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 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).
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 organism can be cultivated in a large-scale fermentation. The recombinant 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 protein is formed at high levels and the producing organism can be expressed under the control of a specific promoter (Cereghino and Cregg, 2000). The recombinant 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 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 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-

### 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 µg/ml of ampicillin. P. ostreatus (Jacq, NRRLO366) 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 CuSO₄.5H₂O 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’-GATCTGCGTCCATGACACAA-3’ and 5’-CTCTAGAGCCGACGTCG-3’. A 2656 bp fragment of pox1 gene was amplified from genomic DNA using synthetic oligos 5’-ATGTTCCAGCGCCACGG-3’ and 5’-TTTTGGATCATATG-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’-ATTGAATTCAATTTCCAGGCGACGG-3’ and 5’-TTTTGGATCATATG-3’ to create an EcoRI site (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 with ligated to pGEM-T easy vector (Promega) were also cleaved with EcoRI. The cloned gene was transformed inside E. coli DH5α cells (Strategene), 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
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.

**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 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*- AY450404) 98%, bilirubin oxidase (*Box*) 83% and phenol oxidase 2 (*pox2*) 83% (Table 1). The isolated genes 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-AY450404) 99%, bilirubin oxidase (*Box*) 83% and 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 supplements. *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*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Percentage homology</th>
<th>Nucleotide Accession no.</th>
</tr>
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<tbody>
<tr>
<td>Pox1</td>
<td>100</td>
<td>Z34847</td>
</tr>
<tr>
<td>Lacc</td>
<td>98</td>
<td>AY485827</td>
</tr>
<tr>
<td>Box</td>
<td>90</td>
<td>AB402026</td>
</tr>
<tr>
<td>Pox2</td>
<td>83</td>
<td>A5472462</td>
</tr>
</tbody>
</table>

**Note:** The cDNA sequence is compared with published sequences of *Pleurotus ostreatus* phenol oxidase 1 (pox1) gene (Accession no. AB514561) and other related genes from *P. ostreatus*.
Figure 2. Nucleotide sequence of the *P. ostreatus* pox1 gene and deduced amino acid sequence of POX1. Introns are shown in lowercase 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.

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<table>
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<tr>
<th>Nucleotide Sequence</th>
<th>Protein Sequence</th>
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<tbody>
<tr>
<td>tccagCGCGTGACGCTGCTCCTCCCTCTTGAATGGATATATGCTGGTGCCACTGAAGATGACCCTACCACGACTTCGTCGACGAGTACCCCCCTTGAGG</td>
<td>ATGGTTCCAGGCGCAGGAGAATGCAGTGCTTCCTACTGCTGATTGTTGCTTAGAGGACCATTCATAGTATACGACCCCTCCGATCCCCA</td>
</tr>
<tr>
<td>CCTGTCCTTGACGCTGCTCCTTCATCTGACTCACCTGACCATGTGTTGGCCCTCAGAATGCAGTGCTTCCTACTGCTGATTGTTGCTTAGAGGACCATTCATAGTATACGACCCCTCCGATCCCCA</td>
<td>CCGACCAACGTCGCTGCTGCTCCTCTCTGCAAGGGGACGACCATCATTACACTTGAAGATTGGTACCATGTTGTGGCCCCTCAGAATGCAGTGCTTCCTACTGCTGATTGTTGCTTAGAGGACCATTCATAGTATACGACCCCTCCGATCCCCA</td>
</tr>
</tbody>
</table>
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*.
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...
expression 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′-TGCRNC-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).

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REFERENCES


