

Full Length Research Paper

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

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Accepted 30 December, 2010

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 O₂ to H₂O (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 glycosylation 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 *Fomes 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 reesei* (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 differences 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 supplementa-

tion on the regulation of the transcription was performed.

MATERIALS AND METHODS

Bacterial strains, fungal strains and plasmid

For standard bacterial cloning, *Escherichia coli* DH5 α (Hanahan, 1983) was grown in Luria-Bertani (LB) medium (Sigma) supplemented with 10 μgml^{-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* DH5 α .

Cultivation of *P. ostreatus*

Incubations were carried out at $28 \pm 2^\circ\text{C}$ by inoculating 100 ml of potato dextrose broth containing 0.5% yeast extract and/or supplemented with different concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 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'-GATCTGCATCCATGACACAA-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'-ATTGAATTCATGTTTCCAGGCGCACGG-3' and 5'-ATTGAATTCCTAAGCTATGCCA CTTTGT-3' to create an *EcoRI* sites (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* DH5 α 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

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MRE3
GATCTGCATCCATGACACAAACATAGATACCAGGGTTCACACCTTTTGTGTCCGGGATACCCATACCAACGCTATGAATAGAAGCACTTTGAGTCTATTTTCGCTCTAATAGCCTTCCA -280
XRE TATA box GC box CAAT box
TTCTTCACGGGCTCTTCTTCGCATCTCCGAATATACCCAGGTTATGCACTGACTCGGGGACGATCATCGCTCCCTTACGACAAACGGATCTTTAACAAACCCGCCCAATTCGGTGAAGA -160
XRE TATA box MRE2
TCCTTGAGATGAGGTACGCCTAACCGAGGCTTCTAATCCGTTTCATTTTCGTTTCATGTCATATCGTTTCTGTAGGTATATTTAAGACGTGCATGGACGACCCGAAAAATATCATCATCAACT - 40
MRE1
TAACACCTCATCCAGCGCTACTGCTACACCTACGAACGATGTTTC 8

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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

ATGTTTCAGGCCCGGATTCTCGCTACGCTTACATTAGCTCTTACCTTTTACATGGGACTCATGCTGCCATTGGGCCACTGGCGACATGTACATCGTCAACGAGGAGCTCTCTCT 120
MetPheProGlyAlaArgIleLeuAlaThrLeuThrLeuAlaLeuHisLeuLeuHisGlyThrHisAlaAlaIleGlyProThrGlyAspMetTyrIleValAsnGluAspValSerPro
 GACGGCTTCACTCGTTGtgaagtgggttctcggcgctcctttcggggcccaactaattttattacagGGCTGTCGTCGCTCGCTTACCCACCACAAATGGGACGTCGGAGACGCTTAC 240
 AspGlyPheThrArgSe INS I rAlaValValAlaArgSerAspProThrThrAsnGlyThrSerGluThrLeuTh
 CGGTGTCCTCGTCAAGGAACAAGGtaaacgtccgcttctgtaagctgctcgctttgttactcttcttagGGCGACAACCTCCAGCTGAACGTTCTCAATCAACTGTCGGACACGAC 360
 rGlyValLeuValGlnGlyAsnLys INS II GlyAspAsnPheGlnLeuAsnValLeuAsnGlnLeuSerAspThrTh
 TATGTTGAAGACCACTAGTATCgtatgtagacaatggttggtagataataactaactacctgcgagcagCATGGCATGGCTTCTTCAATCCGGTCTACGTGGGACAGATGgtatgtcc 480
 rMetLeuLysThrThrSerIle INS III HisTrpHisGlyPhePheGlnSerGlySerThrTrpAlaAspG
 ttcaatcgtgttgcatgctcgtcaattttttttcaagGACCCGCTTCGTGAATCAATGCCCATCGCCTCGGGGAACAGCTTCTGtgagtgtcataacctcatgtttatccttc 600
 INS IV lyProAlaPheValAsnGlnCysProIleAlaSerGlyAsnSerPheLe INS V
 gttcatcgatatttctcagATATGACTTCAACGTCCTCCGACCAAGCTGGCAGCTTCTGtaagtcgacgatcatgaatccattttgttctcctgaccgtatatccagGGTACCATTTCGCAT 720
 uTyrAspPheAsnValProAspGlnAlaGlyThrPheT INS VI rpTyrHisSerHis
 CTTCCACCCAGTATTGTGATGGTCTTAGAGGACCATTCATAGTgtaagcttcataatcgataagaacctaacgacgcccgggttaatcggtccacacagATACGACCCCTCCGATCCCA 840
 LeuSerThrGlnTyrCysAspGlyLeuArgGlyProPheIleVa INS VII lTyrAspProSerAspProHi
 CCTGTCTGTATGACGTTGACAACGgtgagctgtgaaagtattccagcgcgtgcatcgctgctgacggtctcacttgacCGACACCATCATTACACTTGAAGATTGGGtacatctcac 960
 sLeuSerLeuTyrAspValAspAsnA INSVIII laAspThrIleIleThrLeuGluAspTrp
 tcctcatggaataatgtgttctcattcttctgtagTACCATGTTGGCCCCCAGAAATGCAGTCTTCTACTGCTGATAGTACACTCATCAATGGCAAAGGTCGCTCGCTGGG 1080
 INS IX TyrHisValValAlaProGlnAsnAlaValLeuProThrAlaAspSerThrLeuIleAsnGlyLysGlyArgPheAlaGly
 GGGCCTACTTCGGCTTGGCCGTCATCAACGTCGAAAGCAACAAGCGATATCGTTTCAGACTTATCTCGATGCTTTCGCGACCCCAATTTACAGTTCGATCGACGGTCACTCTTGCAG 1200
 GlyProThrSerAlaLeuAlaValIleAsnValGluSerAsnLysArgTyrArgPheArgLeuIleSerMetSerCysAspProAsnPheThrPheSerIleAspGlyHisSerLeuGln
 GTCATCGAGGACGCTGTCAATATGTGCCATTGTCCGgtatgtccttttgcgcttgcttctcactgtctcaattcogctgactacggatggtcgatattgttagTGGATAGTATCAAAT 1320
 ValIleGluAlaAspAlaValAsnIleValProIleValV INS X alAspSerIleGlnIl 240
 CTTCGAGgtaaatgcaacccgcttctcgcgaaactttgctaagccgactttcaagGCCAACGCTATTCCTTCTGCTTGAATGCCAATCAGACTGTGCACAATTACTGGATTTCGCGCA 1440
 ePheAlaG INS XI lyGlnArgTyrSerPheValLeuAsnAlaAsnGlnThrValAspAsnTyrTrpIleArgAla
 GATCCCAACTTGGGATCGACTGgtatggcatcttgaagcaaacactgtgtctcgctgacttctgtaatgccagGCTTCGATGGTGGTATCAATCCGCTATCCTTCGGTATGCTGGTG 1560
 AspProAsnLeuGlySerThrG INS XII lyPheAspGlyGlyIleAsnSerAlaIleLeuArgTyrAlaGlyA
 CCACTGAAGATGACCCACCAGACTTCGTCGACGAGTACCCCTTGGAGGACTAATCTTGTGCCACTTGAAAACTCTGGTGCTCCTGGTCCAGCTGTCCCTGGAGGCGCAGACATCA 1680
 laThrGluAspProThrThrSerSerThrSerThrProLeuGluGluThrAsnLeuValProLeuGluAsnProGlyAlaProGlyProAlaValProGlyGlyAlaAspIleA
 ACATCAATCTCGCTATGGCCTTCGACGTTACTAACTTTGAACGACCATCAACGgtacgcatcttagctcttttcaatgccctggatggtactcatcgaccatgtccagGCTCCCCCT 1800
 snIleAsnLeuAlaMetAlaPheAspValThrAsnPheGluLeuThrIleAsnG INS XIII lySerProP
 TCAAAGCGCCGACTGgtaagctcaactcggcagcgaataaccaaaacaattgatgtgatattcgtgttagCTCCTGTTCTGCTCCAGATTCTGTCGGGTGCCACAACCTGCCCTCACTTC 1920
 heLysAlaProThrA INS XIV laProValLeuLeuGlnIleLeuSerGlyAlaThrThrAlaAlaSerLeuL 360
 TCCCTTCCGGCAGTATATACTCGCTAGAAGCCAACAAGTTGTGCGAGATCTCCATACCCGCTTAGCTGTTGGAGGACCGgtaagcccaagccctggccccagaaactatgtgctgata 2040
 euProSerGlySerIleTyrSerLeuGluAlaAsnLysValValGluIleSerIleProAlaLeuAlaValGlyGlyPro INS XV
 actaccgcgctgaacagCATCCTTTCCATCTTACGGAGtgtagtaagcaatgctcacaattctctacaacagctgatccatcgtatagCACAGTTCGACGTCATCAGGAGTGCGG 2160
 HisProPheHisLeuHisGly INS XVI HisThrPheAspValIleArgSerAlaG 410
 GCTTACTACGTATAACTTCGACACCCCTGCGCGACGCGATGTTGTCAACACTGGAACCTGACGCGAACGACAACTTACTATCCGCTTGTGACGGATAATCCAGGCCCATGGTTCCTCC 2280
 lySerThrThrTyrAsnPheAspThrProAlaArgArgAspValValAsnThrGlyThrAspAlaAsnAspAsnValThrIleArgPheValThrAspAsnGlyProTrpPheLeuH
 ACTGgtaggcatttgacgcaattcttgcgacagcagtaaacagcttccctagCCACATTGACTGGCATCTCGAAATgttaggtggcattctttattgattcaattactcgactcaa 2400
 isCy INS XVII sHisIleAspTrpHisLeuGluI INS XVIII 460
 aggcatttagCGTCTTGGGTCGTTTTTCGCCGAAGATGTGACGTCGATCAGGCCCCACCTGgtacgctcttctgtattttaccgcagcttgtgttctgtagctgacettcatttc 2520
 eGlyLeuAlaValValPheAlaGluAspValThrSerIleThrAlaProProA INS XIX 470

Figure 2. Cont.

tccagCCGCGTGGGACGACTTGTTGTCGGATTTATGATGCTTTGAGCGATTCCGACAAAGGTGGCATAGCTTAGGATATCTCTACCTACTTACAGAGATAAACTCGAAAGAAATAGAACCA 2640
 1aAlaTrpAspAspLeuCysProIleTyrAspAlaLeuSerAspSerAspLysGlyGlyIleAlaEnd
 480 490 500
TCTGCATTCCAACAA 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 ATGTTTCCAGGCGCACGGATTCTCGCTACGCTTACATTAGCTCTTCACCTTTTACATGGGACTCATGCTGCCATTGGGCCACTGGCGACATGTACATCG 100
Pox1-234847 ----- 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 TCAACGAGGACGCTCTCTCTGACGGCTTCACTCGTTCGGCTGTCGTCGCTCGCTCGCTGACCCACCACAAATGGGACGTCGGAGACGC TTACCG 194
Pox1-234847 ----- 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 TGTCCTCGTGCAAGGAAACAAGGGCGACAACCTCCAGCTGAACGTTCTCAATCAACTGTCGGACACGACTATGTTGAAGACCACAGTATCCATTGGGCAT 294
Pox1-234847 ----- 294
Lacc-AY485827 C-----C-----T----- 294
Lacc-AY450404 C--T---T---T---T---T---CG---T---C---G---C---C 300
Box-AB020026 C--T---T---T---T---T---CG---T---C---G---C---C 300
Pox2-AB474261 C--T---T---T---T---T---C---T---G---T---A---G---G---C 300

P. ostreatus-Pox1 GGCTTCTTTCAATCCGGTTCTACGTGGGCAGATGGACCCGGTTCGTGAATCAATGCCCATCGCCTCGGGGAACAGCTTCTATATGACTTCAACGTCC 394
Pox1-234847 ----- 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 CCGACCAAGCTGGCAGCTTCTGGTACCATTGCATCTTTCCACCCAGTATTGTGATGGTCTTAGAGGACCATTATAGTATACGACCCCTCCGATCCCCA 494
Pox1-234847 ----- 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 CCTGTCCTTGATGACGTTGACAACGCCGACACCATCATTACACTTGAAGATTGGTACCATGTTGTGGCCCTCAGAAATGCAGTGTCTTCTACTGCTGAT 594
Pox1-234847 ----- 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 AGTACACTCATCAATGGCAAAGGTCGCTTCGCTGGGGGCGCTACTTCCGCTTTGGCCGTCATCAACGTCGAAAGCAACAAGCGATATCGTTTCAGACTTA 694
Pox1-234847 ----- 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 TCTCGATGCTTGGCACCACAATTACAGTTCTCGATCGAGGTCACCTTTGCAGGTCATCGAGGCAGACGCTGCAATATTGTGCCATTGTCTGCTGGA 794
Pox1-234847 ----- 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 TAGTATTCAAATCTTCGACGGCCAACGCTATTCCTTCGCTTGAATGCCAATCAGACTGTGCACAATTACTGGATTTCGCGCAGATCCCAACTTGGGATCG 894
Pox1-234847 ----- 894
Lacc-AY485827 -----T----- 894
Lacc-AY450404 -----G-----T-----C-----G-----C---T---G-----GA---T----- 900
Box-AB020026 -----G-----T-----C-----TG---A---C---T---G---C-----GA---T----- 900
Pox2-AB474261 -----G-----T-----C-----T-----AG---C---T-----C-----G---GA---C---T-----A 900

P. ostreatus-Pox1 ACTGGCTTCGATGGTGGTATCAATTCCGCTATCCTTCGGTATGCTGGTGCCTGAAGATGACCTACCACGACTTCGTCGACGAGTACCCCCCTTGAGG 994
Pox1-234847 ----- 994
Lacc-AY485827 ---C-----A----- 994
Lacc-AY450404 ---C-----C-----T-----A-----G-----T---A-----C-----AT---GCT-- 1000
Box-AB020026 ---C-----T-----T-----G-----C-----A---C-----C-----T---GCT-- 1000
Pox2-AB474261 ---C-----T-----T---C---T-----A-----G-----C-----A---A-----C-----G---GCT-- 1000

Figure 3. Cont.

<i>P. ostreatus-Pox1</i>	AGACTAATCTTGTGCCACTTGAAAATCCTGGTGCTCCTGGTCCAGCTGTCCCTGGAGGCGCAGACATCAACATCAATCTCGCTATGGCCCTTCGACGTTAC	1094
<i>Pox1-234847</i>	-----	1094
<i>Lacc-AY485827</i>	-----C-----T-C-----	1094
<i>Lacc-AY450404</i>	---C-C---T-G---G---C-----C--A-C-A---T-A-----T-C---	1100
<i>Box-AB020026</i>	---C-C---T-G---G-----C-----C-G---T-A-----T-C---	1100
<i>Pox2-AB474261</i>	---C-C-C-T-G---G-----C--A-C---C---G---T-A-C--T-----T-C---	1100
<i>P. ostreatus-Pox1</i>	TAACITTTGAACTGACCATCAACGGCTCCCCCTTCAAAGCGCCGACTGCTCCTGTTCTGCTCCAGATTCTGTGGGTGCCACAACGCGCCCTCACTTCTC	1194
<i>Pox1-234847</i>	-----	1194
<i>Lacc-AY485827</i>	-----	1194
<i>Lacc-AY450404</i>	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
<i>Box-AB020026</i>	---C--C--T--T-----GTT--T---TCC-A-C---C---C--T--T-A---C--A-A--T-CT---T---G-G-T	1200
<i>Pox2-AB474261</i>	A-C--C--TTA-----TGTT--T---CTTC-A-A---C---C--T-----T-A-A-GT-----T---G---T	1200
<i>P. ostreatus-Pox1</i>	CCTTCCGGCAGTATATACTCGCTAGAACGCCAACAAAGTTGTCGAGATCTCCATACCCGCCCTTAGCTGTTGGAGGACCGCATCCTTTCCATCTTCACGGAC	1294
<i>Pox1-234847</i>	-----	1294
<i>Lacc-AY485827</i>	--C-----	1294
<i>Lacc-AY450404</i>	---T--T-C--T--G---G--GC-T--T-G---T--A---G--G--T--GC-G---C-G---C---A---C---T-	1300
<i>Box-AB020026</i>	---T--T---T--G---G--GC-T--T-G---T--A---G--G--T--GC-G---C-G---C---A---C---C-	1300
<i>Pox2-AB474261</i>	---T--T-C--T--GA--G--G--T-----A---G--G--T--GC-G---G---C---A-----C-	1300
<i>P. ostreatus-Pox1</i>	ACACGTTTCGACGTCATCAGGAGTGC GGCTCTACTACGTATAACTTCGACACCCCTGCGCAGCGATGTTGTCAACACTGGAAGTACGCGGAACGACAA	1394
<i>Pox1-234847</i>	-----	1394
<i>Lacc-AY485827</i>	-----	1394
<i>Lacc-AY450404</i>	---C-----T--T-----T-C-----C---T---T---C---C--A-----C--G-----	1400
<i>Box-AB020026</i>	---C-----T--T-----T-C-----A-C---T---T---C---C--A-----C--G-----	1400
<i>Pox2-AB474261</i>	---T---T--T-----C--T---G---C---T-----C---C--C-----C--G-----	1400
<i>P. ostreatus-Pox1</i>	CGTTACTATCCGCTTTGTGACGGATAATCCAGGCCCATGGTTCCCTCCACTGCCACATTGACTGGCAITCGAAATCGGTCCTGCGGTCGTTTTCGCCGAA	1494
<i>Pox1-234847</i>	-----	1494
<i>Lacc-AY485827</i>	-----	1494
<i>Lacc-AY450404</i>	---C---T---C---C---C---G--G-----T-----T-----T--C--C--T-----	1500
<i>Box-AB020026</i>	---C---T---C---C---C---G--G-----T-----T-----T--C--C--T-----	1500
<i>Pox2-AB474261</i>	---C---T---C---C---C---G-----T-----T-----T--T--G--T-----	1500
<i>P. ostreatus-Pox1</i>	GATGTGACGTCGATCACGGCCCCACTGCCGCTGGGACGACTTGTGTCGGATTTATGATGCTTTGAGCGATTCCGACAAAGGTGGCATAGCTTAG	1590
<i>Pox1-234847</i>	-----	1593
<i>Lacc-AY485827</i>	-----	1593
<i>Lacc-AY450404</i>	---C---A-C--TT-----C--C--C---C--A-----CAA-----A---C-T-CC-TCCT	1600
<i>Box-AB020026</i>	---C---A-C--TT-----C--C--C---G-C-A-----CAA-----A---C-T-CC-TCCT	1600
<i>Pox2-AB474261</i>	---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 (*Pox1*-cDNA), phenol oxidase 1 (*Pox1*), laccase (*Lacc*), bilirubin oxidase (*Box*) and phenol oxidase 2 (*Pox2*) of *P. ostreatus*. The primers used for amplification are underlined. Alignment was done with ClustalW 2.0 software (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

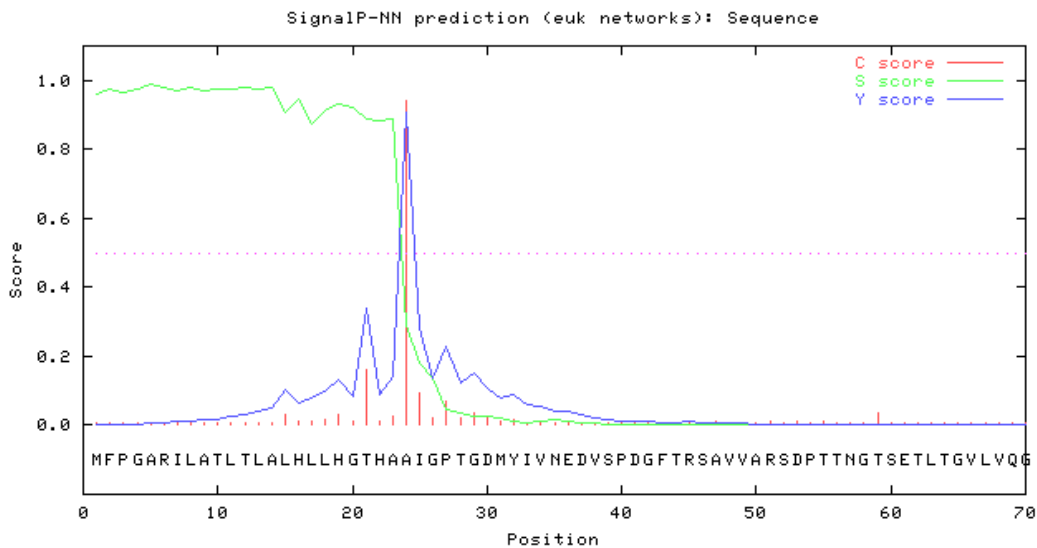


Figure 4. Determination of signal peptide sequence for the predicted amino acid sequence of POX1 from *P. ostreatus*

<i>P. ostreatus</i> -POX1	MFPGARILATLTLALHLLHGTHAAIGPTGDMYIVNEDVSPDGFTRSAVVARSDPTTNGT	59
POX1-Z34847	-----	59
LACC-AY485827	-----	59
LACC-AY450404	-----N-----A-----V-A-DP-P	60
BOX-AB020026	-----N-----A-----V-A-DP-P	60
POX2-AB474261	-----A-----A-N-----A-----V-A-DP-P	60
<i>P. ostreatus</i> -POX1	SETLTGVLVQGNKGNFQNLNVLNQLSDTTMLKTTSI <u>HWHGFFQ</u> SGSTWADGPAFVNQCP	118
POX1-Z34847	-----	118
LACC-AY485827	-----	118
LACC-AY450404	ATVSIP-----V-----A-S-----T---	120
BOX-AB020026	ATVSIP-----V-----A-S-----T---	120
POX2-AB474261	ATASIP-----V-----A-S-----T---	120
<i>P. ostreatus</i> -POX1	IASGNSFLYDFNVPDQAG <u>TFWYHSH</u> LSTQYCDGLRGPFIVYDPSDPHLSLYDVDNADTII	178
POX1-Z34847	-----	178
LACC-AY485827	-----	178
LACC-AY450404	V--D--N-----V--S-----I--V-	180
BOX-AB020026	V--D--N-----V-----I--V-	180
POX2-AB474261	V--D--N-----V-----I--V-	180
<i>P. ostreatus</i> -POX1	TLEDWYHVVPQNAVLPADSTLINGKGRFAGGPTSALAVINVESNKRYRFLRISMSCDP	238
POX1-Z34847	-----	238
LACC-AY485827	-----	238
LACC-AY450404	-----I-----AI--P-----Y-----P-S-----V-----	240
BOX-AB020026	-----I-----AI--P-----Y-----P-SI-----V-----	240
POX2-AB474261	-----I-----AI--P-----Y-----P--I-----V-----	240
<i>P. ostreatus</i> -POX1	NFTFSIDGHSLQVIEADAVNIVPIVVDSIQIFAGQRYSFVLNANQTVDNWIRADPNLGS	298
POX1-Z34847	-----	298
LACC-AY485827	-----	298
LACC-AY450404	-----L-----T-----T-D--G-----N-----	300
BOX-AB020026	-----L-----T-----T-D--G-----N-----	300
POX2-AB474261	-----L-----T-----T--A-----N-----	300
<i>P. ostreatus</i> -POX1	TGFDGGINSAILRYAGATEDDPTTTSSTSTPLEETNLVPLENPGAPGPAVPGGADININL	358
POX1-Z34847	-----	358
LACC-AY485827	----D-----P-----	358
LACC-AY450404	-----L-----T-----	360
BOX-AB020026	-----L-----	360
POX2-AB474261	--V-----L-----P-----	360
<i>P. ostreatus</i> -POX1	AMAFDVTNFELTINGSPFKAPTAPVLLQILSGATTAASLLPSGSIYSLEANKVVEISIPA	418
POX1-Z34847	-----	418
LACC-AY485827	-----S-----	418
LACC-AY450404	----F-T-----V--IP-----SS-----A--P-----M--	420
BOX-AB020026	----F-----V--IP-----SS-----A--P-----M--	420
POX2-AB474261	----F-T-----V--LP-----S-----E-----M--	420
<i>P. ostreatus</i> -POX1	LAVGGP <u>HPFHLHG</u> HTFDVIRSAGSTTYNFDTPARRDVVNTGTDANDNVITIRFVTDNPGPW	478
POX1-Z34847	-----	478
LACC-AY485827	-----	478
LACC-AY450404	-----G-----	480
BOX-AB020026	-----G-----	480
POX2-AB474261	-----G-----	480
<i>P. ostreatus</i> -POX1	FL <u>HCHIDWH</u> LEIGLAVVFAEDVTSITAPPAWDDLCPYDALSDSDKGGIA	529
POX1-Z34847	-----	529
LACC-AY485827	-----	529
LACC-AY450404	-----S-----N-----VPS	533
BOX-AB020026	-----S-----N-----VPS	533
POX2-AB474261	-----S-----N-----N-----VPS	533

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

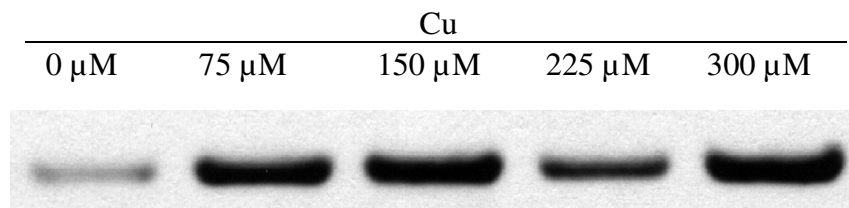


Figure 6. *P. ostreatus pox1* expression is up-regulated during copper supplementations. Total RNA was isolated from *P. ostreatus* following growth for 5 days during copper starvation (0 μ M) and different copper concentrations conditions and subjected to northern analysis.

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). Other laccase 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).

ACKNOWLEDGEMENT

The author thanks Prof. Dr. Hubertus Haas, Division of

Molecular Biology, Biocenter, Innsbruck Medical University, Innsbruck, Austria for providing facilities for this work.

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