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Enzymatic and Expression Profiling of Oxidases Produced by *Pleurotus ostreatus* in Submerged Fermentation in the Presence of Remazol Brilliant Blue R (RBBR) and Yellow Azo (AYG) Dyes

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Abstract: The white-rot fungus *Pleurotus ostreatus* produces laccase (LAC), manganese peroxidase (MnP) and versatile peroxidase (VP) within other oxidases. We studied the effect of the textile dyes Remazol Brilliant Blue R (RBBR) and Yellow Azo (AYG) on enzyme activity and expression profile of the peroxidases produced by *Pleurotus ostreatus* during its growth in submerged fermentation.

In general terms enzymatic activity increased in the presence of both textile dyes that compared to the basal condition being higher for laccase and MnP in RBBR and for VP in AYG fermentation. On the other hand, the gene expression fluctuated in each condition and growth time tested. The laccase isoform *poxa1b* gene seems to be the highest constitutively expressed. The induction effect of dyes on gene expression was clearly observed for the *vp* gene in the presence of RBBR with almost 300 fold level. For the laccase isoforms, the gene showing the highest upregulation was *pox4* with inductions levels ranging from 800 and 25 folds in AYG and RBBR respectively.

On the other hand the induction levels observed for *mnp* gene ranged from 5 to 20 folds in AYG and RBBR respectively.

The enzyme activity and expression profile analysis obtained suggest the coordinate participation of the enzymes under study in the oxidation and probably mineralization of the dyes evaluated. Moreover, the above results show that both dyes act as inducers of the enzyme activity and modify the gene expression profile of the oxidases under study. This effect may be the result of differences in chemical structure and physicochemical properties of these compounds.

Key words. *Pleurotus ostreatus*, oxidases, bioremediation, gene expression

INTRODUCTION

Pleurotus ostreatus is a basidiomycete fungi used for the development of bioremediation methods for xenobiotics and recalcitrant compounds, due to its nonspecific enzymatic system, capable of catalyzing the oxidation and/or hydrolysis in a wide variety of compounds, including dyes[1,2]. The ligninolytic enzyme system secreted by *P. ostreatus* includes laccase and heme peroxidases. Heme peroxidases of this fungus consist of cytochrome c peroxidase and ligninolytic peroxidases, including manganese peroxidase and versatile peroxidase, heme-thiolate peroxidases, and dye-decolorizing peroxidases but not lignin peroxidase. In addition, these enzymes are produced typically as multiple isoenzymes[2-4].

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are glycoproteins classified as multi-copper oxidases that use the distinctive redox ability of copper ions to concomitantly catalyze the oxidation of a wide range of aromatic substrates with the reduction of molecular oxygen to water[5,6]. On the other hand, manganese peroxidase (Mn(II):hydrogen-peroxide oxidoreductase EC 1.11.1.13) is a glycosylated heme protein with molecular masses ranging from 38 to 62.5 kDa, and averaging at 45 kDa. Basically oxidize Mn^{2+} to Mn^{3+} in the presence of H_2O_2 and organic acid chelators. Mn^{3+} in turn, oxidizes a variety of phenolic substrates[1,7] and the versatile peroxidase (VP) Mn(II): H_2O_2 oxidoreductase / diarylpropane: O_2, H_2O_2 oxidoreductase EC 1.11.1.16) which shares catalytic and structural properties with lignin peroxidase (LiP) and manganese peroxidase (MnP) being one of the nine members of the manganese-peroxidase (MnP) gene family.It is able to directly oxidize low redox potential substrates, including phenols, as well as high redox potential dyes that are oxidized by LiP only in the presence of some mediator. VP present both Mn^{2+} binding site and the catalytic tryptophan[8]. The VP encoded by *mnp4* is the responsible for the Mn^{2+}-dependent and Mn^{2+}-independent peroxidase activity under Mn^{2+}-deficient culture conditions[9].

The biodegradation capabilities of white rot fungi including *Pleurotus ostreatus*, for different pollutants are variable, mainly due to physiological differences among them, difference in their genetic makeup and variable pattern and expression of complex lignin mineralizing enzymes in the presence of chemically different compounds[10].

Several studies had demonstrated the influence of dyes structure on the patterns of expression and activity of oxidases produced by *P. ostreatus* during the process of growth and dye oxidation[11,12] .

In order to define the main oxidases produced by this fungi in the presence of dyes, the objective of this investigation was to study the production and expression patterns of the main oxidases produced by *P. ostreatus* during the growth process in submerged fermentations in the presence of remazol brilliant blue R (RBBR) and yellow azo (AYG) dyes.

METHODS

Three fermentations of *P. ostreatus* grown in basal medium (MBF) and in the presence of either 500 ppm of RBBR (remazol brilliant blue R dye, SIGMA) or 500 ppm of AYG (Acetyl yellow G, ALDRICH) were established. The fermentations were performed in 125 ml Erlenmeyer flasks containing 50 ml of basal medium (BM) [13]. The cultures were incubated at 25 °C on a rotary shaker at 120 rpm. After 168 h cultures were supplemented with 500 ppm of either AYG or RBBR. Samples were taken at 175, 187, 199, 211, 223 and 247 h of fermentation. The enzymatic extract (EE) was obtained by filtration of the cultures using filter paper (Whatman No. 4), and stored at -20 °C until it was analyzed, while the mycelium was rinsed with 0.9% NaCl and stored at -70 °C until the total RNA extraction procedure was conducted. RNA was isolated from frozen mycelium produced at different fermentation times, using TRIZOL (Invitrogen) extraction, and was spectrophotometrically quantified by determining the optical density at OD 260/280. RNA was treated with RNAse-free DNase I (Invitrogen) and then resuspended in 20 µl of diethylpyrocarbonate-treated water. cDNA synthesis was performed using oligos dT and Moloney murine Leukaemia virus reverse transcriptase (M-MuLV-RT; Fermentas) in accordance with the manufacturer's instructions. The product of the RT reaction mixture (1 µg) and 10 ρmol of specific primers were mixed for specific PCR amplification using TrueStart TaqDNA polymerase (Thermo Scientific). The denaturation conditions used were 94 °C for 3 min. The program included 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 45 s. The control mixtures for the RT-PCR reaction contained either water or 10 ng of P. ostreatus DNA. The constitutive expression of glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene was used as internal control.

Primers for RT-PCR were designed using the open primer-blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) based on complete sequences of laccase mRNAs reported in the NCBI GeneBank (http://www.ncbi.nlm.nih.gov/nucleotide). (Table 1)

Table 1: Primers used for RT-PCR expression profile

gene	Forward primer	Reversed primer	RNA amplicon bp
mnp	GGCCTGTTGTGTTCTGTTC	GCTGGGAAGTTGACCTCA	198
vp	CTTGVCAGAATGRGTGACG	CTTCTCCCTTGTTGTCDGB	208
lacc	TGTTGAAGACGACCAGTATC	TGGAAAGATGCGAGTGAT	171
pox1	GTCTCTCCTGACGGCTTCAC	ATACTGGGTGGAAAGATGCG	333
pox2	ACGAGCTGGAGGCTAACAAA	TCACGAAGCGAATAGTGACG	204
pox3	GCAGAACTCGACATAGTCA	GTACTGGTCTTCATTGTTGG	167
pox4	TGAACAAAGTGGTCTCCC	TATCGGTGAGCTCATTGAC	147
poxa1b	ACGTCGTTGAGATCACTATG	AAATCGGATGGTGACGTT	182
gpd	TCTGCGGTGTTAACCTTGAGTCGT	TGGTAGCGTGGATGGTGCTCATTA	149

***P.ostreatus* was grown in liquid fermentation at 25°C for 23 days. Samples were taken at regular intervals and filtrated, with the supernatant obtained then used to measure enzyme activities. Enzyme assays for laccase, manganese peroxidase and versatile peroxidase were performed according to [20], [10] and [17] respectively.**

RESULTS AND DISCUSSION

In order to determine the induction effect of the textile dyes (RBBR and AYG) on gene expression profile and enzyme activity of laccase (Lacc), manganese peroxidase (MnP) and versatile peroxidase (VP) produced by *Pleurotus ostreatus*, dyes under study were added at the beginning of the fermentation exponential growth phase (168 h). The transcript profiles of genes *lacc, pox1, pox2, pox3, pox4, poxa1b* as well as *mnp* and *vp* together with the internal control gene glyceraldehyde-3-phosphate dehydrogenase (*gpd*) were determined by RT-PCR, at different fermentation times and in both the presence and absence of dyes. Figure 1 shows the gene expression profiles, it can be seen that both dyes induced the expression of laccase genes (*lacc,pox2 and pox4*), as well as manganese peroxidase (*mnp*) gene, on the other hand versatile peroxidase (*vp*) gene was expressed only in the presence of RBBR at specific times. *poxa1b* showed a constitutively expression in the three conditions and during all the fermentation times analyzed, whereas *pox1* was not expressed in the presence of any dye.

In accordance with our results *pox2* is known to be both constitutively and inducible expressed and particularly *poxa1b* its induction is up regulated by copper, it can be mention that the basal media used in this research contains copper and that may explain the high transcriptional level of this gene in all conditions tested[14].

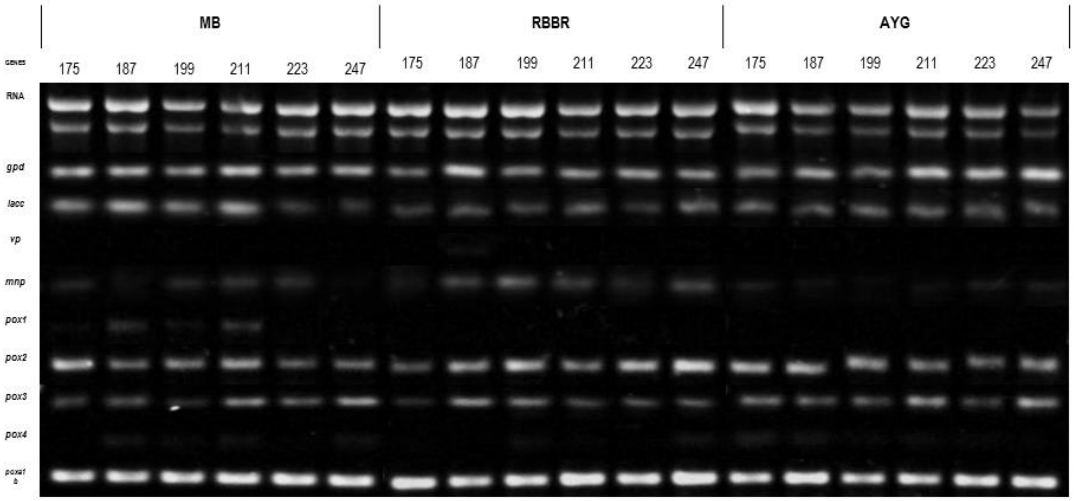

Fig. 1: Expression profile of oxidase genes from *P. ostreatus* grown in basal media (MB), Remazol brilliant blue R (RBBR) and Acetyl yellow G (AYG). Constitutive expression of glyceraldehyde-3-phosphate dehydrogenase (*gpd*) was used as an internal control

Figure 2 shows the relative transcriptional induction level determined by densitometry, the *gpd* gene was used as internal control and transcript profile in MB were considered as the basal level (1 unit) and comparisons were being made with either RBBR or AYG fermentations.

The induction effect of dyes on gene expression was clearly observed for the *vp* in the presence of RBBR with almost 300 fold level. For the laccase isoforms, the greatest effect was observed for gene *pox4* with inductions levels ranging from 800 and 25 folds in AYG and RBBR respectively.

The induction level observed for *mnp* ranged from 5 to 20 folds in AYG and RBBR respectively.

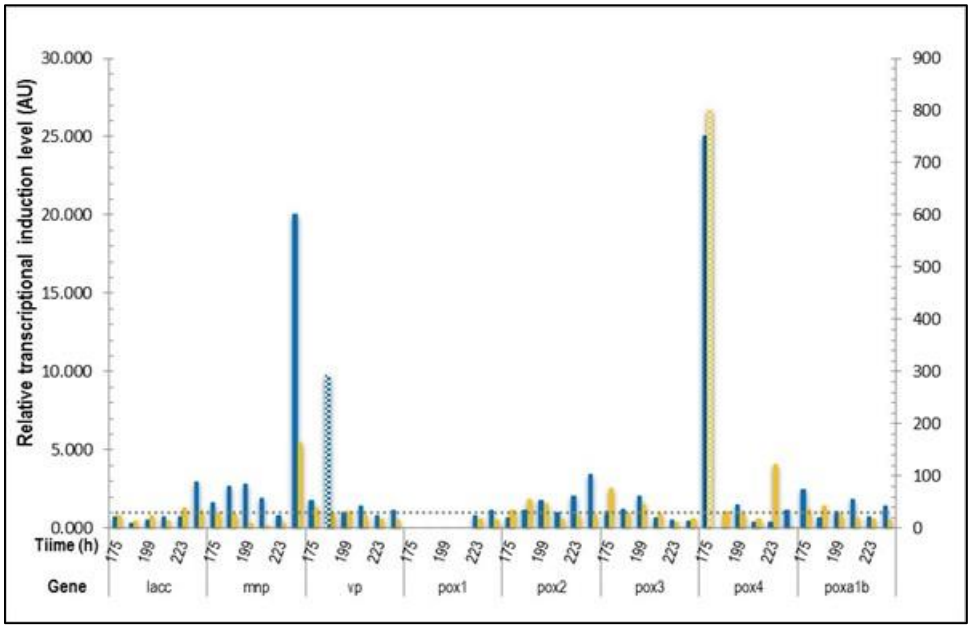

Fig.2: Transcript level of *P. ostreatus* oxidases genes in Remazol brilliant blue R (RBBR) (blue bars) and Acetyl yellow G (AYG)(yellow bars)

Figure 3 shows the relative transcriptional induction and the laccase extracellular activity. Laccase activity seems to be constitutive from the 175 h, showing an increasing level with time, reaching the highest level at 247 h, particularly in RBBR.

It is clear that laccase genes are expressed at different times and levels, depending on the growth conditions and also the enzyme activity not necessary correlate with the expression level.

Palmieri *et al.*[11] reported that the laccase isoenzymes POX2 and POXA3 were able to degrade RBBR in vitro, moreover in liquid media supplemented with veratryl alcohol *P. ostreatus* produced not only laccases but veratryl alcohol oxidase and dye-decolourising peroxidases among other enzymes.

The use of wheat straw extract as inducer of laccase gene family demonstrated that the *pox2* and *poxA3a* genes are the main sources of laccase activity in submerged fermentation[15].

Since the laccase expression is often induced by several different physiological factors, its promoter region may contain elements that take part in fungal metal response mechanism (MRE-metal responsive element), xenobiotics response mechanism (XRE-xenobiotic responsive element) temperature shock response (HSE- heat shock responsive element) or oxidative stress response (ARE- antioxidant response element)[7].

In addition the laccase isoenzyme patterns are more complex as a result of different posttranslational modifications (proteolytic processing, glycosylation, etc.).

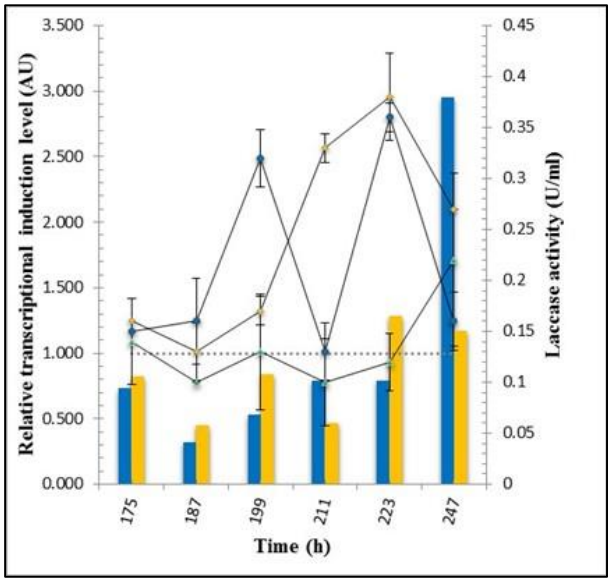

Fig.3: Laccase activity and relative transcriptional induction level in RBBR (blue circle and blue bars) and AYG (yellow diamond and yellow bars)

Moreover for *mnp* (Fig.4), the highest transcriptional induction level (20 folds) was obtained in the presence of RBBR at 247 h. Contrary the highest MnP activity (3 U/ml) was detected at 211 h in AYG, but this does not coincide with the highest level of transcriptional induction in the presence of that dye. Goudopoulos et al.[1] , reported that the patterns of MnP activity and transcripts are in full agreement with the notion that although Mn^{2+} is indispensable required for the production of active MnP, it is not necessary for the accumulation of the corresponding transcripts. This illustrates that not all manganese genes are expressed at any time and under different growth conditions, but moreover that not all manganese protein forms are necessarily present or detectable in supernatants of growing cultures.

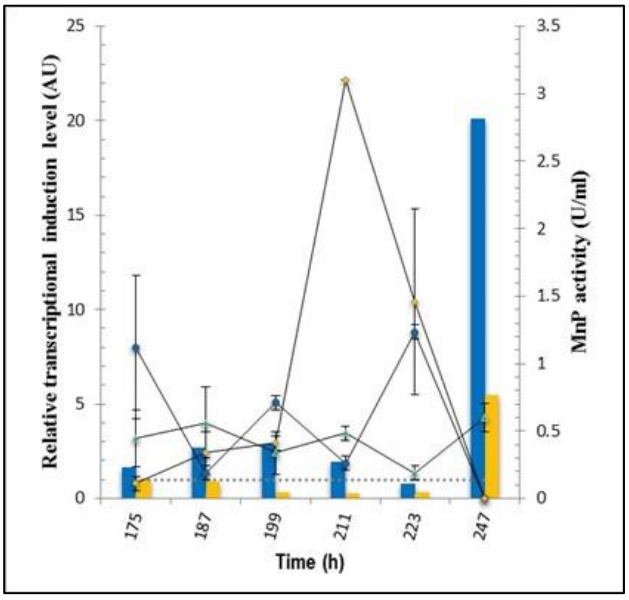

Fig.4: Manganese peroxidase activity and relative transcriptional induction level in RBBR (blue circle and blue bars) and AYG (yellow diamond and yellow bars)

VP transcriptional levels and activity are presented in Figure 5, while it was observed an important transcriptional induction of 294 folds at 187 h in RBBR, the highest VP activities were detected at 187 h and 223 h both in AYG. It has been reported that VP is able to efficiently oxidize phenolic compounds and dyes that are the substrates of generic peroxidases[16, 17].

The effect of environmental parameters (temperature and pH) on the expression of six manganese peroxidase and three versatile peroxidase genes of *P. ostreatus* was studied[18]. The global analysis of the expression patterns divided peroxidase genes into three main groups according to the level of expression at optimal conditions (vp1/mnp3 > vp2/vp3/mnp1/mnp2/mnp6 > mnp4/mnp5).

The differences in the transcription levels of the peroxidase genes when the culture temperature and pH parameters were changed suggested an adaptive expression according to environmental conditions.

Cohen *et al*.[19], studied the effect of Mn^{2+} supplementation on enzymatic and transcript abundance profiles of three VPs and one MnP from *P. ostreatus*. The reduction in VP gene transcript abundance and the increase in *mnp3* transcript level were collinear with the changes observed in the enzyme activity profiles.

These results indicated that the activity of peroxidases is regulated at the transcriptional level. They suggested that the expression of MnP and VP may be differentially regulated by the presence of Mn^{2+}.

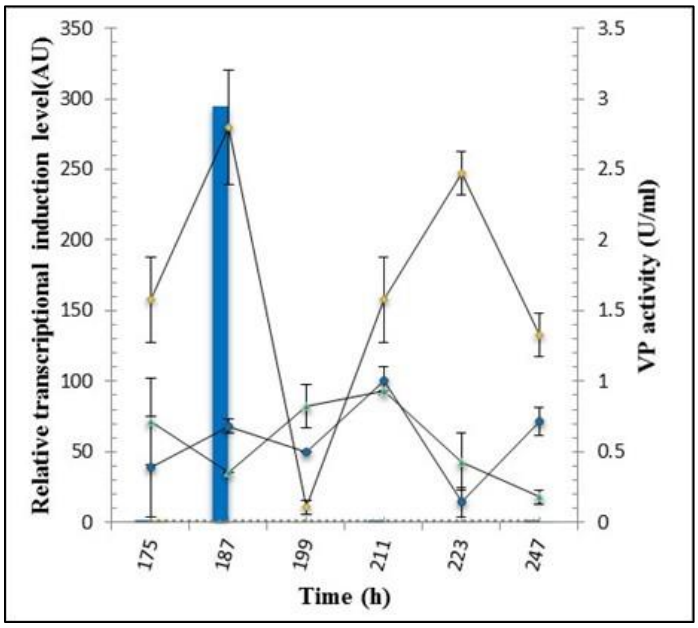

Fig.5: Versatile peroxidase activity and relative transcriptional induction level in RBBR (blue circle and blue bars) and AYG (yellow diamond and yellow bars)

CONCLUSIONS

The inoculation of the textile dyes RBBR and AYG at the beginning of the exponential phase has regulated the expression of the genes under study, with or without the increase of the extracellular enzyme activity. The high percentage of genes with altered transcriptional responses in the presence of dyes reveals a complex regulation mechanism that could be related to the sensitivity of the genes under study to the dyes. This effect may be the result of differences in chemical structure and physicochemical properties of these compounds.

Finally the enzyme activity and expression profile analysis suggest the coordinate participation of the enzymes under study in the oxidation and probable mineralization of the dyes evaluated.

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REFERENCES.

1. A.Goudopoulou, A. Krimitzas, M.A. Typas. Differential gene expression of ligninolytic enzymes in *Pleurotus ostreatus* grown on olive oil mill wastewater. Appl Microbiol Biotechnol, 2010, 88:541-551.
2. F.J. Ruiz-Dueñas, E. Fernández, M.J. Martínez, A.T. Martínez. *Pleurotus ostreatus* heme peroxidases: An in silico analysis from the genome sequence to the enzyme molecular structure.*CR BIOL*, 2011¸334: 795–805.
3. C. Pezzella, V. Lettera, A. Piscitelli, P. Giardina, G. Sannia. Transcriptional analysis of *Pleurotus ostreatus* laccase genes. *Appl Microbiol Biotechnol*, 2013 97:705-717.
4. M. Hofrichter, R. Ullrich, M. Pecyna, C. Liers, T. Lundell. New and classic families of secreted fungal heme peroxidases. *Appl Microbiol Biotechnol*, 2010, *87*(3):871–897.
5. E.I. Solomon, U.M. Sundaram, T.E. Machonkin, Multicopper oxidases and oxygenases. *Chemical Reviews*, 1996, 96(7): 2563-2605.
6. C.F. Thurston. The structure and function of fungal laccases. *Microbiology*, 1994, 140: 19-26
7. G. Janusz, K. Kucharzyk, A. Pawlik, M. Staszczak, A. Paszczynski. Fungal laccase, manganese peroxidase and lignin peroxidase: gene expression and regulation. *Enzyme Microb. Technol*, 2013, 52:1–12
8. F.J. Ruíz-Dueñas, M. Morales, E. García, Y. Miki, M.J. Martínez, A.T. Martínez. Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. *J Experimental Botany*, 2009, 60:441-452.
9. T. Salame, D. Knop, D. Tal, D. Levinson, O. Yarden, Y. Hada. A Gene-Targeting System for *Pleurotus ostreatus*: Demonstrating the Predominance of Versatile-Peroxidase (mnp4) by Gene Replacement. *Appl Environ Microbiol*, 2012, 78(15): 5341–5352.

10. M. Asgher, H.N. Bhatti, M. Ashraf, R.L. Legge. Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme. *Biodegradation*, 2008, 19:771-783.
11. G. Palmieri, G. Cennamo, G. Sannia. Remazol Brilliant Blue R decolourisation by the fungus Pleurotus ostreatus and its oxidative enzymatic system. *Enzyme Microb. Technol*, 2005, 36: 17-24.
12. T. Salame, O. Yarden, Y. Hadar. *Pleurotus ostreatus* manganese-dependent peroxidase silencing impairs decolourization of Orange II. *Microb Biotechnol*, 2010, 3(1):93–106.
13. M. Téllez-Téllez, F.J. Fernández, A.M. Montiel-González, C. Sánchez, G. Díaz-Godínez. Growth and laccase production by *Pleurotus ostreatus* in submerged and solid-state fermentation. *Appl. Microbiol. Biotechnol*, 2008, 81: 675-679.
14. G. Palmieri, G. Cennamo, V. Faraco, A. Amoresano, G. Sannia, P. Giardina. A typical laccase isoenzymes from copper supplemented *Pleurotus ostreatus cultures*. *Enzyme Microb. Technol*, 2003¸ 33: 220-230
15. R. Castanera, G.A. Pérez, A. Omarini, M. Alfaro, A.G. Pisabarro, V. Faraco, A.Amore, L. Ramírez. Transcriptional and Enzymatic Profiling of *Pleurotus ostreatus* Laccase Genes in Submerged and Solid-State Fermentation Cultures. *Appl Environ Microbiol*, 2012, 78(11):4037-4045.
16. P. Giardina, G. Palmieri, B. Fontanella, V. Rivieccio, G. Sannia. Manganese peroxidase isoenzymes produced by *Pleurotus ostreatus* grown on wood sawdust. Arch Biochem Biophys, 2000, 376: 171–179.
17. M. Pérez-Boada, F.J. Ruiz-Dueñas, R. Pogni, R. Basosi, T. Choinowski, M.J. Martínez, K. Piontek, A.T. Martínez. Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigations of three long-range electron transfer pathways. *J Mol Biol*, 2005¸ 354:2385–402.
18. E. Fernández-Fueyo, R. Castanera, F.J. Ruiz-Dueñas, M.F. López-Lucendo, L> Lucía Ramírez, A.G. Pisabarro, A.T. Martínez. Ligninolytic peroxidase gene expression by *Pleurotus ostreatus*: Differential regulation in lignocellulose medium and effect of temperature and pH. *Fungal Genet Biol*, 2014, 72:150-161.
19. R. Cohen, Y. Hadar and O. Yarden. Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by Mn2+. *Environ Microbiol*, 2001, 3: 312–322.

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