Synergic effect of inductors on laccase production by Pycnoporus sanguineus

María Eugenia Eugenio[1*], José María Carbajo[1], Juan Antonio Martín[1], Raquel Martín-Sampedro[1], Aldo Enrique González[2], and Juan Carlos Villar[1]

[1] CIFOR-INIA, Ctra. de La Coruña km 7.5, 28040 Madrid, Spain

CMB-CSIC C/ Nicolás Cabrera 1. 28049. Madrid, Spain

Efecto sinérgico de inductores en la producción de lacasa por Pycnoporus sanguineus

Efecte sinèrgic d'inductors en la producció de lacasa per Pycnoporus sanguineus

Recibido: 27 de enero de 2010; revisado: 2 de marzo de 2010; aceptado: 28 de abril de 2010

RESUMEN

Como la producción de lacasa fúngica depende de algunos componentes que la inducen, hemos estudiado el efecto del etanol, la paja de trigo y el sulfato de cobre de forma independiente y combinada sobre la actividad volumétrica de la lacasa de *Pycnoporus sanguineus*.
La paja de trigo y el sulfato de cobre han dado los mejores resultados, mostrando un efecto sinérgico en el décimo tercer día de incubación (la actividad lacasa fue 3 y 4,5 veces mayor que la actividad obtenida con cada uno de ellos por separado). Por el contrario, el etanol afectó negativamente a la producción de lacasa.
La lacasa producida por *Pycnoporus sanguineus* mantiene su estabilidad en un amplio intervalo de temperatura; estabilidad que depende también del pH. Eligiendo un pH adecuado, el nivel y la estabilidad de la actividad lacasa se mantienen satisfactoriamente a temperaturas entre 25 y 70ºC, lo que diversificaría el uso industrial de esta lacasa.

Palabras claves: inductores, lacasas, *Pycnoporus sanguineus*, estabilidad, efecto sinérgico

SUMMARY

As the fungal laccase production depends on some components which induce it, we have examined the independent effects of ethanol, wheat straw and copper sulphate and their combinations on the volumetric laccase activity of *Pycnoporus sanguineus*.
Wheat straw and copper sulphate gave the best results showing a synergic effect after 13 days of incubation (laccase activity was 3 and 4.5 times greater than the activity generated when they were used alone). Ethanol on the contrary, was found to have a negative effect on laccase production.
The laccase produced by the fungus *Pycnoporus sanguineus* maintains a good stability in a wide range of temperatures; a stability which also depends on the pH. By choosing the adequate pH, the level and stability of the laccase activity are satisfactory in a range of temperatures from 25 to 70ºC, which would allow the diversification of laccase use in industrial applications.

Keywords: inductors, laccases, *Pycnoporus sanguineus*, stability, synergic effect

RESUM

Donat que la producció de lacasa fúngica depèn d'alguns components que la indueixen, s'estudia l'efecte de l'etanol, la palla de blat i el sulfat de coure de forma independent i combinada sobre l'activitat volumètrica de la lacasa de *Pycnoporus sanguineus*.
La palla de blat i el sulfat de coure donen els millors resultats, mostrant un efecte sinèrgic en el tretzè dia d'incubació (l'activitat lacasa és 3 i 4,5 cops superior que l'activitat obtinguda amb cadascun d'ells per separat). Per contra, l'etanol afecta negativament a la producció de lacasa.
La lacasa produïda per *Pycnoporus sanguineus* manté la seva estabilitat en un ampli interval de temperatura; estabilitat que depèn també del pH. Elegint un pH adequat, el nivell i l'estabilitat de l'activitat lacasa es mantenen satisfactòriament a temperatures entre 25 i 70ºC, el que diversificaria l'ús industrial d'aquesta lacasa.

Mots clau: inductors, lacases, *Pycnoporus sanguineus*, estabilitat, efecte sinèrgic

*Corresponding author: PhD. María Eugenia Eugenio,
Tel 34-913476834 Fax 34-913476767
email: mariaeugenia@inia.es

1. INTRODUCTION

White-rot fungi are known for their ability to degrade lignin as well as a wide range of organic pollutants including chlorophenols, polycyclic aromatic hydrocarbons and industrial dyes [1]. In lignin degradation laccases are, in most cases, an important part within the non-specific ligninolytic system by which these microorganisms are able to degrade this polymer completely [2-4]. Laccases are copper-containing glycoproteins involved in a number of natural pathogenic, pigmentation and lignin degradation processes[5]. Moreover, they are able to catalyse the oxidation of various aromatic compounds such as mono-, di-, and polyphenols, aminophenols and diamines by reducing molecular oxygen to water [6]. Furthermore, the fact that laccases require only oxygen to act as catalysts and can readily oxidize phenolic compounds, makes them attractive for biotechnological applications [7-10], including pulp bleaching [11-13].

It is known that the production of laccase is affected by many fermentation factors such as medium composition, carbon and nitrogen sources and their concentrations [14], pH and temperature [15]. Furthermore, many compounds have been widely used to stimulate laccase production. Scientists have used different laccase inductors with different fungi. For instance, Koroljova-Skorobogat'ko et al. [16] found that syringaldazine was an efficient inductor of laccase in *Coriolus hirsutus*. Eggert et al. [17] found that 2,5-xylidine was one of the most effective inducers for the production of laccase from *Pycnoporus cinnabarinus* without modifying the isoenzyme pattern of the enzyme. For the same *Pycnoporus* specie, Herpoël et al. [18] showed that ferulic acid was also an effective inductor. Later, Lomascolo et al. [19] using the same fungus, found that ethanol increased laccase activity up to nine-fold, compared to acid ferulic-induced cultures. For *Trametes sp* AH28-2, Xiao et al. [20] tested several laccase inductors with the following sequence of effectiveness: 2,5 xylidine>guaiacol>ferulic acid. On the contrary, for Trametes pubescens, Galhaup et al. [21] found a different order: guaiacol>ferulic acid>2,5-xylidine. From the latest results, it can be concluded that the intensity of the inductor effect on laccase activity strongly depends on the fungi that are being studied.

Besides, one strain of the basidiomycete *Pycnoporus sanguineus* has been found that produces extracellular laccases among its major ligninolytic enzymes [22]. An important characteristic of this strain is that it produces laccases could capable of enduring temperatures of 60–75 ºC for more than 1 hour. This can be very useful for some industrial purposes [23-24] such as pulp bleaching (which is usually done at temperatures of around 80 ºC), where the need for an excessive number of intermediate cooling and heating steps is a serious disadvantage.

For the *Pycnoporus sanguineus* species, there are only a few works available which study the effect of laccase inductors [22-25], and none of those consider the simultaneous use of various substances to find a hypothetical synergic effect.

Based on the foregoing and the promising prospect of using a laccase which endures high temperatures for industrial applications, this work aims to study the effect of various inductors (individually and in combination) on the increment of laccase activity in submerged cultures of *P. sanguineus*.

2. MATERIALS AND METHODS

2.1. Chemicals.

2,2´-Azino-bis-3-ethylbenzthiazoline-6-sulphonate (ABTS) was purchased from Roche (Madrid, Spain) and Tween 80 from Panreac Química (Barcelona, Spain). All other chemicals were reagent-grade and obtained from Merck (Barcelona, Spain) or Sigma–Aldrich (Madrid, Spain).

2.2. Organism, maintenance and growth conditions.

The basidiomycete *Pycnoporus sanguineus* (B-84) was obtained from the IJFM collection (Instituto Jaime Ferrán de Microbiología-CIB). The fungus culture was grown on malt extract agar slants (2 % malt extract, 2 % Bacto Agar) at 28 °C for 10 days and stored at 4 °C.

A plug (1 cm^2) of fungus was transferred from the slants to the middle of a Petri dish containing malt extract agar medium and grown at 28 ºC for 7 days.

Fungal spores for inoculation of liquid cultures were obtained by washing Petri dishes covered with the fungus with 10 ml of a solution containing 8 g sodium chloride and 1 ml of Tween 80 per litre, and filtering the supernatant through gauze in order to remove the fungus mycelium. The spore concentration in the liquid was determined by counting them in a Neubaüer chamber.

2.3. *Pycnoporus sanguineus* cultures with laccase inductors.

A modified Kirk's medium [14] was used in all experiments. 500 ml Erlenmeyer culture flasks were filled with 200 ml volume of this modified medium and then sterilized. In half of the flasks, an amount of 5 g of wheat straw (a potential laccase inductor) was added under sterile conditions [26]. Then, all flasks were inoculated with 7.6 10^7 spores of *P. sanguineus* [17] and incubated in an orbital shaker at 120 rpm. Based on previous results (not shown), the 6th day of inoculation was chosen to add ethanol and/or copper sulphate, the other potential laccase inductors. Details of the inductor addition are described in table 1. All tests were done in duplicate. The volumetric laccase activity and total protein content were determined in the extracellular fluid on a daily basis over the 17 days of the study.

Table 1. Inductors added in each experiment.

Experiments	Copper Sulphate (%p/v)[a]	Ethanol (%v/v)[b]
Flask 1 (without WS[c])	-	-
Flask 2 (without WS)	0.005	-
Flask 3 (without WS)	-	4.3
Flask 4 (without WS)	0.005	4.3
Flask 5 (with WS)	-	-
Flask 6 (with WS)	0.005	-
Flask 7 (with WS)	-	4.3
Flask 8 (with WS)	0.005	4.3

Inductors concentration as it is described by (a) Collins and Dobson [29] and (b) Lomascolo et al., [19]
(c) WS: wheat straw.

2.4. Volumetric laccase activity.
Laccase activity was determined daily in all samples of extracellular fluid from the fungal cultures, using ABTS as substrate at pH 5 and 25ºC [27]. The reaction mixture consisted of 1:1 (v/v) culture fluid and substrate. One unit of laccase activity was defined as the amount of enzyme needed to obtain 1 µmol of product per minute under the assay conditions. The results were given in relative form.

2.5. Protein assays.
The total protein content was determined on a daily basis by using Kit no. 500-0006 from BioRad, which is based on Bradford's method. Bovine serum albumin was used as the standard. The results were used to calculate the specific laccase activity, which is given in relative form.

2.6. Study of laccase stability
The enzymatic extracellular fluid that showed the maximum laccase activity was isolated from the fungus mycelium by filtration. In the resulting liquid, the laccase activity was measured at different pH values within a range of pH 2.5 to pH 5.5, using citric acid-disodium hydrogen phosphate buffer (200 mM). After establishing the optimum pH value, the laccase activity was determined at different temperatures that ranged between 25 and 80ºC. Then the enzyme stability was studied, measuring the laccase activity every hour during three consecutive hours, and at different combinations of pH (3 and 5) and temperature (25, 40, 50, 60 and 70ºC).

2.7. Zymograms.
A 25 µl aliquot from the extracellular enzymatic liquid isolated before was placed on activity gels to obtain the corresponding zymograms. Polyacrylamide gel electrophoresis was performed at an alkaline pH under non-denaturing conditions in a Mini-Protean electrophoresis cell (BIO-RAD Laboratories, Hercules, CA). The separating gel contained 12 % acrylamide and the buffer solution 375 mM Tris–HCl (pH 8.8). The stacking gel contained 5 % acrylamide and the buffer solution 125 mM Tris–HCl (pH 6.8). The electrode buffer solution contained 25 mM Tris–HCl and 122 mM glycine (pH 8.8). Pre-stained molecular weight standards (Precision Plus Protein Standards, All Blue, from BIO-RAD) were used. The gel had previously been equilibrated in 100 mM acetate buffer at pH 5.0 at room temperature for 15 minutes, washed with the same buffer and stained with a solution of 2,6-dimethoxyphenol (10 mM) in acetate buffer (100 mM, pH 5.0) as substrate [28] to determine laccase activity.

3. RESULTS AND DISCUSSION

3.1. Effect of the different inductors on *Pycnoporus sanguineus* laccase activity
We examined the influence of various inductors (copper sulphate, wheat straw and ethanol) on laccase production by *P. sanguineus*. Figure 1 shows the volumetric laccase activity results, as determined in extracellular fluid from the fungus on a daily basis. It can be observed that the laccase activity found for all experiments with wheat straw (figure 1B) was always considerably higher, after the 4th day of incubation, than that obtained in a medium without straw (figure 1A). On the other hand, in most experiments without straw, maximum laccase activity was reached on the 8th day of incubation, while in the experiments with straw, laccase activity almost always peaked later, around the 13th day of incubation. Therefore, it seems clear that wheat straw has a very strong influence on laccase activity, although it delays its peak.
Regarding the other inductors, only the experiment with copper sulphate showed a higher laccase activity compared to the control, with or without the addition of wheat straw. On the contrary, the experiment containing ethanol, or ethanol plus copper sulphate, didn't increase laccase activity. In fact, these tests even showed a slight decrease in comparison to the control.
From the mycelium dried weight results at the end of the experiments (table 2), it can be explained that ethanol could be a toxic compound for *P. sanguineus* at the tested concentration, since the fungus' growth is inhibited when ethanol, or ethanol plus copper sulphate, are used in the medium. Lomascolo et al. [19] also found an initial inhibition of fungal growth by ethanol using . However, in that experiment, a 155- fold increase in lacasse activity, comparing ethanol-induced cultures with non-induced cultures, was found after some days had passed. The latter behavior was not found in our case. It can be concluded that ethanol could be a fungal inhibitor during the first days of its addition and that its inhibition reversibility will depend on the fungi used.
In the conditions assayed, only copper sulphate and wheat straw act as laccase inductors with *P. sanguineus*. In comparison with the maximum laccase activity obtained without inductor (480 U/l), the addition of copper sulphate and wheat straw to the medium yields a 1.5- fold and 2.5-fold increase in laccase activity, respectively. The induction capacity of copper sulphate had previously been proved by Collins and Dobson [29] using *Trametes versicolor*. The fact that laccase induction by copper occurs at transcriptional level has been demonstrated for *Trametes versicolor* [29] and Pleorotus ostreatus [30] among others. For P. ostreatus a differential regulation has been found [30] and the role of metal responsive elements has been studied [31]. Pérez-Leblic et al. [26] demonstrated that wheat straw is also an efficient laccase inductor using *Pleurotus eryngii*.
When the simultaneous effect of both laccase inductors is studied, it must be pointed out that the maximum laccase activity reached (3250 U/l on day 13 in Fig 1B) doesn't correspond to the sum of the maximum laccase activities where wheat straw and copper sulphate act independently (1090 U/l on day 13 in Fig 1B and 720 U/l on day 8 in Fig 1A respectively). This sum amounts to approximately 55% of the laccase activity which could be reached with the simultaneous use of both inductors. Moreover, if the comparison is made with the results of the 13th day (comparing activities of different days is inappropriate), this percentage is even lower (38%). These facts highlight the important synergic effect of said inductors.
In order to know the laccase enrichment in the enzymatic fluids produced by *P. sanguineus*, the total protein content was analyzed and the specific laccase activity vs. time was plotted (Figure 2). In experiments with wheat straw, the specific laccase activity wasn't assayed. Wheat straw, with a significant content of polyphenols, interferes with the Bradford's method, based on the reaction of phenolic groups of the proteins. As can be seen, *P. sanguineus* produced the extracellular liquid most enriched in laccase on the 8th day of incubation, when copper sulphate was added as inductor. These results concur with those of volumetric laccase activity, where the same experiment

reached the highest laccase activity on the same day of incubation.

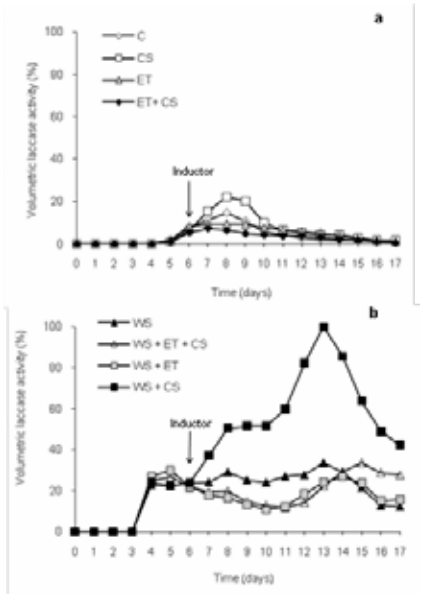

Fig. 1. *Variation of the volumetric laccase activity of Pycnoporus sanguineus at different times using: (a) ethanol (ET) and copper sulphate (CS) as inductors, and (b) ethanol (ET), copper sulphate (CS) and wheat straw (WS) as inductors. C is the control*

Table 2. *Results of mycelium dry weight at the end of the experiments.*

Assay[a]	Mycelium dry weight (g)
without inductors	0.44
with copper sulphate	0.43
with ethanol	0.16
with copper sulphate and ethanol	0.10

(a). Without wheat straw.

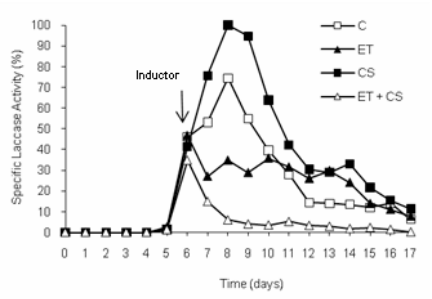

Fig. 2 *Variation of the specific laccase activity of Pycnoporus sanguineus at different times, using as inductors ethanol (ET) and copper sulphate (CS). C is the control*

3.2. Stability study of the extracellular enzymatic fluid produced from *P. sanguineus*

The extracellular enzymatic fluid which showed the highest laccase activity, resulting from the experiment with wheat straw and copper sulphate, was isolated from the fungus mycelium and its volumetric laccase activity variation was studied at different pH and temperature values (figure 3). It can be observed from figure 3A) that the enzymatic fluid reached the highest volumetric laccase activity at pH 3. Similar results were found by Rancano et al. [32] for laccase produced by *Trametes versicolor*. At higher pH, the value gradually decreased until, at pH 5.5, only 20% of the maximum laccase activity remained. On the other hand, figure 3B) demonstrates that, in the range of 25-70ºC, the higher the temperature, the greater the volumetric laccase activity.

Stability studies of the enzymatic fluid at pH 3 and 5 and at temperatures in the range of 25-70ºC are plotted in figure 4. The value pH 3 was chosen because it had proved to be the optimum pH in the aforementioned study; although the selection of pH 5 could be more interesting for some industrial applications. It can be observed that at moderate temperatures (25 to 40ºC) the volumetric laccase activity remained relatively constant, for at least three hours, and even in the most unfavourable conditions within the temperature range (40ºC, pH 3) the remaining laccase activity was close to 80%. Although the differences in laccase stability at this range of temperatures are not very important, the lowest temperature (25ºC) favours laccase stability. The highest initial volumetric laccase activity was shown at pH 3, which subsequently declined slightly more quickly than at pH 5.

A well-known characteristic of this strain *P. sanguineus* is its endurance at moderate to high temperatures. This property implies that the enzymes which it secretes could also endure these conditions. The evolution of volumetric laccase activity at temperatures in the range of 50-70ºC is quite different, depending on the pH. In those testing conditions with the highest acid content (pH 3), volumetric laccase activity rapidly decreases and, at 50ºC, is reduced to half its initial value in about one hour. If the temperature is raised to 60 or 70ºC, volumetric laccase activity after one hour has either become residual or disappeared completely. A similar behaviour was observed by Silva [33] for lacasse from *Trametes sp*. I-62. However, once pH is increased from 3 (the value that gave the highest initial laccase activity) to 5, the volumetric laccase activity shows an entirely different behaviour. At 50 or 60ºC, it virtually maintains its initial value for the first hour and, three hours later, 83% of volumetric laccase activity is still conserved. Even at 70ºC, 71% of the activity is maintained at the end of the first hour, which implies a high enzymatic stability for those applications that do not require longer treatments.

These differences in the stability of laccase activity due to pH and temperature, could mean that those conditions which produce the highest initial volumetric laccase activity (pH 3 and 70ºC), are not as convenient if the enzymatic reaction must go on for a certain length of time. To further clarify which are the optimal performance conditions the average volumetric laccase activity has been calculated, for each combination of pH and temperature, for one and three-hour periods respectively (table 3). In a one-hour period, the average volumetric laccase activity reaches a maximum (3506 UA/ml) at pH 3 and 40ºC, although satisfactory values are also maintained at higher temperatures (around 3000 UA/ml). It might be that laccase treatment requires certain conditions of pH or temperature. This is the case of enzymatic pulp biobleaching, which is preferably carried out at temperatures around 80ºC, to avoid excessive intermediate steps of heating and cooling. Because of this, and if the laccase treatment is maintained for one hour, pH 5 guarantees higher laccase activity at temperatures above 50ºC. Below this temperature, pH 3 is more effective.

A similar situation is found if the laccase treatment is to be maintained for three hours. Again pH 3 and 40ºC give

the best volumetric laccase activity (3223 UA/ml), but after three hours, the average laccase activity in the rest of the conditions is considerably lower (from 2243 to 2693 UA/ml). Again, at low temperatures (40ºC or less), pH 3 produces higher volumetric laccase activity than pH 5, while the opposite occurs at temperatures above 40ºC.

Enzyme purification would prove valuable for further characterization and to concentrate the laccase activity. However, it was not considered in this part of our work aimed at improving pulp biobleaching, because an intermediate purification step may remove compounds which act as laccase mediators that catalyze the delignification with high efficiency.

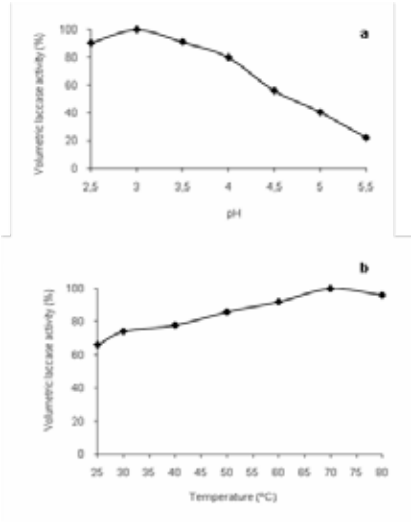

Fig. 3 *Variation of the volumetric laccase activity in the extracellular enzymatic fluid, induced with wheat straw and copper sulphate at different values of pH (a) and temperature (b)*

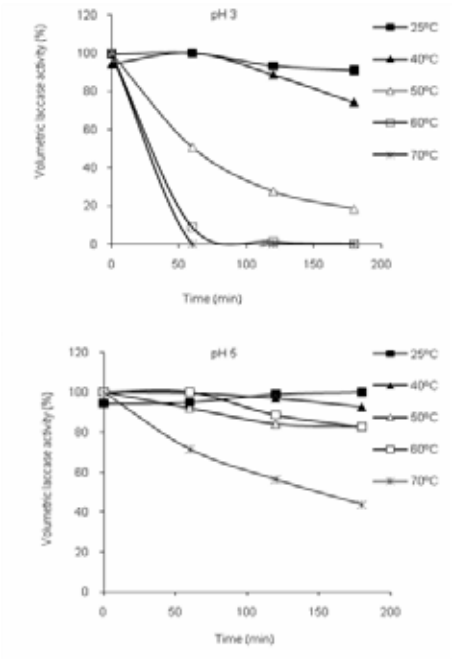

Fig. 4 *Study of the effects of temperature on laccase activity at pH 3 and 5*

Table 3. *Laccase activity at different pHs and temperatures after one and three hours.*

Temperature (ºC)	Average volumetric laccase activity (UA/ml) after 1 hour		Average volumetric laccase activity (UA/ml) after 3 hour	
	pH 3	pH 5	pH 3	pH 5
25	2.636	1.200	2.536	1.231
40	3.506	2.108	3.223	2.055
50	3.240	2.400	2.115	2.243
60	2.500	2.900	1.267	2.693
70	2.700	3.000	1.350	2.375

3.3. Laccase activity zymograms. Effect of different inductors.

Figure 5A) shows the native gel electrophoresis results obtained on the maximum laccase activity day for all experiments, except for the experiment which used wheat straw and copper sulphate together as inductors. The native gel electrophoresis results of this last experiment are shown in figure 5B) on different days of incubation.

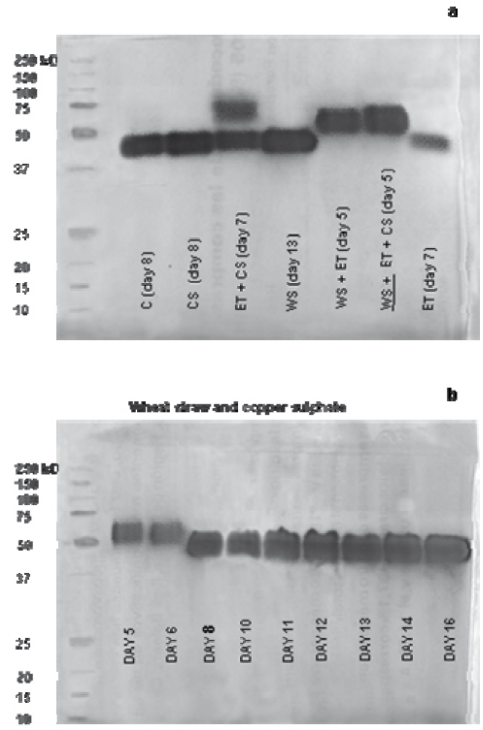

Fig. 5 *Zymograms of laccase activity corresponding to: (a) the day (in brackets) of maximum volumetric laccase activity found in each experiment, and (b) several days in the maximum laccase induction (wheat straw and copper sulphate). C: control; CS: copper sulphate; WS: wheat straw and ET: Ethanol*

As can be seen in figure 5A), all experiments which reached their maximum volumetric laccase activity on the 7th day of incubation or later, showed a laccase size of around 40 kDa. However, the laccase size was higher (60 kDa) in those experiments which peaked before the 7th day of incubation. In the culture with copper sulphate and ethanol, which reached its maximum laccase activity on

day 7, both isoforms are detected, supporting the fact that a larger size laccase is produced during the first days of incubation and a smaller size one during the last days. This result was also observed in a previous work with the same strain [14]. It can also be seen that the weakest sign in the native gel electrophoresis results was yielded by the experiment which only used ethanol as inductor, and the strongest one by all experiments which contained wheat straw in their culture medium.

In figure 5B) it can be seen that all signs show more or less the same intensity, but all are stronger that those found in figure 5A). It is also observed in figure 5B), as in figure 5A), that the signs found after the 7th day of incubation are the strongest.

All these results are consistent with the above described spectrophotometric measurements and support our decision to choose wheat straw and copper sulphate as the best laccase inductors.

4. CONCLUSIONS

It was found that wheat straw and copper sulphate, when used together, gave the best results and led to maximal enzyme activity after 13 days of incubation. These inductors showed a synergic effect, since the laccase activity reached was higher than the sum of their activities when they were used alone in the culture medium.

The laccase produced by the fungus *Pycnoporus sanguineus* maintains stability at a wide range of temperatures (from 25 ºC to 70ºC at an adequate pH), which would allow the diversification of laccase use in industrial applications.

5. ACKNOWLEDGEMENTS

The authors wish to thank the Spanish Ministry of Science and Innovation for funding this work via Project CTQ2009-11268 and the Ramón y Cajal Programme.

6. BIBLIOGRAPHY

1. Pointing, S.B., 2001. Feasibility of bioremediation using white rot fungi. Appl. Microbiol. Biotechnol. 57, 20-33.
2. Kirk,T.K., Farrell ,R.L., 1987. Enzymatic “combustion”. The microbial degradation of lignin. Ann. Rev. Biochem. 41, 465-505.
3. Leonowicz, A., Matuszewska, A., Luterek ,J., Ziegenhagen, D., Wojtas-Wasilewska, M., Cho, N.S., Hofrichter, M., Rogalski, J., 1999. Biodegradation of lignin by white rot fungi. Fungal Genet. Biol. 27, 175-185.
4. Mayer, A.M., Staples, R.C., 2002. Laccase: new functions for an old enzyme. Phytochemistry 60, 551-565.
5. Thurston, C.F. 1994. The structure and function of fungal laccases. Microbiology 140, 19-26.
6. Reinhammar, B., 1984. Laccase, in: Lontie, R. (Ed.), Copper proteins and copper enzymes Vol.3, CRC Press, Boca Raton, Florida pp. 1-35.
7. Bhattacharya, S.S., Banerjee, R., 2008. Laccase mediated biodegradation of 2,4-dichlorophenol using response surface methodology. Chemosphere 73, 81-85.
8. Maijala, P., Kleen, M., Westin, C., Poppius-Levlin, K., Herranen, K., Lehto, J.H., Reponen, P., Maentausta, O., Mettala, A., Hatakka, A., 2008. Biomechanical pulping of softwood with enzymes and white-rot fungus *Physisporinus rivulosus*. Enzyme Microb. Technol. 43, 169-177.
9. Tauber, M.M., Gübitz, G.M., Rehorek, A., 2008. Degradation of azo dyes by oxidative processes – Laccase and ultrasound treatment. Bioresour. Technol. 99, 4213-4220.
10. Zhang, J., Liu, X., Xu, Z., Chen, H., Yang, Y., 2008. Degradation of chlorophenols catalyzed by laccase. Int. Biodet. Biodegr. 61, 351-356.
11. Da Re, V., Papinutti, L., Villalba, L., Forchiassin, F., Levin, L., 2008. Preliminary studies on the biobleaching of loblolly pine Kraft pulp with *Trametes trogii* crude extracts. Enzyme Microb. Technol. 43, 164-168.
12. Fillat, U., Roncero, M.B., 2009. Biobleaching of high quality pulps with laccase mediator system: Influence of treatment time and oxygen supply. Biochem. Eng. J. 44, 193-198.
13. Moldes, D., Vidal, T., 2008. Laccase–HBT bleaching of eucalyptus kraft pulp: Influence of the operating conditions. Bioresour. Technol. 99, 8565-8570.
14. Eugenio, M.E., Carbajo, J.M., Martín, J.A., González, A.E., Villar, J.C., 2009. Laccase production by *Pycnoporus sanguineus* under different culture conditions. J. Basic Microbiol. 49, 433-440.
15. García, T.A., Santiago, M.F., Ulhoa, C.J., 2007. Studies on the *Pycnoporus sanguineus* CCT-4518 laccase purified by hydrophobic interaction chromatography. Appl. Microbiol. Biotechnol. 75, 311-318.
16. Koroljova-Skorobogat’ko, O.V., Stepanova, E.V., Gavrilova, V.P., Morozova, O.V., Lubimova, N.V., Dzchafarova, A.N., Jaropolov, A.I., Makower, A., 1998. Purification and characterization of the constitutive form of laccase from the basidiomycete *Coriolus hirsutus* and effect of inducers on laccase synthesis. Biotechol. Appl. Biochem. 28, 47-54.
17. Eggert, C., Temp, U., Eriksson, K.E.L., 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase. Appl. Environ. Microbiol. 62, 1151-1158.
18. Herpoël, I., Moukha, S., Lesage-Meessen, L., Sigoillot, J., Asther, M., 2000. Selection of *Pycnoporus cinnabarinus* strains for laccase production. FEMS Microbiol. Lett. 183, 301-306.
19. Lomascolo, A., Record, E., Herpoël-Gimbert, I., Delattre, M., Robert, J.L., Georis, J., Dauvrin, T., Sigoillot, J.C., Asther, M.J., 2003. Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. J. Appl. Microbiol. 94, 618-624.
20. Xiao, Y.Z., Chen, Q., Hang, J., Shi, Y.Y., 2004. Selective induction, purification and characterization of a laccase isozyme from the basidiomycete *Trametes sp*. AH28-2. Mycologia 96, 26-35.
21. Galhaup, C., Wagner, H., Hinterstoisser, B., Haltrich, D., 2002. Increased production of laccase by the wood-degrading basidiomycete Trametes pubescens. Enzyme Microb. Technol. 30, 529-536.
22. Eugenio, M.E., Carbajo, J.M., Terrón, M.C., González, A.E., Villar, J.C., 2008. Bioremediation of lignosulphonates by lignin-degrading basidiomycetous fungi. Bioresour. Technol. 99, 4929-4934.

23. Trovaslet, M., Enaud, E., Guiavarc'h, Y., Corbisier, A.-M., Vanhulle, S., 2007. Potential of a *Pycnoporus sanguineus* laccase in bioremediation of wastewater and kinetic activation in the presence of an anthraquinonic acid dye. Enzyme Microb. Technol. 41, 368-376.
24. Urbina, H., Reyes, A., Fusella, E., Gonzalez, M., Leon, V., Naranjo, L., 2007. *Pycnoporus sanguineus* IDEA, a laccase-overproducing fungi with high potential in partial enzymatic conversion (PEC-Technology) of Venezuelan extra-heavy crude oil. J. Biotechnol. 131, S94-S95.
25. Garcia, T.A., Santiago, M.F., Ulhoa, C.J., 2006. Properties of laccases produced by *Pycnoporus sanguineus* induced by 2,5-xylidine. Biotechnol.Lett. 28, 633–636.
26. Pérez-Leblic, M.I., Martínez, A.T., Martínez, M.J., 2005. Análisis proteómico preliminar de las proteínas extracelulares producidas por *Pleurotus eryngii* en diferentes condiciones de cultivo, in: *Congreso Nacional* de Microbiología, Cáceres, pp. 490.
27. Mansur, M., Flärdh, M., Arias, M.E., Copa-Patiño , J.L., González, A.E., 2003. The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specifities. Mycologia 95, 1013-1020.
28. Calvo, A.M., Copa-Patiño, J.L., Alonso, O., González, A.E., 1998. Studies of the production and characterisation of laccase activity in the basidiomycete *Coriolopsis gallica*, an efficient decolorizer of alkaline effluents. Arch. Microbiol. 171, 31-36.
29. Collins, P.J., Dobson, A.D.W., 1997. Regulation of laccase gene transcription in *Trametes versicolor*. Appl. Environ. Microbiol. 63, 3444-3450.
30. Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., Sannia, G., 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 66, 920–924.
31. Faraco, V., Giardina, P., Sannia, G., 2003. Metal-responsive elements in *Pleurotus ostreatus* laccase gene promoters. Microbiology 149, 2155–2162.
32. Rancano, G., Lorenzo, M., Molares, N., Couto, S.R., Sanroman, M.A., 2003. Production of laccase by *Trametes versicolor* in an airlift fermentor. Process Biochem. 39, 467-473.
33. Silva, R., 2002. Obtención de enzimas ligninolíticas producidas por hongos basidiomicetos. Evaluación de su aplicación al blanqueo de pastas de madera. Doctoral Thesis, Universidad Politécnica de Madrid, Madrid.