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Full Length Research Paper

Cloning and sequencing of phenol oxidase 1 (*pox1*) gene from *Pleurotus ostreatus*

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The gene (*pox1*) encoding a phenol oxidase 1 from *Pleurotus ostreatus* was sequenced and the corresponding *pox1*-cDNA was also synthesized, cloned and sequenced. The isolated gene is flanked by an upstream region called the promoter (399 bp) prior to the start codon (ATG). The putative metal-responsive elements (MREs) were determined in the promoter region, where MRE 1, 2 and 3 were located in positions -20, -62 and -389, respectively. Functional TATA consensus sequences were recognized in positions -78 and -245, while CAAT consensus sequence was recognized in position -171. The putative GC boxes consensus sequences were recognized in positions -175 and -344, and xenobiotic-responsive elements (XREs) in positions -100 and -270. The *pox1*-DNA gene consists of 2656 bp, with the coding sequence being interrupted by 19 introns. The nucleotide sequence of cDNA (*pox1*-cDNA) was found to contain an ORF of 1590 bp capable of coding for a protein of 529 amino acid residues. The signal peptide was predicted to be 23 amino acids in length using SIGNALP 3.0 program. Northern blot analysis revealed that strong transcriptional induction was observed in the copper-supplemented cultures for *pox1* gene.

Key words: *Pleurotus,* cDNA, *pox1*, gene promoter, putative sequences, northern blot analysis, copper.

INTRODUCTION

Lignin is the second most abundant renewable organic compound in the biosphere after cellulose and its biodegradation is a rate limiting step in the carbon cycle (Bumpus and Aust, 1987). White rot fungi secrete ligninolytic enzymes which are able to generate radical species that allow the complete biodegradation of the lignin polymer (Evans et al., 1994). Because of the complex structure of lignin, the biodegrading system is highly non-specific and so, ligninolytic enzymes can be employed in the degradation of structurally different environmental pollutants (Barr and Aust, 1994; Field et al., 1993). Among ligninolytic enzymes, laccases (EC 1.10.3.2) are phenol oxidases that catalyse one-electron oxidation of many aromatic substrates (Polyphenols, methoxy substituted monophenols, aromatic amines, etc.) with the concomitant reduction of O_2 to H_2O (Thurston, 1994). Moreover, the substrate range of these enzymes can be extended to include non-phenolic lignin subunits in the presence of readily oxidizable primary substrates, which can act as electron-transfer mediators (Bourbonnais and Paice, 1990).

Laccases belong to the class of blue oxidases and contain four copper atoms/ molecule distributed in three different types. The type-1 site is responsible for the intense blue color 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 centers and is responsible for a band near 330 nm (Solomon et al., 1996). These ligninolytic enzymes are secreted in multiple isoforms, depending on the fungal species and environmental growth conditions (Bollag and Leonowicz, 1984).

Abbreviations: MRE, Metal-responsive element; **ATG,** start codon; ***pox 1,*** phenol oxidase 1; **DNA,** deoxyribonucleic acid; **ORF,** opening reading frame; **PCR,** polymerase chain reaction; **RNA,** ribonucleic acid; **ABI,** applied biosystems; **XRE,** xenobiotic-responsive elements.

Laccases may have a number of biotechnological applications, including pulp delignification, detoxification of recalcitrant biochemicals, polycyclic aromatic hydrocarbons degradation, wastewater, and soil bioremediation and organic synthesis (Mayer and Staples, 2002). In particular, fungal laccases can decolorize and detoxify industrial dyes *in vitro* (Kandelbauer et al., 2004; Palmieri et al., 2005; Zille et al., 2005) and that the substrate specificity of the enzyme can be broadened in the presence of redox mediators (Claus et al., 2002).

Enzyme production on industrial scale is feasible when the protein is formed at high levels and the producing organism can be cultivated in a large-scale fermentation. Thus, the genes for many industrially important enzymes have been inserted in a heterologous host such as filamentous fungi and yeasts. A frequently used expression organism is the methylotrophic yeast *Pichia pastoris* that can grow on methanol as sole carbon and energy source (Cereghino and Cregg, 2000). The recombinant proteins can be expressed under the control of a strong tightly regulated promoter, the methanol induced alcohol oxidase P_{AOX1}. *P. pastoris* has the potential for high expression levels, efficient secretion of extracellular proteins, post translational modifications, such as glycolsylation and growth at high cell densities on defined minimal medium. Laccase genes from *Trametes versicolor* (Jönsson et al., 1997; O'Callaghan et al., 2002), *Pycnoporus cinnabarinus* (Otterbein et al., 2000), *Pleurotus sajor-caju* (Soden et al., 2002) and *Fome lignosus* (Liu et al., 2003) have been expressed in *P. pastoris* indicating the suitability of this system for laccase production. A number of laccase genes has been also expressed in the filamentous fungi, *Trichoderma reseei* (Kiiskinen et al., 2004) and *Aspergillus* (Larrondo et al., 2003; Record et al., 2003); although, filamentous fungi are generally good hosts for protein secretion, they are more time consuming to work with compared to yeast.

Laccase isoenzymes produced by *Pleurotus ostreatus*, a white rot basidiomycete fungus, have been studied extensively. One of these, POXC (where POX is phenol oxidase), is the most abundantly produced under all the growth conditions examined (Giardina et al., 1996). Two other isoenzymes, secreted by the mycelium, have also been purified and characterized (POXA1w and POXA2) (Palmieri et al., 1997). POXA1w shows peculiar differentces with regard to metal content. This enzyme contains two zinc, one iron and only one copper atom/molecule. Moreover, POXA1w shows greater stability with respect to temperature and pH than the other two isoenzymes (Palmieri et al., 1997).

In this study, the author designed primers to amplify the *pox1* gene (DNA and cDNA) and also for the promoter region that extend 5´-upstream of the initiation codon ATG to analyze and determined the putative sequences that control transcription of the gene. Also, northern blot analysis to determine the effect of copper supplementation on the regulation of the transcription was performed.

MATERIALS AND METHODS

Bacterial strains, fungal strains and plasmid

For standard bacterial cloning, *Escherichia coli* DH5α (Hanahan, 1983) was grown in Luria-Bertani (LB) medium (Sigma) supplemented with 10 $\mu g ml^{-1}$ of ampicillin. *P. ostreatus* (Jacq. NRRL0366) was maintained by periodic transfer at 4°C on potato dextrose agar (3.9%, Oxoid, UK) plates in the presence of 0.5% yeast extract (Oxoid). The pGEM®-T Easy Vector system I (Promega, Madison, USA) was used in cloning of *pox1*-cDNA. Plasmids were propagated in *E. coli* DH5α.

Cultivation of *P. ostreatus*

Incubations were carried out at 28 ± 2°C by inoculating 100 ml of potato dextrose broth containing 0.5% yeast extract and/or supplemented with different concentrations of $CuSO_4.5H_2O$ solution in 500 ml flasks with three discs of *P. ostreatus*. The cultures were incubated in dark on a rotary shaker (100 $rpm.min^{-1}$). At different incubation times, the mycelia were harvested by filtration and kept at -40°C till used.

Isolation of genomic DNA and PCR amplification of pox1 gene

Standard molecular biology techniques was used for DNA manipulations as described by Sambrook et al. (1989). DNA was isolated by mixer mill isolation protocol as described by Moussa (2009). A 407 bp fragment of *pox1* promoter was amplified from *P. ostreatus* genomic DNA using synthetic oligos 5´-GATCT GCATCCATGACACAA-3´ and 5´-CCTACGAACGATGTTTCC-3´. A 2656 bp fragment of *pox1* gene was amplified from genomic DNA using synthetic oligos 5´-ATGTTTCCAGGCGCACGG-3´ and 5´-TTGTTT GGAATGCAGATGG-3´.

Isolation of total RNA and PCR for pox1- cDNA

Total RNA was isolated using RNA isolation solution (Omega Bio-Tek Inc.). A 1590 bp fragment of *pox1-cDNA* was amplified from *P. ostreatus* total RNA using oligos 5´-ATTGAATTCATGTTTCCAGG CGCACGG-3´ and 5´-ATTGAATTCCTAAGCTATGCCA CCTTTGT-3´ to create an *EcoRI* sits (underlined) in the start codon and after the stop codon, respectively.

cDNA cloning and transformation

The PCR product was eluted from gel using MicroElute™ gel extraction kit (Omega Bio-Tek Inc.) and cleaved while ligated to pGEM-T easy vector (Promega) were also cleaved with *EcoRI*. The cloned gene was transformed inside *E. coli* DH5α cells (Stratagene), following standard procedures (Ausubel et al., 1992).

Miniprep plasmid procedure

The isolated colonies indicating positive transformation were selected and miniprep was carried out as described by Moussa (2009).

Sequencing of pox1 gene

Nucleotide sequences were determined using the ABI Prism Big

```
      MRE3                                          GC box
GATCTGCATCCATGACACAAACATAGATACCAGGGTCACACCTTTTTGTGTCCGCCGGATACCCATACCAACGCTATGAATAGAAGCACTTTGAGTCTATTTCGCTCTAATAGCCTTCCA -280
     XRE                          TATA box                                                    GC box CAAT box
TTCCTCACGGGCTCTTCTTCGCATCTCCGAATATACCCAGGTTATGCACTGACTCGCGGACGATCATCGCTCCCTTACGACAAACGGATCTTTAACAACACCGCCCAATTCGGTGCAAGA -160
                                                 XRE          TATA box       MRE2
TCCTTGAGATGAGGTACGCCTAACCGAGGCTTCCTAATCCGTTCATTTTCGTTCTCATGCATATCGTTCTGTAGGTATATTTAAGACGTGCATGGACGACCGAAAATATCATCATCAACT - 40
              MRE1
TAACACCTCATCCAGCGCGTACTGCTACACCTACGAACGATGTTTCC 8
```

Figure 1. Nucleotide sequence of *P. ostreatus pox1* promoter region, extending 399 bp upstream of the start codon (ATG) (bold). The putative TATAs, GCs and CAAT boxes, MREs and XREs are underlined.

Dye Terminator Cycle Sequencing kit (Applied biosystems) on ABI automated sequencers (ABI 3100), carried out at Biocenter, Innsbruck Medical University, Austria. The genomic nucleotide sequences for the *pox1* gene is available on GenBank database with accession no. AB514559 (Assigned to *pox1* gene promoter), accession no. AB514560 (Assigned to *pox1*-DNA) and accession no. AB514561 (Assigned to the *pox1*-cDNA). Nucleotide and amino-acid-sequence similarity searches used the BLAST method (Altschul et al., 1990) on the National Center for Biotechnology Information databases (NCBI).

Northern blot analysis

Ten micrograms of total RNA was electrophoresed on 1.2% (w/v) agarose-2.2 M formaldehyde gels and blotted onto Hybond N membranes (Amersham Biosciences) and hybridized with digoxigenin-labeled probes (Boehringer Mannheim). Hybridization probes were generated by PCR amplification using oligonucleotides 5´-ATGTTTCCAGGCGCAC GG-3´ and 5´-CTAAGCTATGCCACCTT TGT-3´ for *pox1*.

RESULTS

Isolation and analysis of the phenol oxidase 1 (*pox1*) genomic sequence were performed. Three PCR products were obtained, which showed strong homology to known basidiomycete phenol oxidase genes. Based on the sequence analysis, two fragments were formed; one was for about 407 bp and extended to the 5´-non-coding region and the other fragment was about 2656 bp which is the coding sequence of *pox1* gene. Putative regulatory sites such as metal-responsive elements (MREs) and xenobiotic-responsive elements (XREs) were identified in the *pox1* promoter region, which extended about 399 bp upstream of the start codon, are shown in Figure 1. The alignment of sequence analysis of this fragment on DNA database led to the definition of *pox1* gene promoter (*pox1* promoter region, accession no. AB514559). The putative MREs were determined in the promoter region, where MRE 1, 2 and 3 were located in positions -20, -62 and -389, while XREs were located in positions -100 and -270. Functional TATA consensus sequences were recognized in position -78 and -245, while CAAT consensus sequence was recognized in position -171. The putative GC boxes consensus sequences were recognized in positions -175 and -344 (Figure 1). The entire structure of the *pox1* gene (*pox1*-DNA, accession no. AB514560) of *P. ostreatus* could be determined; the coding sequence was 2656 bp long, interrupted by 19 introns varying in size from 47 to 64 bp (Figure 2), when compared with the PCR fragment for *Pox1*-cDNA sequence. All of the introns splice junctions corresponded to the GT----AG rules (Figure 2).

First-strand cDNA was reverse transcribed from mRNA of a 5-day-old mycelium culture. An amplification experiment was performed using primer-*EcoRI*. The fragment which counted for the *pox1*-cDNA was cloned using pGEM-T easy vector. The nucleotide sequence of *pox1* was found to contain an ORF of 1590 bp (Figure 3), capable of coding for a protein of 529 amino acid residues. Using SIGNALP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/), the signal peptide was predicted to be 23 amino acids in length (Figure 4). The alignment of sequence analysis of this fragment with the previously determined nucleotide sequence led to the definition of the gene (*pox1*-cDNA, accession no. AB514561). *Pox1*-cDNA gene shared significant homology with phenol oxidase 1 (*pox1*) 100%, laccase (*Lacc*-AY485827) 98%, laccase (*Lacc*- AY450404) 83%, bilirubin oxidase (*Box*) 83% and phenol oxidase 2 (*pox2*) 83% (Table 1).

The isolated gene codes for a protein of 529 amino acids. The encoded amino acid sequence is reported in Figure 5. The multiple alignment of deduced amino acids sequence of POX1 shared high homology with phenol oxidase 1 (POX1) 100%, laccase (Lacc- AY485827) 99%, laccase (Lacc- AY450404) 90% phenol oxidase 2 (POX2) 90% and bilirubin oxidase (BOX) 90% of *P. ostreatus* in Databank (Table 1). The copper-binding domain structure found in other laccase genes is conserved in the *P. ostreatus* phenol oxidase 1 protein (Figure 5).

The results of northern blot experiments are shown in Figure 6. Strong transcriptional induction was observed in the copper-supplemented cultures for *pox1* gene. The amount of mRNA decreased after 2 days and there was an increase from the third day onwards for copper supplementations. *P. ostreatus-pox1* expression was found to be up-regulated by copper supplementations.

DISCUSSION

Although, laccase production in white rot fungi is known to be influenced by a number of factors, little work has been done to study the regulation of laccase gene ex-

Table 1. Comparison of the phenol oxidase 1 (pox1) cDNA sequence (Accession no. AB514561) with published sequences of this gene and other related genes from *Pleurotus ostreatus*

Gene	Percentage homology		Nucleotide Accession no.
	nt level	aa level	
Pox1	100	100	Z34847
Lacc	98	99	AY485827
Lacc	83	90	AY450404
Box	83	90	AB020026
Pox2	83	90	AB474261

Figure 2. Cont.

```
tccagCCGCGTGGGACGACTTGTGTCCGATTTATGATGCTTTGAGCGATTCCGACAAAGGTGGCATAGCTTAGGATATCTCTACCTACTTACAGAGATAAACTCGAAAGAAATAGAACCA 2640
     laAlaTrpAspAspLeuCysProIleTyrAspAlaLeuSerAspSerAspLysGlyGlyIleAlaEnd
       480                         490                         500
TCTGCATTCCAAACAA 2656
```

Figure 2. Nucleotide sequence of the *P. ostreatus pox1* gene and deduced amino acid sequence of POX1. Introns are shown in lower-case letters and indicated by INS. The putative signal peptide is bold and underlined (Recognized using signalP 3.0). The forward and reverse primers are bold and underlined.

```
P. ostreatus-Pox1 ATGTTTCCAGGCGCACGGATTCTCGCTACGCTTACATTAGCTCTTCACCTTTTACATGGGACTCATGCTGCCATTGGGCCCACTGGCGACATGTACATCG 100
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 100
Lacc-AY485827     ---------------------------------------------------------------------------------------------------- 100
Lacc-AY450404     ------------------------T-----------C--G--------T--------------------------C------------A------------ 100
Box-AB020026      ------------------------T-----------C--G--------T--------------------------C------------A------------ 100
Pox2-AB474261     ------------------------T-----------C--G--C-----T-----G------G--------------------G-----A-------T---- 100

P. ostreatus-Pox1 TCAACGAGGACGTCTCTCCTGACGGCTTCACTCGTTCGGCTGTCGTCGCTCGCTCTGACCCCACCACAAATGGGACGTCGGAGACGC      TTACCGG 194
Pox1-Z34847       ---------------------------------------------------------------------------------      ------- 194
Lacc-AY485827     ---------------------------------------------------------------------------A-------      ------- 194
Lacc-AY450404     ----------------------T--T---G----------G--T-----------A-TG---G-----G--CC----C-T-C---AGTATCCA--C-T-- 200
Box-AB020026      ----------------------T--A---G----------G--T-----------G-TG---G-T---G--CC----C-T-C---AGTATCCA--C-T-- 200
Pox2-AB474261     ----------------------T--T---G----------T-----------A-TG---G-----G--CC----C-T-C---AGCATCTA--C-T-- 200

P. ostreatus-Pox1 TGTCCTCGTGCAAGGAAACAAGGGCGACAACTTCCAGCTGAACGTTCTCAATCAACTGTCGGACACGACTATGTTGAAGACCACTAGTATCCATTGGCAT 294
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 294
Lacc-AY485827     C-------------------------------------------------C--------------------------------T------------------ 294
Lacc-AY450404     C--T-----T--------------T--T-----------------CG--------T-------------C-----------G--C--------------C 300
Box-AB020026      C--T-----T-----------------T-----------------CG--------T-------------C-----------G--C--------------C 300
Pox2-AB474261     C--T-----T-----------------T--------------T---G-T-----------A--------------------G-----------------C 300

P. ostreatus-Pox1 GGCTTCTTTCAATCCGGTTCTACGTGGGCAGATGGACCCGCGTTCGTGAATCAATGCCCCATCGCCTCGGGGAACAGCTTCCTATATGACTTCAACGTCC 394
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 394
Lacc-AY485827     ---------------------------------------------------------------------------------------------------- 394
Lacc-AY450404     --T-----C---G----A---T-------T-----T-----T-------CC---------G-------T---G-T--T-----G--CA-T-----T---- 400
Box-AB020026      --T-----C---G----A---T-------T-----T-----T-------CC---------G-------T---G-T--T-----G--CA-T-----T---- 400
Pox2-AB474261     --T-----C---G----A---T-------------C-----T-------CC---------G-------T---G-T--T-----G--CA-T-----T---- 400

P. ostreatus-Pox1 CCGACCAAGCTGGCACGTTCTGGTACCATTCGCATCTTTCCACCCAGTATTGTGATGGTCTTAGAGGACCATTCATAGTATACGACCCCTCCGATCCCCA 494
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 494
Lacc-AY485827     ------------------------------------------------A--------------------------------------------------- 494
Lacc-AY450404     -A-----------A-----T-----T--C----------------A----------C--C------------G-G---------T----G-----G-- 500
Box-AB020026      -A-----------A-----T-----T--C----------------A--------C--C--C------------G-G--------------G-----G-- 500
Pox2-AB474261     -G-----------A-----T-----T--C----------------A-----C--C--C--C-----G-----TG-G--------T-----G-----G-- 500

P. ostreatus-Pox1 CCTGTCCTTGTATGACGTTGACAACGCCGACACCATCATTACACTTGAAGATTGGTACCATGTTGTGGCCCCTCAGAATGCAGTGCTTCCTACTGCTGAT 594
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 594
Lacc-AY485827     ---------------------------------------------------------------------------A-----G------------------ 594
Lacc-AY450404     -T--AGT-----C--TA----------------GG-------G-----G--C-----T---A-C----------A-----G-C-A-C--C--CC-G--- 600
Box-AB020026      -T--AGT--A--C--TA----------------GG-------G-----G--------T---A-C-----T--C--A--C--G-CAA-C--A---C-G--- 600
Pox2-AB474261     -T-AAGT--A-----CA----------T----GG-------G-----G----------A-C----------A--C--G-C-A-C--C--CC-A--- 600

P. ostreatus-Pox1 AGTACACTCATCAATGGCAAAGGTCGCTTCGCTGGGGGGCCTACTTCCGCTTTGGCCGTCATCAACGTCGAAAGCAACAAGCGATATCGTTTCAGACTTA 694
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 694
Lacc-AY485827     ----------------------------------------------------------------------------------------------C- 694
Lacc-AY450404     -----T----------T--------T-A---C-----C-----C---C-----T----------------------------C--------------CG 700
Box-AB020026      -----C----------T--------T-AT--C-----C-----C--TC-----T--A-------------------------C--------------TG 700
Pox2-AB474261     -----C----------T--------T-A---C-----C--------CC-------TA-------------------------C--------------TG 700

P. ostreatus-Pox1 TCTCGATGTCTTGCGACCCCAATTTCACGTTCTCGATCGACGGTCACTCTTTGCAGGTCATCGAGGCAGACGCTGTCAATATTGTGCCCATTGTCGTGGA 794
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 794
Lacc-AY485827     ------------------------------------------------------------------------A-----------------C-------- 794
Lacc-AY450404     ----A----------T--T-----------------------------------TC-----T--A-----T--------C----------CAC------ 800
Box-AB020026      ----A--------T--------------------------------------TC-----T--A-----T--------C-----T-----CAC------ 800
Pox2-AB474261     ----A--------T--------------------------------------TC-----T--A-----T--------C-----A-----CAC------ 800

P. ostreatus-Pox1 TAGTATTCAAATCTTCGCAGGCCAACGCTATTCCTTCGTCTTGAATGCCAATCAGACTGTCGACAATTACTGGATTCGCGCAGATCCCAACTTGGGATCG 894
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 894
Lacc-AY485827     ------------------------------------------------------------T--------------------------------------- 894
Lacc-AY450404     ---------G--------T----------C-------------C----G-------C--T-G----------------GA----T------------ 900
Box-AB020026      ---------G--------T----------C-------------C---TG----A--C--T-G---C--------------GA----T------------ 900
Pox2-AB474261     ---------G--------T----------C-----T-------C---T-----AG-C--T-----C----------G--GA-C--T----------A 900

P. ostreatus-Pox1 ACTGGCTTCGATGGTGGTATCAATTCCGCTATCCTTCGGTATGCTGGTGCCACTGAAGATGACCCTACCACGACTTCGTCGACGAGTACCCCCCTTGAGG 994
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 994
Lacc-AY485827     --C-------------A-------------------------------------------------------------------C------------- 994
Lacc-AY450404     ----------C--------------------T-----A----------------G----------T--A--------------C-----AT-GCT-- 1000
Box-AB020026      ---------------------------------T----------------------G--------C-----A--C----------C------T-GCT-- 1000
Pox2-AB474261     ----------T---------------T--C--T-----A----------------G--------C-----A--A----------C-----G--GCT-- 1000
```

Figure 3. Cont.

```
P. ostreatus-Pox1 AGACTAATCTTGTGCCACTTGAAAATCCTGGTGCTCCTGGTCCAGCTGTCCCTGGAGGCGCAGACATCAACATCAATCTCGCTATGGCCTTCGACGTTAC 1094
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 1094
Lacc-AY485827     ----------C-----T-C------------------------------------------------------------------------------- 1094
Lacc-AY450404     ----C--C-----T--G-----G--------C---------C---A-C--A-----T--A-----------------------------------T-C-- 1100
Box-AB020026      ----C--C-----T--G-----G----------------C-----C--G-----T--A-----------------------------------T-C-- 1100
Pox2-AB474261     ----C--C--C--T--G-----G--------C-----A--C---C----G-----T--A--C--T-------------------------------T-C-- 1100

P. ostreatus-Pox1 TAACTTTGAACTGACCATCAACGGCTCCCCCTTCAAAGCGCCGACTGCTCCTGTTCTGCTCCAGATTCTGTCGGGTGCCACAACTGCCGCCTCACTTCTC 1194
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 1194
Lacc-AY485827     ----------------------------------------------------------------------------------------------A------------ 1194
Lacc-AY450404     A-C---C---T----T---------GTT--T----TTC-A--A-----C-----C--T--T--A-----C--A--A---T-CT-G--T-----G--G--T 1200
Box-AB020026      ------C---T----T---------GTT--T----TCC-A--C-----C-----C--T--T--A-----C--A--A---T-CT----T-----G--G--T 1200
Pox2-AB474261     A-C---C---TTA----------TGTT--T---CTTC-A--A-----C-----C--T----------T--A--A--GT-------T-----G-----T 1200

P. ostreatus-Pox1 CCTTCCGGCAGTATATACTCGCTAGAAGCCAACAAAGTTGTCGAGATCTCCATACCCGCCTTAGCTGTTGGAGGACCGCATCCTTTCCATCTTCACGGAC 1294
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 1294
Lacc-AY485827     --C----------------------------G---------------------------------------------------------------- 1294
Lacc-AY450404     -----T--T--C--T---G----G--GC-T--T--G-----T--A-----G--G--T--GC-G-----C--G-----C-----A--------C-----T- 1300
Box-AB020026      -----T--T-----T---G----G--GC-T--T--G-----T--A-----G--G--T--GC-G-----C--G-----C-----A--------C-----C- 1300
Pox2-AB474261     -----T--T--C--T---GA---G--G--T----------A-----G--G--T--GC-G--------G-----C-----A----------C- 1300

P. ostreatus-Pox1 ACACGTTCGACGTCATCAGGAGTGCGGGCTCTACTACGTATAACTTCGACACCCCTGCGCGACGCGATGTTGTCAACACTGGAACTGACGCGAACGACAA 1394
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 1394
Lacc-AY485827     ----------------------------------------------------------------------------------G----------------- 1394
Lacc-AY450404     ----C--------T--T----------T--C--------C-----T-----T--------C-----C--A----------C----G------------ 1400
Box-AB020026      ----C--------T--T----------T--C-----A--C-----T-----T--------C-----C--A----------C----G------------ 1400
Pox2-AB474261     ----T-----T--T----------C--T-----G-----C-----T--------------C-----C--C----------C----G------------ 1400

P. ostreatus-Pox1 CGTTACTATCCGCTTTGTGACGGATAATCCAGGCCCATGGTTCCTCCACTGCCACATTGACTGGCATCTCGAAATCGGTCTTGCGGTCGTTTTCGCCGAA 1494
Pox1-Z34847       ---------------------------------------------------------------------------------------------------- 1494
Lacc-AY485827     ---------------------------------------------------------------------------------------------------- 1494
Lacc-AY450404     ---C-----T-----C-----C-----C-----G--G-------------T--------T-------------T--C--C--T--------------- 1500
Box-AB020026      ---C-----T-----C-----C--C--C-----G--G-------------T--------T-------------T--C--C--T--------------- 1500
Pox2-AB474261     ---C-----T-----C-----C--C--C--------G-------------T--------T-------------T--T--G--T--------------- 1500

P. ostreatus-Pox1 GATGTGACGTCGATCACGGCCCCACCTGCCGCGTGGGACGACTTGTGTCCGATTTATGATGCTTTGAGCGATTCCGACAAAGGTGGCATAGCTTAG      1590
Pox1-Z34847       ----------------------------------------------------------------------------------------GA-T 1593
Lacc-AY485827     ----------C-----------------------------------------------------C-------------------------GA-T 1593
Lacc-AY450404     --C-----A--C--TT-------------------------------C--C--C-----C--A--------CAA---------A-----C-T-CC-TCCT 1600
Box-AB020026      --C-----A--C--TT-------------------------------C--C--C---G-C--A--------CAA---------A-----C-T-CC-TCCT 1600
Pox2-AB474261     --C-----T--C--TT-------------------------------C--C--C---A-C--T--------CAA---------A-----C-T-CC-TCCT 1600
```

Figure 3. Multiple sequence alignment of nucleotide sequences of phenol oxidase 1 (*Pox*1-cDNA), phenol oxidase 1 (*Pox*1), laccase (*Lacc*), bilirubin oxidase (*Box*) and phenol oxidase 2 (*Pox2*) of *P.ostreatus*. The primers used for amplication are underlined. Alignment was done with ClustalW 2.0 software (http://www.ebi.ac.uk/ Tools/clustalw2/ index.html).

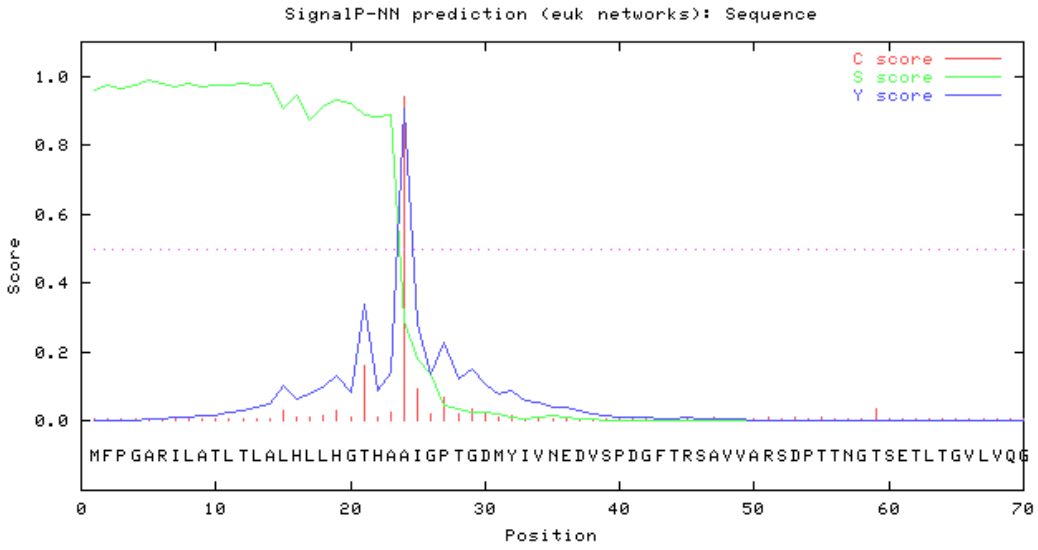

Figure 4. Determination of signal peptide sequence for the predicted amino acid sequence of POX1 from *P.ostreatus*

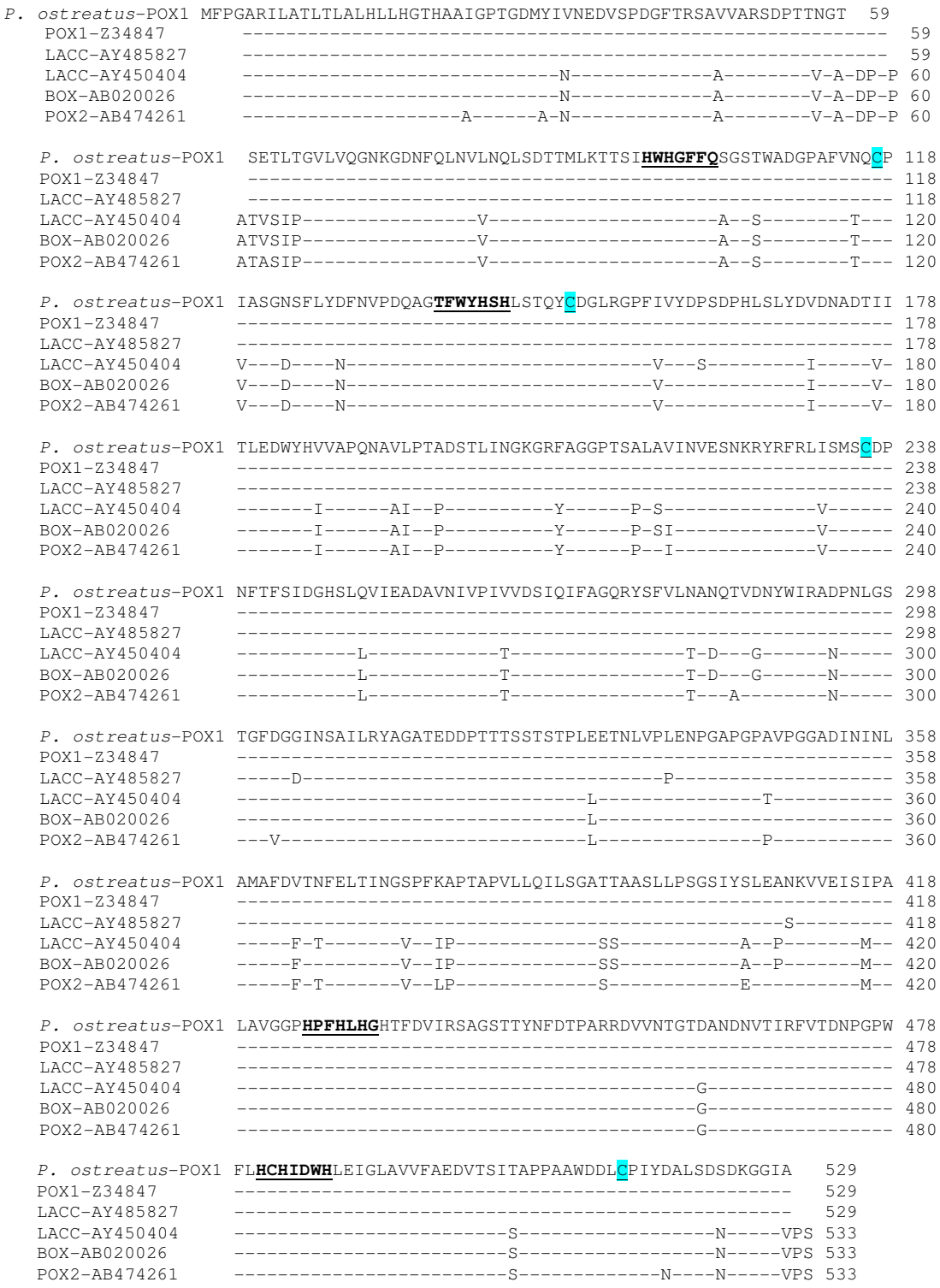

Fig. 5: Multiple sequence alignment of predicted amino acid sequence of phenol oxidase (POX1-cDNA), Phenol oxidase 1 (POX1), bilirubin oxidase (BOX), laccase (LACC) and phenol oxidase 2 (POX2) of *Pleurotus ostreatus*. Four potential copper-binding domains are bold and underlined. Four Cys residues involved in the formation of two disulphide bridges are shaded and underlined. Alignment was done with ClustalW 2.0 software (http://www.ebi.ac.uk/Tools/clustalw2/ index.html).

decreases the binding affinity of the adjacent MRE, affecting its interactions with fungal protein factors

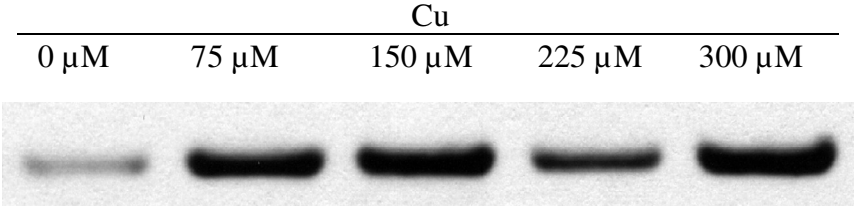

Figure 6. *P. ostreatus pox1* expression is up-regulated during copper supplementations. Total RNA was isolated from *P. ostreatus* following growth for 5 days during copper starvation (0 µM) and different copper concentrations conditions and subjected to northern analysis.

pression at the molecular level (Kalisz et al., 1986; Thurston, 1994). In the 5´-flanking region of *pox1* promoter, several sequences have been identified that match closely the consensus of regulatory elements, in particular, a metal-responsive element (Thiele, 1992) and a xenobiotic-responsive element (Fujisawa-Sehara et al., 1988). In the *poxa1b* promoter, a GC-rich region, homologous to the core binding site for transcription factor Sp1, (Faraco et al., 2003).

Nucleotide sequences of the *poxc* and *poxa1b* promoter regions, extending about 400 nt upstream of the start codon (ATG), have been analyzed and multiple putative regulatory sites such as metal-responsive elements (MREs), xenobiotic-responsive elements and heat-shock elements have been identified in them. The sequences of all MREs are similar to the core MRE consensus sequence (5'-TGCRCNC-3') identified in metallothionein (*mt*) gene promoters (Thiele, 1992). Otherlaccase promoters have been reported to contain multiple were based on comparison with the published sequences (Kojima et al., 1990; Saloheimo et al., 1991; Coll et al., 1993b; Morohoshi, 1993) and consensus sequences for 59 splicing GT(AG)(AT)GT and 39 splicing (CT)AG junctions present in filamentous fungi (Balance, 1986).

Laccase genes have been isolated from several basidiomycetes (Kojima et al., 1990; Saloheimo et al., 1991; Giardina et al., 1995; Berka et al., 1997). The sequences of these genes display a common pattern and they encode polypeptides of approximately 520 to 550 amino acid residues including an N-terminal signal peptide (Coll et al., 1993a; Giardina et al., 1995; Eggert et al., 1998).

In addition, the single cysteine residue and 10 histidine residues involved in binding the four catalytic cupric ions found in each laccase molecule are conserved, together with a small amount of sequence around the four regions in which the copper ligands are clustered (Thurston, 1994; Eggert et al., 1998). It is in the copper-binding amino acid residues and their general distribution in the polypeptide chain that the laccases are all similar (Coll et al., 1993a; Giardina et al., 1995; Eggert et al., 1998). Alignment of the polypeptide sequence derived from *lac1* with the sequences derived from other basidiomycete laccase genes shows that the domain structure of Lac1 protein is conserved. Lac1 showed the conserved sequences in the single cysteine residue and 10 histidine residues. The N-terminal *lac1* sequence is separated from the C-terminal catalytic domain by a hinge region (Thurston, 1994). The latter appears to be duplicated but is typically rich in serine residues.

Laccases are copper-containing oxidases which catalyze the four-electron oxidation of a variety of phenolic compounds and a simultaneous four-electron reduction of oxygen to water. The PCR strategy used in this study is based on the use of degenerate primers corresponding to the consensus sequences conserved in the copper-binding regions in the N-terminal domains of known basidiomycete laccases (Kojima et al., 1990; Messerschmidt and Huber, 1990; Saloheimo et al., 1991; Coll et al., 1993a; Morohoshi, 1993; Perry et al., 1993; Thurston, 1994).

Northern blot analyses clearly revealed that, copper had a marked effect on induction of *pox1* gene transcription. In addition, the *pox1* transcript was the most abundant transcript in the copper-supplemented cultures at all of the times analyzed. *P. ostreatus pox1* expression was found to be up-regulated by copper supplementations. Collins and Dobson (1997) have found that, the expression of laccase in *T. versicolor* was regulated at the level of gene transcription by copper and nitrogen. As the concentration of copper or nitrogen in fungal cultures was increased, an increase in laccase activity corresponding to increased laccase gene transcription was observed. Zhao and Kwan (1999) used HN medium supplemented with copper, to study the effects of physiological parameters on laccase expression in *Lentinula edodes*. The addition of copper sulphate to *P. ostreatus* growth medium causes a marked increase of total laccase activity and a transcription induction of poxc and mostly, *poxa1b* genes (Palmieri et al., 2000).

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