1}\,cm^{-1}$). The enzyme activity was expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min. Protein concentration was determined using the BioRad Protein Assay, with bovine serum albumin as a standard.

2.5. Enzyme preparations

Enzyme solutions were concentrated by ultrafiltration and dialyzed in sodium phosphate buffer (50 mM, pH 7.0). Samples were centrifuged at 8000 rpm, 4 °C in Amicon tubes (Amicon Ultra centrifugal filters 0.5 mL, Ultracel 30 kDa membrane – Millipore).

2.6. Zymograms of native PAGE

Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under non-denaturing conditions. The separating and stacking gels contained, respectively, 9% and 4% acrylamide and 50 mM Tris–HCl (pH 9.5) and 18 mM Tris–HCl (pH 7.5) as buffers. The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). Visualization of the bands was achieved by the addition of ABTS 20 mM to the gel submerged in sodium citrate buffer (0.1 M, pH 3.0) after electrophoresis.

2.7. Preparation of enzymes for protein identification

Enzymes from the samples selected for enzyme identification were extracted according to item 2.2 and concentrated by ammonium sulfate precipitation (80% saturation, 4 °C, overnight) followed by centrifugation (8000 rpm, 4 °C, 40 min). Protein precipitate was suspended in 1 mL sodium phosphate buffer (50 mM, pH 6.5) and extensively dialyzed against the same buffer in a 12,000–14,000 Daltons membrane (Delchimica dialysis tubing – visking, code DTV 12000), until the ammonium sulfate was completely removed. Activity and protein concentration in the enzyme suspension were determined according to item 2.4. Semi-denaturating SDS–PAGE was performed by loading the protein suspension in 0.1% SDS buffer not containing 2-mercaptoethanol and without boiling. The separating and stacking gels contained, respectively, 12.5 and 4% acrylamide and 375 mM Tris–HCl plus 0.1% SDS (pH 8.8) and 125 mM Tris–HCl plus 0.1% SDS (pH 6.8) as buffers. The electrode reservoir solution contained 25 mM Tris, 190 mM glycine and 3.5 mM SDS (pH 8.4). Visualization of the bands was achieved by Coomassie brilliant blue staining.

2.8. Protein identification

Coomassie blue-stained protein bands were excised from the gels and washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubating the bands with 10 mM dithiothreitol (DTT) for 45 min at 56 °C. Cysteines were alkylated by incubation in 5 mM iodoacetamide for 15 min at room temperature in the dark. The bands were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/μL) in 50 mM ammonium bicarbonate buffer, pH 8.5. Gel particles were incubated at 4 °C for 2 h. The buffer solution was then removed and a new aliquot of buffer solution was added for 18 h at 37 °C. Peptides were then extracted by washing the gel particles with 0.1% (v/v) formic acid in 50% (v/v) acetonitrile at room temperature and lyophilized. Peptide mixtures were analyzed by LC–MS/MS, using a HPLC–Chip LC system (Agilent 1200) connected to a Q-TOF 6520 (Agilent Technologies). Lyophilized samples were resuspended in 10 μL of 0.1% (v/v) formic acid. After loading, the peptide

mixtures were concentrated and washed at 4 μL/min in a 40 nL enrichment column with 0.2% (v/v) formic acid in 2% (v/v) acetonitrile. Fractionation was carried out on a C-18 reverse phase column (75 μm × 43 mm) at a flow rate of 0.4 μL/min with a linear gradient of eluent B (95% v/v acetonitrile and 0.2% v/v formic acid) in eluent A (2% v/v acetonitrile and 0.1% v/v formic acid) from 7% to 80% in 51 min. Mass spectrometry analyses were performed using data dependent acquisition MS scans (mass range 300–2400 m/z), followed by MS/MS scans (mass range 100–2000 m/z) of the 4 most intense ions of a chromatographic peak. Raw data from LC–MS/MS were converted to m/z data, and searched against the PleosPC15 database available at the Joint Genome Institute's website (http://genome.jgi-psf.org/PleosPC15_1) using the licensed version of Mascot 2.1 (Matrix Science).

3. Results and discussion

3.1. Evaluation of the effect of copper sulfate and ferulic acid addition on laccase activity production level and laccase isoenzymes pattern

Results in Table 1 showed a peak of activity at the 5th day, highest values being obtained with ferulic acid: 167 U/g with 5 g/L YE and 86.8 U/g with 2 g/LYE. Other values reported in literature for laccase production by *P. ostreatus* in solid state fermentation are 65.42 U/g with copper as inducer (Mishra and Kumar, 2007) and 36 U/g without inducers (Iandolo et al., 2011). According to the visual analysis of the native PAGE zymogram (Fig. 1), the isoenzyme POXC was the most abundantly produced under all conditions evaluated, which is in accordance with Giardina et al. (1996, 1999). The expression of the isoenzyme POXA3 (Palmieri et al., 2003) was induced by copper sulfate and even more by the combination of copper sulfate and ferulic acid, when comparing the three samples obtained at the 8th day. However, samples of the 10th day presented a more intense POXA3 band, indicating an increase of this isoenzyme production. The band corresponding to the isoenzyme POXA1b (Giardina et al., 1999) could be visualized only in those samples containing ferulic acid. Besides the three known isoforms POXA1b, POXA3 and POXC, three more bands were visualized. However, an isoenzyme with electrophoretic mobility similar to that of the isoform 3 has already been identified by Lettera et al. (2010) as LACC12. Moreover, isoforms with similar electrophoretic mobility to that of the isoforms 1 and 2 have already been detected during solid state fermentation of *P. ostreatus* ATCC on tomato pomace (Iandolo et al., 2011).

It has been previously demonstrated that the addition of copper sulfate 150 μM to a *P. ostreatus* liquid culture medium causes a 30-fold increase in total laccase activity and induces the isoenzymes POXC and POX A1b in *P. ostreatus* at the level of gene transcription (Palmieri et al., 2000). Analysis of the *poxc* and *poxa1b* promoter regions revealed the presence of multiple putative metal responsive elements (Faraco et al., 2002). In fact, in this experiment, the total laccase activity obtained in the presence of copper was 38-fold higher in comparison with the basal medium when analyzing the activities obtained at the 5th day at the same concentration of yeast extract (Table 1). Different studies have shown that laccase production is regulated by metal ions such as Cu^{2+} and Fe^{3+} by gene expression induction or through translational or post-translational regulation (Fonseca et al., 2010). Baldrian and Gabriel (2002) concluded that Cu^{2+} not only induces laccase by the expression of laccase genes in *P. ostreatus*, but it also positively affects activity and stability of the enzyme. Aromatic compounds such as ferulic acid have shown to stimulate laccase production in many different strains (Elisashvili and Kachlishvili, 2009). Haars and Hüttermann (1983) demonstrated that the surface of the fungal hyphae contains specific receptors for small phenolic compounds that stimulate *de novo* synthesis of laccase.

Table 1
Maximum level of laccase activity produced by the strain *P. ostreatus* 22 Em on sugarcane bagasse in different conditions of yeast extract (YE) concentrations and inducers – Cu^{2+} and ferulic acid (Fer).

YE (g/L)	Inducer	Time (days)	U/g dry substrate
2	0	5	1.06
		8	14.6
		10	11.6
2	Cu^{2+} 150 μM	5	40.5
		10	22.3
		12	18.0
2	Cu^{2+} 150 μM + Fer 2 mM	5	86.8
		10	32.2
		12	17.6
5	Cu^{2+} 150 μM	5	51.7
		10	21.4
		12	10.7
5	Cu^{2+} 150 μM + Fer 2 mM	5	167
		10	57.1
		12	37.6

According to the zymogram reported in Fig. 2, the intensity of the band related to POXC was not affected by different concentrations of yeast extract and the presence of both inducers, and its intensity decreased only after 15 days (sample 5Fer15). The expression of the isoenzyme POXA3 was induced by ferulic acid, in accordance with the previous results, for both concentrations of organic nitrogen, and the expression kinetics was dependent on the concentration of yeast extract. For the isoenzyme POXA1b, the higher concentration of organic nitrogen had an apparent negative effect. It is important to remark that this isoenzyme is very sensitive to protease degradation (Palmieri et al., 2000), so this effect could be possibly explained by the relation between the presence of proteases and yeast extract concentration. Ferulic acid did not significantly induce the expression of POXA1b from the 10th day on, in comparison with copper sulfate alone, when the concentration of yeast extract was 2 g/L, however for the concentration of 5 g/L this effect was observed from the 12th day on. The new band 1 was more clearly visualized when the higher concentration of yeast extract was used (5 g/L), and increased with fermentation time in the presence of ferulic acid. However, in the absence of ferulic acid, the most intense band was obtained at the 10th day of fermentation. The isoforms 2 and 3 were also more intense in the presence of higher concentration of yeast extract, and ferulic acid showed an observable positive effect on their expression only at this condition. Also the reference strain *P. ostreatus* ATCC produced three new bands on solid state fermentation of sugarcane bagasse, indicating that this pattern is more related to the fermentation conditions than to the strain in this case.

3.2. Comparison of P. ostreatus 22 Em with the standard strain P. ostreatus ATCC in liquid culture

Cultivation in liquid medium was performed in order to verify if the strain *P. ostreatus* 22 Em would reveal a pattern of laccase isoforms different of that produced in solid state fermentation. Fig. 3 represents the zymogram of laccases produced in liquid culture by the strains *P. ostreatus* 22 Em and *P. ostreatus* ATCC. The new bands revealed during solid state fermentation were not observed, corroborating the conclusion of the previous experiments that the expression of the laccase isoforms 1, 2 and 3 depends on the form of conducting the fermentation – on solid or liquid state, and also on the culture medium composition. Also the isoenzyme POXA1b was not detected in the medium fermented by *P. ostreatus* 22 Em. It is worth noting that *P. ostreatus* 22 Em produced an isoenzyme (isoform 4) very close to the band corresponding to

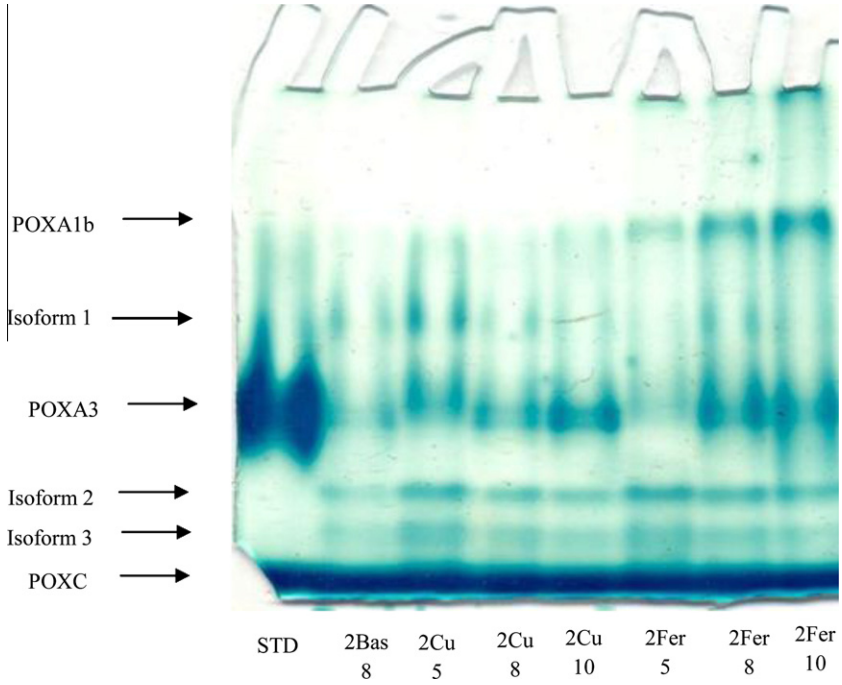

Fig. 1. Zymogram of laccases produced under different conditions of solid state fermentation by the strain *P. ostreatus* 22 Em, with 2 g/L yeast extract. STD – standards (POXA1b, POXA3 and POXC secreted by Pl ATCC); Bas – Basal medium (without inducers); Cu – with Cu^{2+} 150 μM; Fer – with Cu^{2+} 150 μM and ferulic acid 2 mM; numbers below represent the time of fermentation (5, 8 and 10 days). All enzymes were loaded with an activity of 0.0075 U.

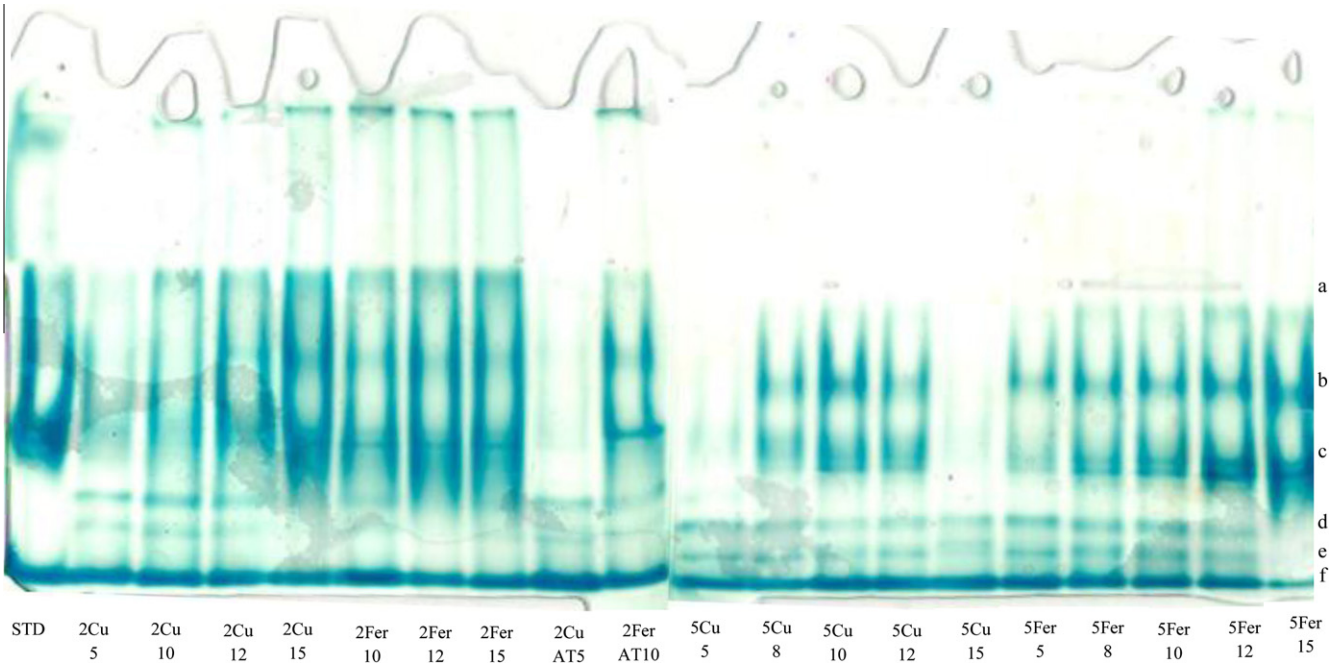

Fig. 2. Zymogram of laccases produced under different conditions of solid state fermentation by the strain *P. ostreatus* 22 Em. STD – standards ([a]POXA1b, [c]POXA3 and [f]POXC); [b]Isoform 1; [d]Isoform 2; [e]Isoform 3; 2Cu – with yeast extract 2 g/L and Cu^{2+} 150 μM; 2Fer – with yeast extract 2 g/L, Cu^{2+} 150 μM and ferulic acid 2 mM; 5Cu – with yeast extract 5 g/L and Cu^{2+} 150 μM; 5Fer – with yeast extract 5 g/L, Cu^{2+} 150 μM and ferulic acid 2 mM; AT represents that the strain *P. ostreatus* ATCC was used for comparison; numbers below represent the time of fermentation (5, 8, 10, 12 and 15 days). All enzymes were loaded with an activity of 0.015 U.

the isoenzyme POXC, with similar electrophoretic mobility to one reported by Lettera et al. (2010) in different growth conditions.

3.3. Protein identification

A sample containing the six isoforms of laccases (condition: 5 g/L yeast extract, with $CuSO_4$ 150 μM and ferulic acid 2 mM, after 10 days) was selected to get an enzyme preparation to perform identification by mass spectrometry. Proteomic analysis confidently assessed the presence of the isoenzymes POXA3 and POXC. Interestingly a glyoxal oxidase and three galactose oxidases, which are enzymes linked to lignin degradation, were also identified (Table 2). No POXA1b isoenzyme could be identified; its absence can be possibly explained by its sensitivity to extracellular protease degradation, as already reported for *P. ostreatus* (Palmieri et al., 2000).

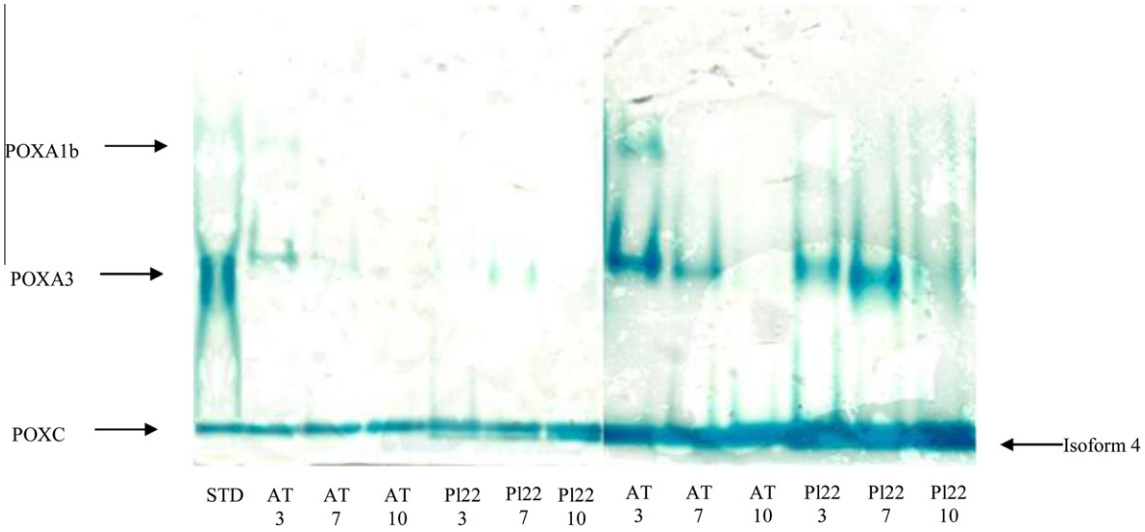

Fig. 3. Zymogram of laccases (same gel at two times of staining) produced in liquid culture (PDY medium with Cu^{2+} 150 μM) by the strains *P. ostreatus* 22 Em and *P. ostreatus* ATCC. STD – standards (POXA1b, POXA3 and POXC); AT – strain *P. ostreatus* ATCC; Pl22 – strain *P. ostreatus* 22 Em; numbers below represent the time of fermentation (3, 7 and 10 days). All enzymes were loaded with an activity of 0.015 U.

Table 2
Protein identification by searching PleosPC15 genome database with MS/MS ion search Mascot software (Matrix Science), oxidation on Met, cyclization of Gln at N-terminus of the peptides to Pyro-Glu, as variable modifications.

Protein name (Accession Number)	Number of peptides	Sequence coverage (%)
Glyoxal oxidase (52532)	11	26
Laccase POXA3 (32778)	8	15
Galactose oxidase (24058)	5	9
Galactose oxidase (28647)	6	14
Galactose oxidase (174951)	6	7
Laccase POXC (36257)	2	2

Note: Only proteins identified with at least two peptides were considered as significant. Peptides with individual ion scores >20 were considered.

4. Conclusions

The highest laccase activity (167 U/g) was achieved at the 5th day of solid state fermentation with yeast extract 5 g/L, $CuSO_4$ 150 μM and ferulic acid 2 mM. Six laccase isoforms (POXA1b, POXA3, POXC and three other isoforms) were observed in the native PAGE zymograms. Ferulic acid induced the expression of POXA3 and POXA1b, and the expression of POXC was not affected by nitrogen concentration and inducers. Higher nitrogen concentration together with ferulic acid induced the expression of isoforms 1, 2 and 3. Mass spectrometry confirmed the presence of POXC and POXA3, and also identified a glyoxal oxidase and three galactose oxidases.

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