Atypical laccases from the white-rot fungus *Pleurotus ostreatus* and their application for the treatment of industrial coloured effluents

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1. Introduction

1.1. *Laccases*

White-rot fungi are the most efficient decomposers of lignocellulose because of their capability to synthesize the relevant hydrolytic (cellulases and hemicellulases) and oxidative (laccases, lignin-peroxidases and Mn-peroxidases) extracellular enzymes required to degrade the major components of substrates (cellulose, hemicellulose, and lignin) into low-molecular-weight compounds that can be assimilated in fungal nutrition [1]. Recently, extensive research on these fungi has been conducted with the aim of isolating new organisms able to secrete new enzymes with capability to be used in industrial applications, such as bioremediation of polluted soils and industrial waste-waters, biobleaching and biopulping in pulp and paper industries, textile and food industries, *etc*.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
 ISBN 84-9769-107-5

Fungal laccases (benzenediol: oxygen oxidoreductases; EC1.10.3.2) are ligninolytic enzymes that have been isolated from various fungi [2]. They belong to the class of the blue oxidases containing 4 copper atoms/molecule distributed in three different copper binding sites [3, 4]. The type-1 site is responsible for the intense blue colour of the enzyme due to a maximum absorbance at 605 nm; the type-2 site does not exhibit signals in the visible absorbance spectrum; and the type-3 site incorporates two copper centres and is responsible for a band near 330 nm. All these copper ions are involved in the catalytic mechanism. Laccases reduce oxygen to water and simultaneously perform a one electron oxidation of aromatic substrates (polyphenols, methoxysubstituted monophenols, aromatic amines, etc.). These enzymes are present in multiple isoforms, depending on the fungal species and environmental growth conditions [5, 6].

1.2. Pleurotus ostreatus *laccases*

Laccase isoenzymes produced by *Pleurotus ostreatus*, a white rot basidiomycete fungus, have been extensively studied. Five different isoenzymes have been purified and characterized: POXC, POXA1b, POXA1w, POXA3a and POXA3b; seven different genes and the corresponding cDNAs have been cloned and sequenced [7, 8, 9].

POXC is the most abundantly isoenzyme produced under all the growth conditions examined [7] (maximum production level 25 mg/l); it shows all typical laccase characteristics: four copper ions/molecule, acidic pI, and stability to proteolytic degradation. All the others *P. ostreatus* laccases (POXA1w, POXA1b, POXA3a and POXA3b) are atypical phenol-oxidases [10, 8, 11].

POXA1w shows a remarkable high stability with respect to both pH and temperature if compared with that of POXC and of other known laccases, and it exhibits a neutral pI (6.7). The most striking characteristic of this protein is the lacking of the typical blue colour and its unusual metal content. UV/visible spectrum, atomic absorption and polarography proved that this enzyme contains only 1 copper atom/molecule- instead of the usual 4 atoms- and furthermore, 2 zinc atoms and 1 iron atom. Nevertheless, the classification of this enzyme as laccase was based on: i) the high degree of identity of

the determined stretches of primary structure with the corresponding sequences of known laccases; ii) the use of O_2 as oxidative substrate and the lack of formation of H_2O_2 as a product in the catalysed reaction; iii) the almost standard pattern of substrate specificity.

POXC production is strongly increased by the presence of copper ions, whilst POXA1w production is substantially unaffected under this condition; furthermore a new neutral laccase isoenzyme (POXA1b) is produced in copper supplemented cultures. POXA1b shows the same characteristics of POXA1w concerning the stability with respect to pH and temperature. Furthermore POXA1b is only partly secreted [12].

In this study we review purification and characterisation of two closely related isoenzymes, POXA3a and POXa3b, and their role in decolourisation of Remazol Brilliant Blue R (RBBR), a molecule frequently used as starting material in the production of many polymeric commercial dyes [13]. We also describe optimal conditions for using selected purified laccases or immobilized crude laccase mixtures in the treatment of this model dye [14].

2. Materials and Methods

2.1. *Organism and culture conditions*

White-rot fungus, *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) was maintained through periodic transfer at 4 °C on PDY agar plates (2.4% potato dextrose and 0.5 yeast extract, Difco Laboratories, Detroit, MI). Incubations were carried out as previously described [10] and laccase production was induced by addition of 150 μM copper sulphate.

2.2. *Enzyme purification*

Secreted proteins were precipitated from the filtered medium by addition of $(NH_4)_2SO_4$ up to 80% saturation and, after extensive dialysis, loaded onto a DEAE Sepharose Fast Flow (Pharmacia Biotech Inc.) column as previously described [10]. Two fractions containing laccase activity, recovered with the equilibrating buffer, were separately pooled, concentrated on an Amicon

PM-10 membrane and equilibrated in Tris-HCl 50mM pH 8.0. Each pool was loaded onto an anion exchange Mono Q HR5/5 (Pharmacia) column in a fast protein liquid chromatography system (FPLC, Pharmacia) equilibrated with the same buffer. The active fractions were pooled, concentrated and loaded onto a gel filtration Superdex 75 PC 3.2/30 column in a SMART System (Pharmacia); the active fractions were pooled and desalted.

2.3. *Enzyme assays*

Laccase activity was assayed at 25 ºC, using 2,2'-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), DMP and syringaldazine as substrates as previously described [10].

2.4. *Remazol Brilliant Blue R transformation by laccase*

The decolourising experiments were performed using the dye RBBR 50 μM, and the purified laccase POXC or POXA3 (1 U/ml) in sodium acetate 20 mM, pH 4.5 buffer. The same incubation was performed using a laccase mixture of POXC and POXA3 (1:1, U:U ratio) from 1 to 10 U/ml final activity. Control samples without enzyme, were run in parallel under identical conditions. All reactions were incubated at 20 ºC for 100 min.

2.5. *Effect of temperature and pH on dye decolourisation*

The effect of temperature on dye decolourisation was studied incubating the reaction mixture, prepared as above described, at 20, 30 or 40 ºC. The effect of pH on dye decolourisation was studied performing the experiments in the pH range 4-7 using the Mc Ilvaine buffer. In all cases, dye decolourisation was monitored as above described.

2.6. *Immobilization method*

The immobilization procedure was carried out at 4 ˚C. Different amounts of crude laccase preparation (from 0.2 to 660 U) were mixed with 5 ml of 3% sodium alginate solution (low viscosity, Sigma), centrifuged at 4,000 rev

min-1 for 5 min to remove air bubbles, and extruded drop by drop through a needle (0.4 mm internal diameter) into a 0.15 M $CuSO_4$ aqueous solution (pH 4.0) under continuous agitation. The resulting spherical blue beads were left to solidify for at least 30 min in the copper solution and then washed exhaustively with distilled water until pH 5.0-5.5 was reached. The total wet weight of beads obtained from 5 ml of sodium alginate solution was about 3.5 g. The beads were stored wet at 4 °C. The immobilization yield was calculated as the fraction of laccase activity found after dissolution of alginate beads (by incubation for 3 h at 4 °C in 50 mM sodium phosphate buffer pH 7.0, 50 mM EDTA) with respect to the activity of the enzyme added to the soluble alginate preparation.

2.7. *Continuous packed-bed reactor*

A glass column (130 mm x 17 mm, working volume 25 ml) was filled with 24 g of copper alginate beads. The flow rate was 0.2 ml/min, and the feed solution was 50 μM RBBR in 20 mM sodium acetate buffer, pH 4.5. Samples were collected after the passage of at least 25 ml of feed solution to reach the concentration equilibration (time 0). All reactor systems were operated at room temperature (approximately 20 °C).

2.8. *Chitosan treatment of alginate beads*

A 24 g portion of copper alginate beads was transferred into a solution of 0.15% (w/v) chitosan, in 20mM sodium acetate buffer pH 4.5 containing 0.1 M CuSO4, and incubated for 20 h at 4 °C on a rotary shaker. After that, the alginate chitosan beads were extensively washed with 20 mM sodium acetate buffer pH 4.5 and used to fill the glass vessel reactor as described above.

3. Results

3.1. *Purification of laccase isoenzymes POXA3a and POXA3b*

Two active different protein fractions (POXA3a and POXA3b) were recovered after chromatography (fig 1) of the ammonium sulphate precipitate of

culture broth collected after 10 days. Both isoenzymes were purified to homogeneity (gel filtration chromatography, fig 2, native PAGE and isoelectrofocusing). Isoelectric points of POXA3a and POXA3b are 4.3 and 4.1, respectively, and the molecular mass, determined by gel filtration chromatography, is 56 kDa for both proteins. Specific activities towards ABTS of POXA3a and POXA3b are 2000 and 1050 U/mg, respectively. However purified isoenzymes displayed a more complex pattern when analysed by SDS-PAGE, in fact three bands (MW 67, 18 and 16 kDa, respectively) were observed for both proteins (fig 3).

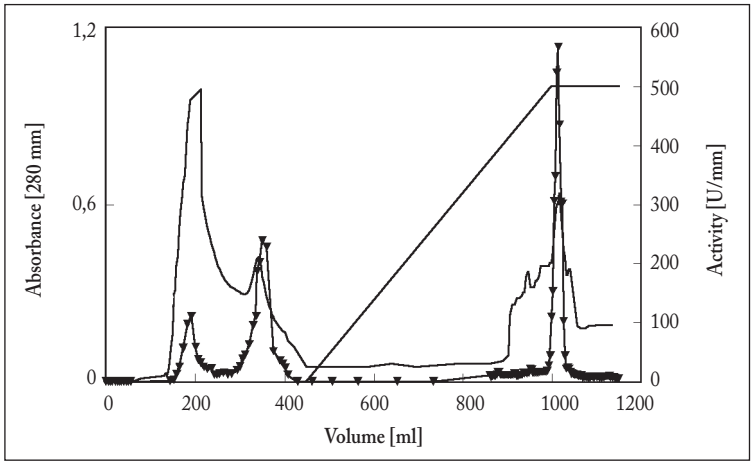

Figure 1. DEAE-Sepharose chromatography of P. ostreatus-secreted proteins. DEAE-Sepharose fast flow elution profile of proteins and laccase isoenzymes secreted by *P. ostreatus* is shown with three different active protein fractions (POXA3a, POXA3b, and POXC).

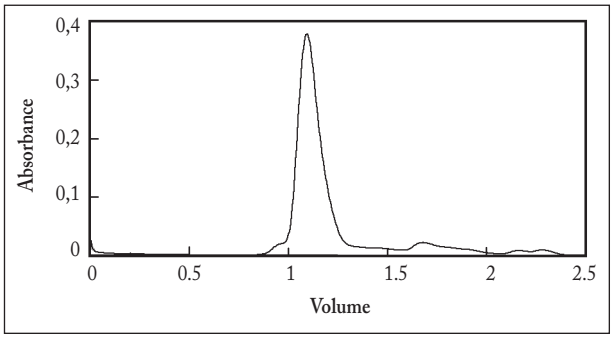

Figure 2. Gel filtration chromatography of POXA3a fraction from MONOQ: a single peak is detectable

3.2. *Metal content analyses*

UV-Vis absorption spectra of POXA3a and POXA3b display the characteristic absorbance peak at 600 nm, due to the presence of type I copper; copper content, determined by atomic absorption, results to be 3.3 copper/protein (mol/mol) for both proteins.

3.3. *Structural analyses*

The two isoenzymes were analysed by MALDI-MS: the mass spectra showed a peak exhibiting a molecular mass centred at about 60,860.4 Da for POXA3a and 60,700.6 for POXA3b; moreover the spectra showed the occurrence of two other components whose molecular masses were measured as 16,870.5 and 18,103.8 Da for POXA3a and 16,838.1 and 18,090.1 Da for POXA3b. The N-terminal sequences of the 60 kDa subunits are identical for both POXA3a and POXA3b, while the same analyses, carried out on the four 16 and 18 kDa subunits, revealed that all subunits have a blocked N-terminus.

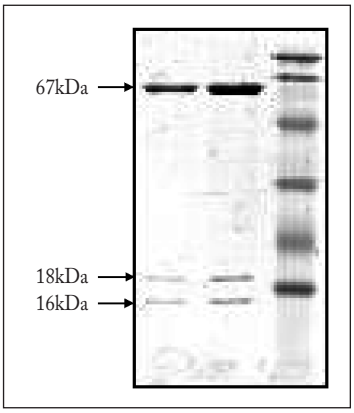

Figure 3. Coomassie stained SDS–PAGE of purified POXA3a and POXA3b laccase isoenzymes.

Mass spectrometric analyses (peptide fingerprints) and N-terminus sequencing did not reveal any significant differences between POXA3a and POXA3b large subunits. Furthermore these analyses show that differences between the 18 and 16 kDa subunits of both isoenzymes are only due to the presence of a glycosidic moiety on the 18 kDa subunits. None of the sequenced peptides from small subunits shows significant homology with proteins in data banks.

3.4. *Cloning and sequencing of poxa3 gene and cDNA*

Oligonucleotide-primer mixtures were designed on the basis of POXA3a tryptic peptide sequences. The 500 bp amplified fragment, homologous to

known laccase genes, was used to screen a *P. ostreatus* genomic library. Two oligonucleotides were designed using the predicted N and C termini of the protein and were used to amplify POXA3 encoding cDNA. The 1500 bp amplified fragment was cloned and sequenced allowing the determination of *poxa3* gene whole structure. The coding sequence is interrupted by 21 introns.

3.5. *POXA3a and POXA3b activation*

POXA3 specific activity increases during fungal growth, probably due to proteolytic activation. POXA3 isoenzymes, purified from broth supplemented with Phenylmethanesulfonyl fluoride (PMSF), were incubated with the serine protease from *P. ostreatus* (*Po*Sl) (fig 4). Under this condition, an increase of POXA3 activity was obtained (about 30%), confirming the generation of more active POXA3 isoform(s) due to *Po*Sl-induced proteolysis.

3.6. *Biotransformation of RBBR by* P. ostreatus *laccases*

Decolourisation experiments by *P. ostreatus* in solid and liquid media were performed using PDY broth in the presence of veratryl alcohol, added with RBBR at two different concentrations (5 and 50 μM). *P. ostreatus* is able to decolourise the dye in all cultural conditions examined, with a temporal relationship between laccase production and dye biotranformation (fig. 5).

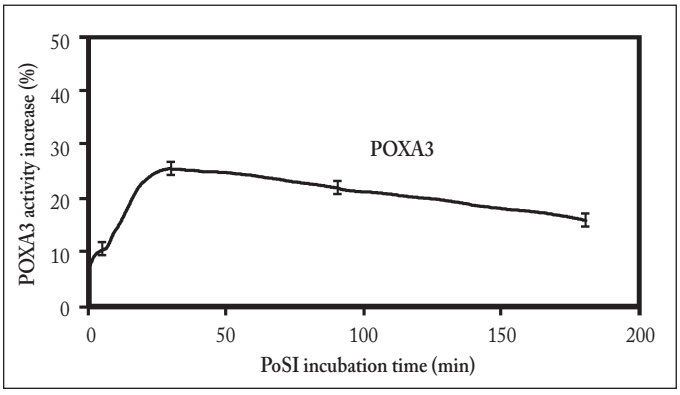

Figure 4. *In vitro* incubation of POXA3 with an extracellular subtilisin-like *Pleurotus ostreatus* protease (*Posl*).

In order to elucidate the role of the oxidative activities produced during biodegradation of RBBR by *P. ostreatus*, purified POXA3 (1 U/ml) or POXC (1 U/ml) isoenzymes were incubated at 20°C with RBBR: after 100 min of incubation, 30 and 50% of decolourisation was obtained by POXC and POXA3, respectively. No more than 60% of decolourisation was obtained even when the enzyme concentration or the incubation temperature was increased. Kinetic parameters of the two laccases were also determined using RBBR as substrate: the *K*m values (0.054 and 0.051 mM for POXA3 and POXC, respectively) are very similar whilst the catalytic efficiency (*k*cat/*K*m) of POXA3 ($9.0x10^6$ mM^{-1} min^{-1}) is six-fold higher than that of POXC ($1.5x10^6$ mM^{-1} min^{-1}). Therefore, the difference in decolourisation efficiency of the two laccases could be due to a variation in enzymatic turnover rather than in substrate affinity.

The effect of a 1 U/ml (final laccase activity) POXC/POXA3 mixture (1:1; U:U) on RBBR was tested. As shown in figure 6, the enzymatic mixture determines an increase both in the rate and in the final level of dye decolourisation (74%) with respect to each isoenzyme working separately. Also enzyme concentration and incubation temperature affected dye decolourisation.

3.7. *RBBR decolourisation by crude laccase complex mixture.*

Crude enzymatic mixture obtained after ammonium sulphate precipitation of culture broth supplemented with $CuSO_4$ and ferulic acid was found to be more efficient in RBBR decolourisation than any single component.

The effect of enzyme concentration on dye degradation was studied: increasing the amount of the crude preparation used, from 2 to 100 U/ml of laccase activity a more efficient dye decolourisation (up to 70%) was observed (fig 7).

3.8. *Immobilization of crude laccase mixture in copper alginate beads and its performance in dye decolourisation*

Optimal conditions for crude laccase mixture immobilization by entrapment in copper alginate beads were set up. Immobilization yield was 65% when laccase concentrations ranging from 15 to 120 U/ml of sodium alginate solution were used, obtaining beads whose laccase activity ranged from 20 to 100 units per gram of beads (U/g). Immobilized protein mixture in copper

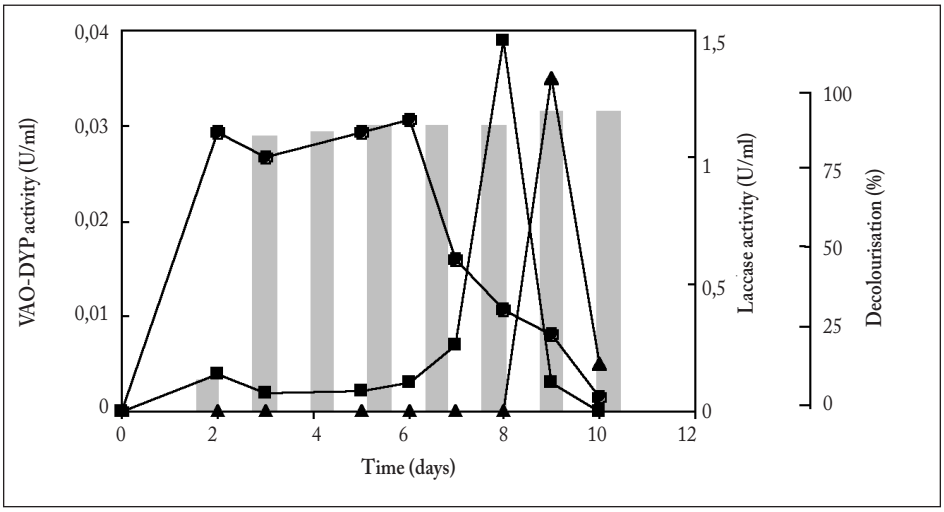

Figure 5. Decolourisation of RBBR in *P. ostreatus* culture supplemented by 50 μM of Remazol Brilliant Blue R. Symbols: laccase activity (•); DYP activity (▲); AAO activity (+); percentage of decolourisation ( ).

alginate beads is more stable if compared with free enzyme mixtures. Optimal conditions for batch decolourisation process were determined (pH 4.5, temperature 20°C, 100 Units per gram of beads).

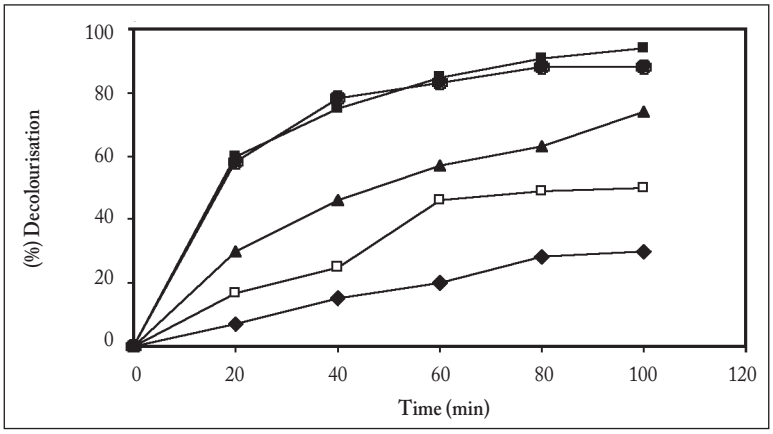

Figure 6. Remazol Brilliant Blue R decolourisation after treatment with laccase isoenzymes in different reaction conditions; POXC (1 U/ml; 20 °C) (❑); POXA3 (1 U/ml; 20 °C) (◆); POXC/POXA3 mixture (1:1, U:U; 1 U/ml; 20 °C) (▲); POXC/POXA3 mixture (1:1; U:U; 10 U/ml; 20 °C) (■); POXC/POXA3 mixture (1:1, U:U; 1 U/ml; 30 °C) (•).

3.9. *Performance of immobilized laccase mixture in a fixed-bed reactor*

The immobilized enzyme mixture was used over 7 days in a fixed-bed reactor, under the optimal conditions set up in batch experiments, operating at a RBBR loading rate of 20.9 mg L^{-1} h^{-1} (fig 8). In these conditions (bioreactor 1) the final decolourisation percentage was 20% after elution of 100 V/V_R (volumes of dye solution respect to the reactor retention volume, V_R=18 ml). The leached laccase activity, determined during this continuous operation, quickly increased at the beginning of the decolourisation process, and the total activity washed out amounted to 1000 U. Attempts were performed to decrease enzyme release.

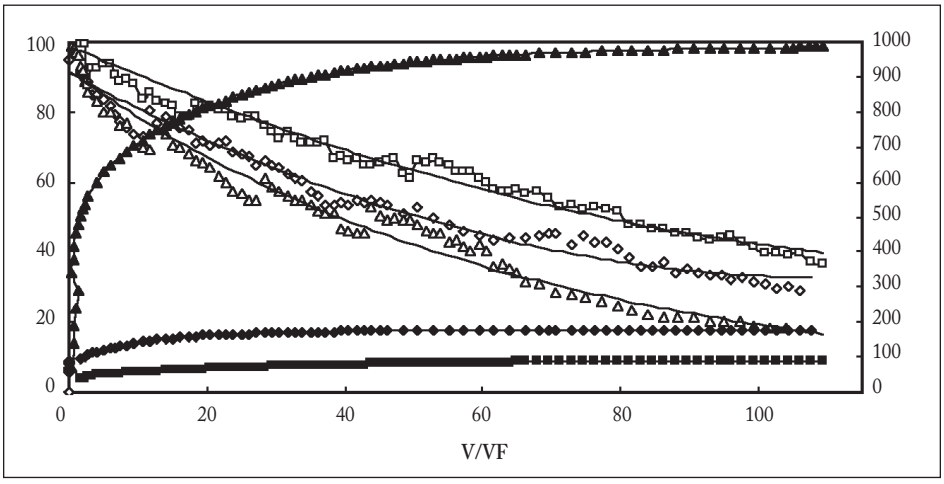

Figure 7. RBBR decolorization in continuous experiments by packed bed bioreactors. Bioreactor 1 (∅): 100 U laccase activity/g copper alginate beads. Bioreactor 2 (◊): 50 U/g. Bioreactor 3 (❑): 50 U laccase activity/g copper alginate beads treated with chitosan. Laccase leaching was measured (▲, bioreactor 1; ◆ bioreactor 2; ■ bioreactor 3).

An experiment (bioreactor 2) was performed using a lower amount of immobilized enzyme mixture (50 U/g). As shown in figure 8, the final decolourisation percentage obtained by this bioreactor was enhanced (about 30%) and laccase leaching was 5-fold decreased with respect to the previously mentioned

continuous experiment (bioreactor 1). Further improvements in the final decolourisation level (40%) and in laccase retention were obtained using chitosan treated beads (bioreactor 3). As a fact, chitosan is known to improve beads mechanical resistance and significantly to reduce leaching [15, 16].

4. Discussion

POXA3a and POXA3b, two closely related laccase isoenzymes, are produced in *P. ostreatus* copper supplemented cultures. SDS-PAGE and MALDI-MS analyses of purified POXA3a and POXA3b reveal the presence of three different polypeptides of 67, 18 and 16 kDa, whereas the native proteins behave homogeneously (as demonstrated by gel filtration chromatography, isoelectrofocusing and native-PAGE analysis). None of the previously characterised *P. ostreatus* laccase isoenzymes shows similar behaviour, and all of them are monomeric proteins. On the other hand, it has been reported that some laccase enzymes from *Phellinus ribis* [17], *Trametes villosa* [18] and *Rhizoctonia solani* [19] show homodimeric structure.

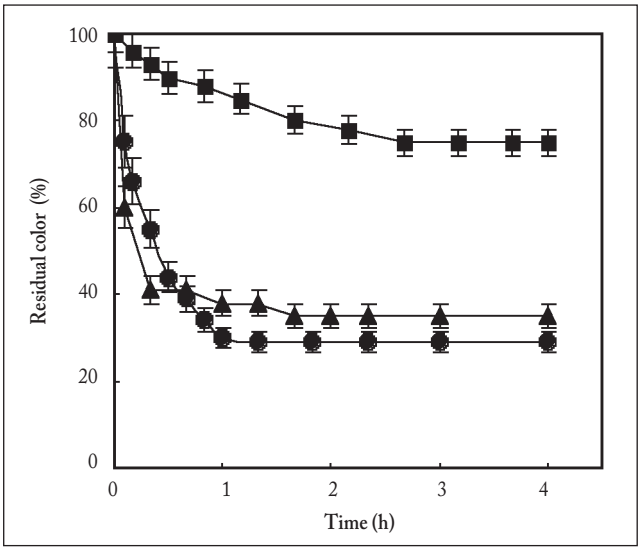

Figure 8. RBBR decolourisation obtained by incubation with different amounts of crude laccase mixture. •º, 2 U/ml laccase activity, 20 ˚C; ■ 20 U/ml laccase activity, 20 ˚C; ▲100 U/ml laccase activity , 20 ˚C.

An unique *poxa3* gene has been identified and protein sequence deduced by cDNA has been verified by means of MALDI-MS mapping against the POXA3a and POXA3b large subunits. It is not possible to univocally associate this gene to either POXA3a and/or POXA3b, or to exclude the existence of another *poxa3* gene. Deduced amino acidic sequence contains all putative copper-binding residues, as well as the five Cys residues found in all the known laccase sequences.

Sequence data from the 18 kDa POXA3a subunit, accounting for about 50% of entire sequence, did not give information on the nature of this subunit because of the absence of significant homology with other known proteins. Furthermore, no sequence encoding these peptides have been recognised in the 3' and 5' flanking region of the *poxa3* gene, thus excluding that small subunit could be originated from maturation of a single polypeptide chain containing the largest one.

Due to their enzymatic properties and relatively low production cost, laccases represent a promising tool for applications in the textile industry effluent bioremediation.

POXC and POXA3 laccases are able to perform RBBR transformation *in vitro*. In particular, POXA3 shows higher decolourisation efficiency with respect to POXC, as also confirmed by kinetics constants determined using RBBR as substrate. It has been also verified that a more efficient process take place in the presence of a mixture of POXC and POXA3 suggesting that RBBR degradation *in vivo* could be due to a concerted action of the two isoenzymes. Hence, we demonstrated that a complete RBBR transformation can be obtained using a simple mixture of two laccase isoenzymes in the absence of any redox mediators and selecting optimal enzyme concentration, temperature and pH values. These findings differ from those reported for other laccases, which also transform RBBR in the absence of redox mediators, but in this case only an incomplete decolourisation is obtained [20, 21, 22]. On the other hand, other authors [23, 24] reported that RBBR is decolourised only when small molecular weight redox mediators are added to the laccase enzymes. These results suggest a strict correlation between decolourisation and detoxification. Reasonable basis for development of a cheaper biotechnological colour reduction process have been provided: a crude laccase mixture preparation was used to decolourise RBBR. Once the potentiality of the enzymatic system had been assessed, the mixture was im-

mobilized by entrapment in copper alginate beads. RBBR decolourisation efficiency was about 70% even after 20 cycles of stepwise dye additions in batch operations. Different strategies for continuous decolourisation in a fixed-bed bioreactor were analysed. The best performances were obtained by decreasing enzyme loading and improving laccase retention by coating the alginate beads with chitosan.

5. Acknowledgement

This work was supported by grants from the Ministero dell'Università e della Ricerca Scientifica (Progetti di Rilevante Interesse Nazionale, PRIN) and from EC FP6 Project SOPHIED (NMP2-CT-2004-505899).

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