

## Cloning and Characterization of a Laccase Gene from Biotechnologically Important Basidiomycete *Cerrena Unicolor*

Janusz, Grzegorz

Department of Biochemistry, Maria Curie-Skłodowska University

Mazur, Andrzej

Checinsks, Aleksandra

Department of Biochemistry, Maria Curie-Skłodowska University : Environmental Biotechnology  
Institute, Environmental Science Program, University of Idaho

Malek, Wanda

Department of Genetics and Microbiology, M. Curie-Skłodowska University

他

<https://doi.org/10.5109/22046>

---

出版情報 : 九州大学大学院農学研究院紀要. 57 (1), pp.41-49, 2012-02. Faculty of Agriculture,  
Kyushu University

バージョン :

権利関係 :

## Cloning and Characterization of a Laccase Gene from Biotechnologically Important Basidiomycete *Cerrena Unicolor*

Grzegorz JANUSZ<sup>1</sup>, Andrzej MAZUR<sup>2</sup>, Aleksandra CHECINSKS<sup>1,a</sup>, Wanda MALEK<sup>2</sup>, Jerzy ROGALSKI<sup>1</sup> and Shoji OHGA\*

Laboratory of Forest Resources Management, Division of Forest Environmental Sciences,  
Department of Agro–environmental Sciences, Kyushu University,  
Sasaguri, Fukuoka 811–2415, Japan

(Received October 31, 2011 and accepted November 9, 2011)

The laccase gene *lac1* and its corresponding full-length cDNA from white-rot fungi *Cerrena unicolor* with high yield of laccase production and potential use in electrochemical studies, was cloned and characterized. The 1533 bp full-length cDNA of *lac1* encoded a mature laccase protein containing 510 amino acids was preceded by a signal peptide of 20 amino acids. The deduced protein sequence of *lac1* shared similarity with other known fungal laccases and contained four copper-binding conservative domains of typical laccase protein. An inferred TATA box and several putative CAAT, MRE, XRE consensus sequences were identified in the *lac1* promoter region. A number of putative consensus sequences such as those binding AP1, AP2, creA and NIT2 transcription factors, involved in nitrogen and carbon regulation in different fungi, were also present in the promoter region of *Cerrena unicolor* laccase gene.

**Key words:** *Cerrena unicolor*, laccase, laccase gene promoter region

### INTRODUCTION

Filamentous fungi play an important role in biotechnology due to the production of low-molecular-weight compounds and enzymes (Mander *et al.*, 2006). The importance of cellulolytic, hemicellulolytic and lignolytic enzyme's complexes, that are dominant in the saprophytic mode of life of filamentous fungi, has been described in a considerable amount of literature. Along with other hydrolases, these enzymes find important applications in biotechnology and industry (Ng, 2004).

Over the last decades, laccases, the major enzymes in ligninolytic complex, have received much attention especially due to their ability to oxidize both phenolic and nonphenolic lignin related compounds as well as highly recalcitrant environmental pollutants. Such applications include: degradation or/and detoxification of industrial effluents, mostly from the pulp and paper, textile and petrochemical industries, medical diagnostic tools and as a bioremediation agents to clean up herbicides, pesticides and certain explosives in soil (Rodriguez-Couto and Toca-Herrera, 2006). However, expressing high levels of stable and functional proteins remains the bottleneck of many scientific and biotechnological endeavors, including the determination of structures in a high-throughput fashion, design, engineering and directed evolution of tailor-made proteins (Roodvelt *et al.*, 2005).

*Cerrena unicolor* was found by Leonowicz *et al.* (1997) as a new potential source of a unique laccase that produces the enzyme under non-induced conditions at the same rate as other superior laccase producers after the induction (Rogalski *et al.*, 1999). Latter experiments followed optimization of medium composition concerning carbon and nitrogen sources content as well as copper ion concentration and fermentor culture parameters leading to higher production of laccase (Janusz *et al.*, 2007). Several attempts of laccase purification from *Cerrena unicolor* resulted in different number of isoenzymes, ranging from two to four and were followed by complete enzyme characteristics (Kim *et al.*, 2002; Michniewicz *et al.*, 2006; Rogalski and Janusz, 2010). Laccase from *Cerrena unicolor* was found to be useful in degradation of lignosulfonate (Cho *et al.*, 2004), bio-scouring of linen fabrics (Sójka-Ledakowicz *et al.*, 2007), electrochemistry (Klis *et al.*, 2007; Shleev *et al.*, 2007; Nazaruk *et al.*, 2010), detection of mono- and diphenols (Jarosz-Willkolazka *et al.*, 2005), demethoxylation of milled wood lignin (Leonowicz *et al.*, 2000) and decolorization of aqueous solution of dyes (Michniewicz *et al.*, 2003). Despite many potential applications of laccases, genes encoding such enzymes in *Cerrena unicolor* have been poorly characterized. There are only two GenBank records concerning patented sequences and one more sequence from the unpublished data. Apart from those, there were no attempts to analyze *Cerrena unicolor* laccase gene nor the promoter region. In this study, we fully characterized *Cerrena unicolor* laccase gene and described a promoter region. We have also examined previous works on medium optimization enhancing laccase production and enzyme purification.

<sup>1</sup> Department of Biochemistry, Maria Curie-Skłodowska University, Akademicka 19 St., 20–033 Lublin, Poland

<sup>2</sup> Department of Genetics and Microbiology, M. Curie-Skłodowska University, Akademicka 19 St., 20–033 Lublin, Poland

<sup>a</sup> Present address: Environmental Biotechnology Institute, Environmental Science Program, University of Idaho, Moscow, Idaho, 83844

\* Corresponding author: (E-mail: ohga@forest.kyushu-u.ac.jp)

## MATERIAL AND METHODS

### DNA manipulation techniques

Standard techniques for plasmid isolation, restriction enzyme digestion, agarose gel electrophoresis and DNA cloning were employed (Sambrook *et al.*, 1989). Automatic Sequencing was performed using BigDye™ Terminator Cycle Sequencing Kit and ABI PRISM 310 sequencer or ABI PRISM 3730 XL (Applied Biosystems, Carlsband, CA, USA).

### Culture conditions

*Cerrena unicolor* strain C-139 was obtained from the culture collection of the Regensburg University. The fungus was maintained on 2% (w/v) malt agar slants. As an inoculum, pieces of agar were grown in Lindenberg and Holm medium (Lindeberg and Holm, 1952) in non-agitated conical flasks for 7 days at 28°C.

### Genomic DNA isolation

A culture of *C. unicolor* was grown stationary in Lindenberg and Holm medium (Lindeberg and Holm, 1952) at room temperature (25°C) for 7 days. Mycelia were harvested through Miracloth (Merck, Whitehouse Station, NJ, USA), washed twice with TE buffer, and frozen in liquid nitrogen. DNA was isolated according to Borges *et al.* (1990). The purity and quantity of the DNA samples were evaluated using ND-1000 spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA).

### Preparation of total mRNA

*Cerrena unicolor* was grown in 100-ml conical flasks containing 40 ml Lindenberg and Holm medium suitable for laccase production (Janusz *et al.*, 2007) at 25°C with shaking at 180 rpm. Synthesis of laccase mRNA was induced by adding 10 mM of CuSO<sub>4</sub> during the 3<sup>rd</sup> and 6<sup>th</sup> day as in Janusz *et al.*, 2007. Isolation of total RNA was performed on the 7<sup>th</sup> day using GenElute Direct mRNA Miniprep Kit (Sigma-Aldrich, Tokyo, Japan). The purity

and quantity of the RNA samples were evaluated using ND-1000 spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA).

### PCR amplification of ITS region

PCRs were performed using Sigma RedTaq in a Tpersonal thermal cycler (Biometra, Goettingen, Germany). To confirm the identity of the fungus, the ITS region in the nuclear ribosomal repeat unit was determined by direct sequencing of the PCR products amplified with ITS1-ITS2 and ITS3-ITS4 primers as described previously (White *et al.*, 1990; Gardes and Bruns, 1993).

### cDNA synthesis and amplification

Total mRNA (2.0 µg) and 80 pmol of the oligo(dT) primer were used to synthesize the first strand of complementary DNA (cDNA) with Transcriptor High Fidelity cDNA synthesis Kit (Roche, Sydney, Australia). To amplify laccase's cDNA, primers identical to the ones used for amplification of respective DNA fragments, were used (Table 1). All PCR amplifications were carried out using Sigma RedTaq in a Tpersonal thermocycler (Biometra, Goettingen, Germany). Specific PCR products were purified using the Clean-up kit (A&A Biotechnology, Gdynia, Poland) and inserted into pTZ57R/T vector from InstAclone kit (Fermentas, Glen Burnie, MA, USA). Clones with target fragments were analyzed by sequencing.

### Amplification and cloning of *lac1* gene

To amplify the laccase gene, pairs of primers were designed on the basis of the conservative sequences of other fungal laccases (Table 1). All PCR amplifications were carried out using Sigma RedTaq in a Tpersonal thermal cycler (Biometra, Goettingen, Germany). Specific PCR products were purified using the Clean-up kit (A&A Biotechnology, Gdynia, Poland) and inserted into pTZ57R/T vector from InstAclone kit (Fermentas, Glen Burnie, MA, USA). Clones with target fragments were analyzed by sequencing.

**Table 1.** Gene-specific primer sequences and their annealing temperatures

| Primer | Sequence 5' – 3'                     | Tm [°C]     |
|--------|--------------------------------------|-------------|
| LCR    | GTGGGSAAGGTRAACGTGCC                 | 55.9 – 57.9 |
| LCF    | GAYGGTTCCTGTACRACCTCCAKGT            | 56.0 – 61.0 |
| Fwzew  | CCCATCKTSTCTTGTATCCTGTGAAG           | 58.0 – 59.5 |
| Rwzew  | TAATAGAGTAKACACA KAWTGTAGACYTCAAAG   | 56.0 – 59.6 |
| cDNAFw | CCCCCTTCGGTTCCTGTT                   | 55.4        |
| cDNARw | TTGTGAGGATCTGAAGGGTCGTAG             | 57.4        |
| SDFP 4 | CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNCGCGT | 63.3 – 71.3 |
| ND     | CAGTTCAAGCTTGTCCAGGAATTC             | 55.7        |
| BSP    | CGTCGCCCCGTGATATAAAATGGTTCGTG        | 61.3        |
| NSP    | ATCGGGGGCAATGGTATCGT                 | 53.8        |

BSP, biotinylated specific primer; SDFP, semi-degenerate flanking primer; ND, nondegenerate; NSP, nested-specific primer; N – A, T, G or C

### Amplification and cloning of laccase promoter region using AFR-PCR

To amplify the laccase promoter region amplified flanking region-PCR (AFR-PCR) technique described by Soden and Dobson (2003) was used. Biotinylated specific primer was designed on the basis of laccase gene sequence (Table 1). All PCR amplifications and Dynabead-streptavidin purification were carried out according to Soden and Dobson (2003). Specific PCR products were purified using the Clean-up kit (A&A Biotechnology, Gdynia, Poland) and inserted into pTZ57R/T vector from InsTAclone kit (Fermentas, Glen Burnie, MA, USA). Clones with targeted fragments were analyzed by sequencing.

### Nucleotide sequence accession numbers

The following GenBank accession numbers were assigned to the nucleotide or amino acid sequences determined in this study (Table 2).

### Bioinformatic tools

Data from sequencing was analyzed with Lasergene v.8.0 analysis software (DNASTAR, Inc). Database searches were performed with the BLAST and FASTA programs at the National Centre for Biotechnology Information (Bethesda, MD, USA) and European Bioinformatic Institute (Hinxton, UK). The DNA and proteins sequence's multiple alignments were performed with Clustal-W algorithm (Thompson *et al.*, 1994). Phylogenetic trees visualization was performed using TreeView applet (Page, 1996). Glycosylation sites were detected with NetNGlyc v.1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

### N-terminal sequencing

The N-terminal amino acid sequence of the purified laccase was determined in Biocentrum in Cracow (Poland) by step-wise Edman degradation with Procise 491 protein Micro Sequencer (Applied Biosystems, Carlsband, CA, USA) equipped with a high performance liquid chromatography system for the on-line phenylthiohydantoin-amino acid identification. Proteins separated by SDS-PAGE were electroblotted into a polyvi-

nylidene difluoride membrane as in Walker (2002) and the excised bands were directly analyzed.

### Mass spectrometry (MS)

Protein samples obtained during enzyme purification were separated on SDS-PAGE gels in 10% Tris-HCl as in Walker (2002) and visualised by Coomassie Brilliant Blue R-250 staining according to Walker (2002). After the electrophoretic separation of samples, equal pieces of 2×7 mm were cut out from experiment gel lanes. The spectrometric analysis of the polypeptides was carried out in the Environmental Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw (Poland). The samples were analyzed by HPLC coupled with tandem mass spectrometry (liquid chromatography/two stage mass spectrometry - LC-MS/MS), according to Kordan *et al.* (2007). The output list of precursor and product ions was compared with the protein database of the National Center for Biotechnology (NCBI, USA), using the MASCOT local server.

## RESULTS

### PCR amplification of ITS region

Two products of 260 bp and 370 bp were obtained from PCR with ITS1-ITS2 and ITS3-ITS4 primers, respectively, and followed by direct sequencing. The complete sequences of these products (648 bp) indicated over 99 % identity to the *Cerrena unicolor* ITS sequences.

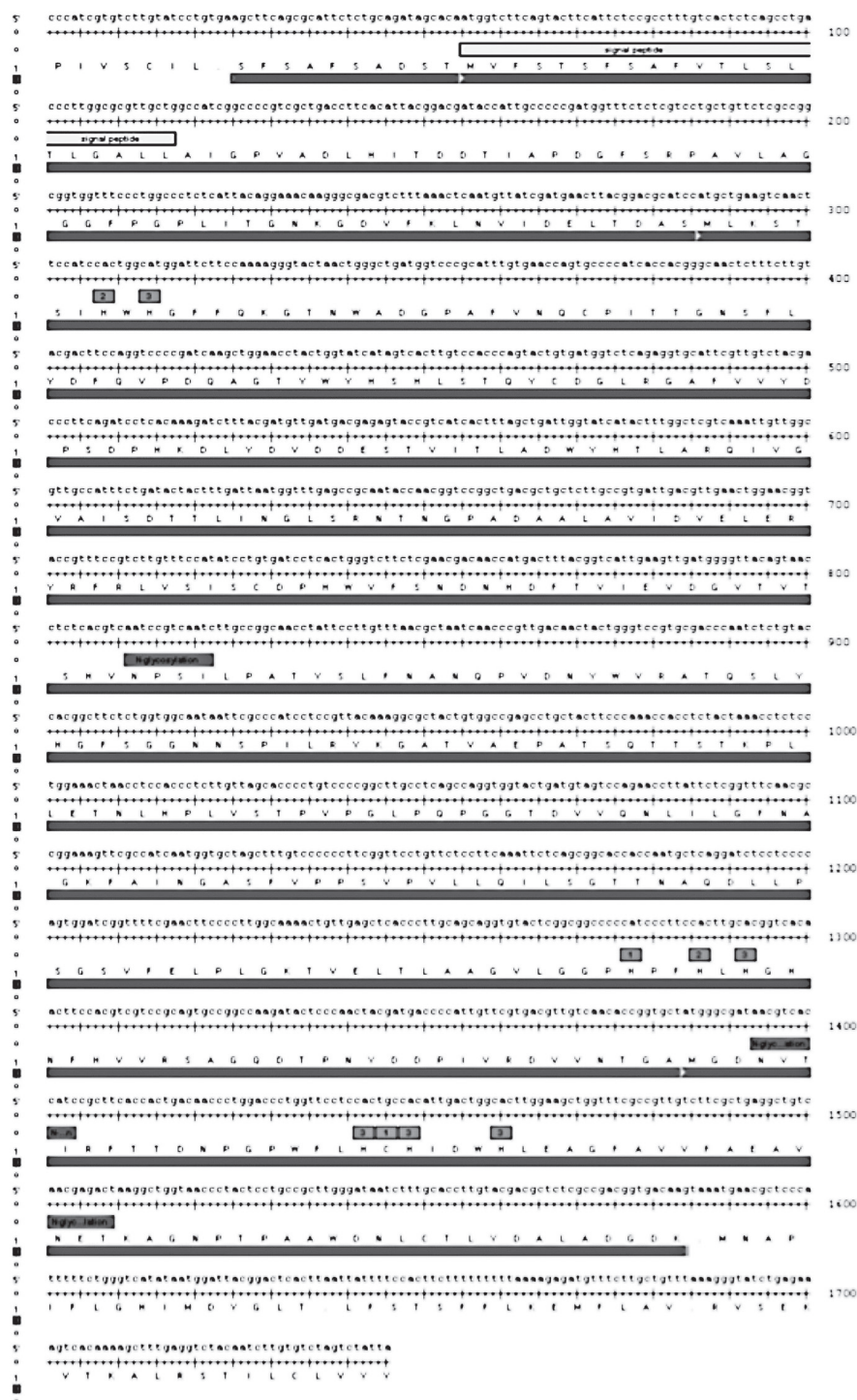
### Nucleotide sequence of *lac1* cDNA

Full length of the *lac1* cDNA from *Cerrena unicolor* was amplified, cloned and the sequence was determined. The analysis of obtained results revealed one open reading frame of 1533 bp with G-C content of 52.51% and 510 amino acids including a signal sequence within the first 20 amino acids (Fig. 1). The predicted molecular weight (mol. Wt) of investigated laccase was calculated for 54837 Da and had the isoelectric point of 4.69.

Three possible N-glycosylation sites (Asn-X-Thr/Ser) were estimated by the NetNGlyc v.1.0 software (Fig. 1). A potential N-glycosylation site was found at positions

**Table 2.** GenBank accession numbers of the nucleotide or amino acid sequences determined in this study

| GenBank accession number | Definition   |
|--------------------------|--|
| DQ056858                 | <i>Cerrena unicolor</i> voucher FCL 139 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence |
| FJ594064                 | <i>Cerrena unicolor</i> strain FCL139 Lac1 ( <i>lac1</i> ) mRNA, complete cds  |
| ACL93462                 | Lac1 ( <i>Cerrena unicolor</i> )   |
| P85308                   | <i>Cerrena unicolor</i> lac1b N-terminal sequence  |
| JF927721                 | <i>Cerrena unicolor</i> strain FCL139 lac1 gene and promoter region  |

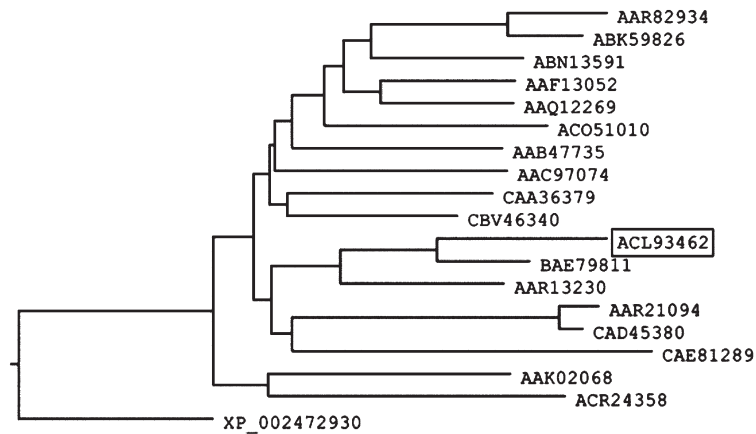


**Fig. 1.** Nucleotide sequence of a complete *lacI* ORF with the estimated protein sequence shown with single-letter symbols below their capitalized codon. The translated amino acids are shown with single-letter symbols below their capitalized codon. The signal peptide sequence has been boxed. Residues implicated in copper coordination are enclosed in single boxes and numbered depending on the type of copper that they coordinate. Hypothetical N-glycosylation sites are underlined.

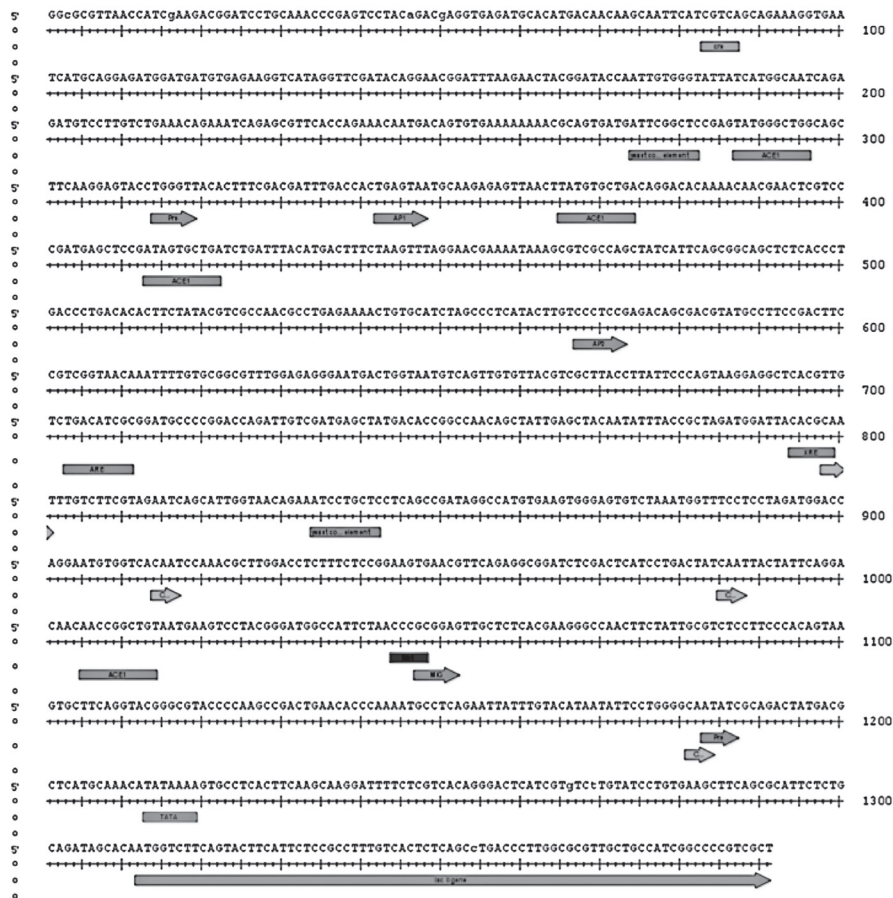
Asn-253, Asn-447 and Asn-483.

The  $\text{Cu}^{2+}$  ligands binding sites were found in all blue copper oxidases, consisting of conservative 10 histidines and 1 cysteine, were detected in *lacI* laccase in positions: H85, H87, H130, H132, H408, H411, H413, H463, H465, H469, and C464.

The dendrogram obtained from the alignments of 18 laccase amino acid sequences of *Polyporales* (Fig. 2), order to which *Cerrena unicolor* is assigned to, indicated that genes of these fungi are monophyletic including laccases from *Spongipellis sp.* and *Panus rudis*, which seem to be the closest to *C. unicolor*. However,



**Fig. 2.** Unrooted UPGMA based phylogenetic trees constructed with: 19 protein sequences. The dendrogram of several laccases from fungi of *Polyporales* generated by neighbour-joined method using the MegAlign (DNASTAR) program. AAR82934–*Ganoderma lucidum*, ABK59826–*Ganoderma fornicatum*, ABN13591–*Polyporus brumalis*, AAF13052–*Pycnoporus cinnabarinus*, AAQ12269–*Trametes* sp. I-62, ACO51010–*Pycnoporus sanguineus*, AAB47735–*Trametes villosa*, AAC97074–*Gelatoporia subvermispota*, CAA36379–*Phlebia radiata*, CBV46340–*Meripilus giganteus*, BAE79811–*Spongipellis* sp. FERM P-18171, AAR13230–*Panus rudis*, AAR21094–*Pleurotus ostreatus*, CAD45380–*Lentinus sajor-caju*, CAE81289–*Rigidoporus microporus*, AAK02068–*Coriolopsis galica*, ACR24358–*Polyporus gramocephalus*, XP\_002472930–*Postia placenta*.



**Fig. 3.** The nucleotide sequence of the promoter regions of the *Cerrena unicolor lac1* gene. Various response elements were identified as follows: putative (PRE, cAMP response element (creA), nitrogen response elements AP2, mammalian enhancer element (Sp1), carbon response element (Mig) and the putative antioxidant response element AP1. At the site around 900 bp upstream of the ATG, an element closely matching the ACE1 transcription factor consensus sequence was found.

all three sequences belong to the cluster that included laccases from *Pleurotus ostreatus*, *Lentinus (Pleurotus) sajor-caju* and *Rigidoporus microporus*.

### Nucleotide sequence of *lac1* genomic DNA

To precisely determine location of introns in the *lac1*, genomic DNA from *Cerrena unicolor* was amplified by PCR using primers designed based on the nucleotide sequence of the *lac1* cDNA as described in Materials and methods section. A fragment of 2341 bp was amplified by PCR and sequenced. The position of putative introns within the laccase gene was determined by comparison of the genomic DNA and cDNA sequences. Ten introns that ranged in size from 52 to 70 bp were found to be present inside the *lac1* gene and fall into the GT-AG rule (Padgett *et al.*, 1984).

### N-terminal peptide sequence analysis

*Cerrena unicolor* laccase protein was purified according to the previously described procedure (Rogalski and Janusz, 2010) and sequenced by Edman method. The first 9 amino acid residues at the N-terminus were AIGPVADLH (Accession number P85308.1). This sequence was identical to residues from 21 to 30 of the precursor protein encoded by *lac1* (Fig. 1) and implied that the protein undergoes posttranslational proteolytic cleavage at position 20.

### Analysis of putative promoters for consensus elements

The 1310 bp 5'-flanking sequence upstream of the start codon ATG in *lac1* gene was obtained by AFR-PCR and then analyzed for the presence of putative *cis*-acting elements involved in transcriptional regulation. The putative promoter region of *lac1* gene extending 1310 bp upstream of the start codon was shown in Fig. 3. The TATA box was located at positions -108 bp upstream from the start codon ATG and four CAAT boxes were located at -128, -324, -395, and -511 position, respectively. Additionally, some other putative response elements were also found in the promoter region (Fig. 3). A putative heat shock element (NGAANNITCN) (Bonner *et al.*, 1994) at -356, one putative metal-responsive element (MRE) adhering to the consensus sequence TGCRCNC (Thiele, 1992) was detected and centered at bp positions -251, cAMP response element was located at position -1225 (Soden and Dobson, 2003). One of putative xenobiotic-responsive elements (XREs) matching the consensus sequence CACGCW (Rushmore *et al.*, 1990) was located at position -513, four ACE elements adhering to the consensus sequence HWHNNGCTGD or NTNNHGCTGN at positions -298, -890, -938, -1016, respectively (Fan *et al.*, 2011). Two TGGGTT motifs were present at positions -993 bp, the motif ATATC occurring at position -125, which also occurs regularly in the upstream regions of laccase genes The mammalian

**Table 3.** Summary of matched peptide queries for laccase in lac1b band

| Primer | Sequence 5' - 3'                     | Tm [°C]     |
|--------|--------------------------------------|-------------|
| LCR    | GTGGGSAAGGTRAACGTGCC                 | 55.9 - 57.9 |
| LCF    | GAYGGTTCCTGTACRACCTCCAKGT            | 56.0 - 61.0 |
| Fwzew  | CCCATCKTSTCTTGTATCCTGTGAAG           | 58.0 - 59.5 |
| Rwzew  | TAATAGAGTAKACACA KAWTGTAGACYTCAAAG   | 56.0 - 59.6 |
| cDNAFw | CCCCCTTCGGTTCCTGTT                   | 55.4        |
| cDNARw | TTGTGAGGATCTGAAGGGTCGTAG             | 57.4        |
| SDFP 4 | CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNCGCGT | 63.3 - 71.3 |
| ND     | CAGTTCAAGCTTGTCCAGGAATTC             | 55.7        |
| BSP    | CGTCGCCCCGATATAAAATGGTCGTG           | 61.3        |
| NSP    | ATCGGGGGCAATGGTATCGT                 | 53.8        |

**1** MVFSTSFSAF VTLSLTLGAL LAIGPVADLH ITDDTIAPDG FSRPAVLGG  
**51** GFPGLITGN KGDVFKLNI DELTDASMLK STS IHWHGFF QKGTNWADGP  
**101** AFVNQCPI TT GNSFLYDFQV PDQAGTYWYH SHLSTQYCDG LRGAFVVYD  
**151** SDPHKDLVDV DDESTVI TLA DWYHTLARQI VGVAI SDTTL INGLSRNTNG  
**201** PADAALAVID VELERYRFR L VSISCDPHWV FSNDNHDFTV IEVDGVTVTS  
**251** HVNPSILPAT YSLFNANQPV DNYWVRATQS LYHGFSGGNN SPILRYK GAT  
**301** VAEPATSQTT STKPLLETNL HPLVSTPVPV LPQPGGTDVV QNLILGFNAG  
**351** KFAINGAS FV PPSVPVLLQI LSGTTNAQDL LPSGSVFELP LGKTVELTIA  
**401** AGVLGGPHPF HLHGHNFHV RSAGQDTPNY DDPIVRDVVN TGAMGDNVTI  
**451** RFTTDNPGPW FLHCHIDWHL EAGFAVFAE AVNETKAGNP TPAAWDNLCT  
**501** LYDALADGDK

**Fig. 4.** Complete sequence of laccase (ACL93462) with matched peptides in bold (sequence coverage: 18%).

Sp-1 transcription factor recognition site CCCGC was located at position -264 in the *lac1* gene promoter (Soden and Dobson, 2003) and antioxidant response elements (ARE) with the consensus sequence TGACNNGC at position -601 in *lac1* promoter together with putative AP1 at -964 and AP2 at -739 (Rushmore *et al.*, 1991; Soden and Dobson, 2003).

#### Analysis of Proteins by SDS-PAGE and LC-MS/MS

To identify laccase isoforms of *C. unicolor* determined by SDS-PAGE, gel slices were excised, digested with trypsin and analyzed by LC-MS spectrometry. The MS/MS raw data were used to search against NCBI protein database. A protein was identified when MASCOT probability-based score ( $p < 0.05$ ) was greater than 52. Laccase from *C. unicolor* (Accession no. ACL93462) was detected in each gel slice, with the highest score for the band *lac1b* (Rogalski and Janusz, 2010), that corresponds to the isoform sequence available in the protein database. The identified protein had (Table 3) MASCOT score of 756 and sequence coverage of 18% (Fig. 4). The molecular mass of 55,089 Da of the identified laccase was very close to the molecular mass determined by SDS-PAGE ( $54.49 \pm 3.38$ ) (Rogalski and Janusz, 2010).

## DISCUSSION

The purification and characterization of four laccase isoforms from *Cerrena unicolor* C-139 was already published by Rogalski and Janusz (2010). However, the laccase gene sequences of tested organism had not been reported yet. In our work, we demonstrate cloning of the *lac1* cDNA and promoter sequence that allows for a more complete and advanced examination of the laccase gene in this biotechnologically important fungus.

Taking into consideration *in silico* laccase characteristics as well as protein N-terminal sequence and LC-MS analysis we found that *lac1* gene seems to be coding for *lac1b*-type laccase described earlier by Rogalski and Janusz (2010). Deducing from the cDNA sequences, the calculated molecular weight of the mature protein of 510 amino acids - *lac1b* was 54.8 kDa. Despite considering glycosylation content (1.84%), the calculated and measured molecular weight slightly differed from each other in the range of 4.9 to 7.3% ( $\pm 1.84\%$ ) (Rogalski and Janusz 2010).

The active site of laccases contained four copper ions: a mononuclear "blue" copper ion (T1 site) and a three nuclear copper clusters (T2/T3 site) consisting of one T2 copper ion and two T3 copper ions (Morozowa *et al.*, 2007). It was earlier proved by an atomic absorption spectroscopy (AAS) method that the purified laccase contained four copper ions (Rogalski and Janusz, 2010). Here, we demonstrated using *in silico* analysis the presence in the laccase cDNA sequence, the exact motif of 10 histidines and 1 cysteine, which is conserved in fungal laccases.

The amino acid residue at 10 amino acids downstream to the conserved cysteine had an important effect on the redox potential of the type 1 copper at the active

site. Based on the amino acids differences of this residue, laccases were proposed to be classified into three types: class 1 (Met), 2 (Leu), and 3 (Phe). It was suggested that the presence of Phe at this position is necessary for a high redox potential in fungal laccases. Moreover, the Leu-Glu-Ala triad at positions +6 to +8 downstream to the conserved Cys was also considered to be important for a high redox potential (Canters and Gilardi 1993; Xu *et al.*, 1996; Eggert *et al.*, 1998). To our knowledge *Cerrena unicolor* laccase belongs to class 3 and it seems to be an enzyme of a high potential (Shleev *et al.*, 2007).

Ten introns ranging in size from 52 to 70 bp in the laccase gene with splicing junctions and internal lariat formation sites adhered to the GT-AG rule (Padgett *et al.*, 1984). *Lac1* gene seemed to be comparable with those of other fungal laccases, which were characterized by presence of various numbers of introns from 5 in *A. nidulans yA* up to 19 introns in *P. ostreatus* (strain Florida) *pox1* and *pox2* with the average length 69 bp in all fungal genes (Kinghorn and Turner, 1992; Okamoto *et al.*, 2003).

*Cerrena unicolor* was capable of producing constitutive form of laccase, i.e. without any extracellular inducers, which are used to stimulate many other fungal species (Rogalski *et al.*, 1999). Different patterns expression of laccase isozymes coded by gene families was regulated, depending on the growth conditions (Bollag and Leonowicz, 1984; Rogalski and Leonowicz, 1992) and physiological states of fungi (Mansur *et al.*, 1998; Rogalski *et al.*, 1993; Pezzella *et al.*, 2009). Many works have proved that white rot fungi laccase is dependent not only on carbon and nitrogen concentrations but also on copper ion ones (Collins *et al.*, 1997; Dittmer *et al.*, 1997; Palmieri *et al.*, 2000). Thus, it is probable that some specific transcriptional activation mechanisms are involved (Soden and Dobson, 2003).

Hence, in the effort to identify potential response elements involved in laccase gene regulation, the promoter region of *C. unicolor lac1* gene was cloned. We proved that production of laccases in *Cerrena unicolor* depends on carbon, nitrogen and copper ion concentrations in the growth medium (Janusz *et al.*, 2007). The analyses of *lac1* promoter sequence seemed to confirm our earlier observations. The regulatory elements such as Mig, CRE, NIT1 and NIT2, known to be involved in carbon and nitrogen regulation of fungal gene expression and ACE1 transcription factor responsible for laccase synthesis due to copper induction (Soden and Dobson 2001, Soden and Dobson, 2003), were found in the promoter of *lac1*. Moreover the analysis of *lac1* promoter sequence showed a metal response element which supports the results of cadmium induction described by Jarosz-Wilkolazka *et al.* (2006). MRE are typically found in metallothionein gene promoters in higher eukaryotes, suggesting that their expression could be induced by heavy metal ions such as Cd, Cu and Zn (Imbert *et al.*, 1990; Hamer, 1995, Soden and Dobson, 2003). Additionally, finding of HSE in *lac1* promoter confirmed that laccase production may be up scaled in response to



heat induction (Fink-Boots *et al.*, 1997). Our current analysis proved the observation of Rogalski *et al.*, (1999) indicated that the transcription of *Cerrena unicolor* laccase genes could be slightly activated by aromatic compounds (xylidine, ferulic acid and veratric acid). From our data, it is indicative that *lac1* promoter contain sequence with perfect homology to XRE.

According to our knowledge, there is yet no known explanation on the high production of laccase in *C. unicolor* without any additional inducers (Janusz *et al.*, 2007). Further studies concerning other *Cerrena unicolor* laccases genes, their promoters and the expression system would help understanding the constitutive nature of laccase production of this biotechnologically interesting fungus.

#### ACKNOWLEDGEMENTS

This work was partially financially supported by BS/Bioch/UMCS grant and research program “Tailored Lipidic Mesophases as Novel Functional Nanomaterials in Bioenergetics and Biosensing” under the framework of Polish–Swiss Research Programme PSPB–079/2010

#### REFERENCES

- Bollag, J.-M. and A. Leonowicz 1984 Comparative studies of extracellular fungal laccases. *App. Environ. Microbiol.*, **48**: 849–854
- Bonner, J. J., C. Ballou and D. L. Fackenthal 1994 Interactions between DNA-bound trimers of the yeast heat shock factor. *Mol. Cell. Biol.*, **14**: 501–508
- Borges, M. I., M. O. Azevedo, R. Bonatelli, M. S. S. Felipe and S. Astolfi-Filho 1990 A practical method for the preparation of total DNA from filamentous fungi. *Fungal Gen. Newslett.*, **10**: 11
- Canters, G. W. and G. Gilardi 1993 Engineering type 1 copper sites in proteins. *FEBS Lett.*, **325**: 39–48
- Cho, N.-S., W. Shin, S.-W. Jeong and A. Leonowicz 2004 Degradation of lignosulfonate by fungal laccase with low molecular mediators. *Bull. Korean Chem. Soc.*, **25**(10): 1551–1554
- Collins, P. J. and A. D. W. Dobson 1997 Regulation of laccase gene transcription in *Trametes versicolor*. *App. Environ. Microbiol.*, **63**: 3444–3450
- Dittmer, J. K., N. J. Patel and S. W. Dhawale 1997 Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. *FEMS Microbiol. Lett.*, **149**: 65–70
- Eggert, C., P. R. LaFayette, U. Temp, K. E. L. Eriksson and J. F. D. Dean 1998 Molecular analysis of a laccase gene from the white rot fungus *Pycnoporus cinnabarinus*. *App. Environ. Microbiol.*, **64**: 1766–1772
- Fan, F., R. Zhuo, S. Sun, X. Wan, M. Jiang, X. Zhang and Y. Yang 2011 Cloning and functional analysis of a new laccase gene from *Trametes* sp. 48424 which had the high yield of laccase and strong ability for decolorizing different dyes. *Bioresour. Technol.*, **102**: 3126–3137
- Fink-Boots, M., M. Jaszek and A. Leonowicz 1997 Heat Shock Stimulation of Laccase in *Abortiporus biennis* and *Cerrena unicolor*. *Second TAPPI Biological Sciences Symposium, San Francisco, October 19*, **23**: 395–396
- Gardes, M. and T. D. Bruns 1993 ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Mol. Ecol.*, **2**: 113–118
- Hamer, D. H. 1995 Metallothionein. *Annu. Rev. Biochem.*, **55**: 913–951
- Imbert, J., V. Culotta, P. Fürst, L. Gedamu and D. Hammer 1990 Regulation of metallothionein gene transcription by metals. In “Metal-ion Induced Regulation of Gene Expression”, Vol. 8., ed by G. L. Eichorn and L. G. Marzilli, Elsevier Science Publishing Inc, New York, pp. 139–164
- Janusz, G., J. Rogalski and J. Szczodrak 2007 Increased production of laccase by *Cerrena unicolor* in submerged liquid cultures. *World J. Microb. Biotechnol.*, **23**(10): 1459–1464
- Jarosz-Wilkolazka, A., M. Grz, B. Braha, S. Menge, D. Schlosser and G.-J. Krauss 2006 Species-specific Cd-stress response in the white rot basidiomycetes *Abortiporus biennis* and *Cerrena unicolor*. *BioMetals*, **19**: 39–49
- Jarosz-Wilkolazka, A., T. Ruzgas and L. Gorton 2005 Amperometric detection of mono- and diphenols at *Cerrena unicolor* laccase-modified graphite electrode: correlation between sensitivity and substrate structure. *Talanta*, **66**(5): 1219–1224
- Kim, Y., N.-S. Cho, T.-J. Eom and W. Shin 2002 Purification and characterization of a laccase from *Cerrena unicolor* and its reactivity in lignin degradation. *Bull. Korean Chem. Soc.*, **23**: 985–989
- Kinghorn, J. R. and G. Turner 1992 *Applied molecular genetics of filamentous fungi*. Blackie Academic & Professional, Glasgow
- Klis, M., E. Maicka, A. Michota, J. Bukowska, S. Sek, J. Rogalski and R. Bilewicz 2007 Electroreduction of laccase covalently bound to organothioliol monolayers on gold electrodes. *Electrochimica Acta* **52**: 5591–5598
- Leonowicz, A., J. Rogalski, E. Malarczyk, K. Grzywnowicz, G. Ginalska, J. Lobarzewski, S. Ohga, N. Pashenova, S.S. Lee and N.-S. Cho 2000 Demethoxylation of milled wood lignin and lignin related compounds by laccase from white-rot fungus *Cerrena unicolor*. *J. Korean Wood Sci.*, **28**(4): 29–40
- Leonowicz, A., L. Gianfreda, J. Rogalski, M. Jaszek, J. Luterek, M. Wojtaś-Wasilewska, E. Malarczyk, A. Dawidowicz, M. Fink-Boots, G. Ginalska and N.-S. Cho 1997 Extracellular laccase from the selected, best enzyme producing white-rot fungus *Cerrena unicolor*. *Proc. Int. Sem. for Sci.*, Chungbuk Nat. Univ., Chengju, Korea, 15–34
- Lindeberg, G. and G. Holm 1952 Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some *Hymenomycetes*. *Physiol. Plant*, **5**: 100–114
- Mander, G. J., H. Wang, E. Bodie, J. Wagner, K. Vienken, C. Vinuesa, C. Foster, A. C. Leeder, G. Allen, V. Hamill, G. G. Janssen, N. Dunn-Coleman, M. Karos, H.G. Lemaire, T. Subkowski, C. Bollschweiler, G. Turner, B. Nüsslein and R. Fischer 2006 Use of laccase as a novel, versatile reporter system in filamentous fungi. *App. Environ. Microbiol.*, **72**(7): 5020–5026
- Mansur, M., T. Suárez and A. E. González 1998 Differential gene expression in the laccase gene family from basidiomycete I-62 (CECT 20197). *App. Environ. Microbiol.*, **64**: 771–774
- Mayer, A. M. and R. C. Staples 2002 Laccase: new functions for an old enzyme – a review. *Phytochem.*, **60**: 551–565
- Michniewicz, A., R. Ullrich, S. Ledakowicz and M. Hofrichter 2006 The white-rot fungus *Cerrena unicolor* strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. *App. Environ. Microbiol.*, **69**(6): 682–688
- Michniewicz, A., S. Ledakowicz, T. Jamroz, A. Jarosz-Wilkolazka and A. Leonowicz 2003 Decolorization of aqueous solution of dyes by the laccase complex from *Cerrena unicolor*. *Biotechnologia*, **4**: 194–204
- Morozova, O. V., G. P. Shumakovich, M. A. Gorbacheva, S. V. Shleev and S. V. Yaropolov 2007 “Blue” laccases. *Biochemistry (Moscow)* **72**(10):1136–1150
- Nazaruk, E., K. Sadowska, J. F. Biernat, J. Rogalski, G. Ginalska and R. Bilewicz 2010 Enzymatic electrodes nanostructured with functionalized carbon nanotubes for biofuel cell applications. *Anal. Bioanal. Chem.*, **398**: 1651–1660
- Ng, T. B. 2004 Peptides and proteins from fungi. *Peptides*, **25**: 1055–1073
- Okamoto, K., Y. Ito, I. Shigematsu, S. O. Yanagi and H. Yanase 2003 Cloning and characterization of a laccase gene from the white-rot basidiomycete *Pleurotus ostreatus*. *Mycoscience*, **44**: 11–17

- Padgett, R. A., M. M. Konarska, P. J. Grabowski, S. F. Hardy and P. A. Sharp 1984 Lariat RNAs as intermediates and products in the splicing of messenger RNA precursors. *Science*, **225**: 898–903
- Palmieri, G., P. Giardina, C. Bianco, B. Fontanella and G. Sannia 2000 Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. *App. Environ. Microbiol.*, **66**: 920–924
- Pezzella, C., F. Autore, P. Giardina, A. Piscitelli, G. Sannia and V. Faraco 2009 The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. *Curr. Gen.*, **55**: 45–57
- Rodríguez-Couto, S. and J. L. Toca-Herrera 2006 Industrial and biotechnological applications of laccases: A review. *Biotechnol. Advan.*, **24**: 500–513
- Rogalski, J., A. Dawidowicz, E. Józwik and A. Leonowicz 1999 Immobilization of laccase from *Cerrena unicolor* on controlled porosity glass. *J. Mol. Catal. B: Enzymatic*, **6**: 29–39
- Rogalski, J., A. Hatakka, B. Longa and M. Wojtas-Wasilewska 1993 Hemicellulolytic enzymes of the ligninolytic white-rot fungus *Phlebia radiata*—influence of phenolic compounds on the synthesis of hemicellulolytic enzymes. *Acta Biotechnol.*, **13**: 53–57
- Rogalski, J. and A. Leonowicz 1992 *Phlebia radiata* laccase forms induced by veratric acid and xyloidine in relation to lignin peroxidase and manganese-dependent peroxidase. *Acta Biotechnol.*, **12**: 213–221
- Rogalski, J. and G. Janusz 2010 Purification of extracellular laccase from *Cerrena unicolor*. *Prep. Biochem. Biotechnol.*, **40**: 242–255
- Rushmore, T. H., M. R. Morton and C. B. Pickett 1991 The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.*, **266**: 11632–11639
- Rushmore, T. H., R. G. King, K. E. Paulson and C. B. Pickett 1990 Regulation of glutathione S-transferase Ya subunit gene expression, identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. *Proc. Nat. Acad. Sci. USA*, **87**: 3826–3830
- Sambrook, J., E. F. Fritsch and T. Maniatis 1989 *Molecular cloning: a laboratory manual*, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory Press, New York
- Shleev, S., M. Klis, Y. Wang, J. Rogalski, R. Bilewicz and L. Gorton 2007 Comparative spectroelectrochemical studies of lyophilized and nonlyophilized laccases from *Cerrena unicolor* Basidiomycete. *Electroanalysis*, **19**(10): 1039–1047
- Soden, D. M. and A. D. W. Dobson 2001 Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. *Microbiology*, **147**: 1755–1763
- Soden, D. M. and A. D. W. Dobson 2003 The use of amplified flanking region-PCR in the isolation of laccase promoter sequences from the edible fungus *Pleurotus sajor-caju*. *J. App. Microbiol.*, **95**: 553–562
- Sójka-Ledakowicz, J., J. Lichawska-Olczyk, S. Ledakowicz and A. Michniewicz 2007 Bio-scouring of linen fabrics with laccase complex from *Cerrena unicolor*. *Fibres & Textiles in Eastern Europe* 15, No. 4(63): 86–89
- Thiele, D. J. 1992 Metal-regulated transcription in eukaryotes. *Nucl. Acids Res.*, **20**: 1183–1191
- Walker, J. M. 2002 *The protein protocols- Handbook*. 2<sup>nd</sup> ed, Humana Press Inc., Totowa
- White, T. J., T. Bruns, S. Lee and J. W. Taylor 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In “PCR Protocols: A Guide to Methods and Applications” ed. by M.A Innis, D. H. Gelfand, J. J. Sninsky and Whiteededs, Academic Press, Inc., New York. pp. 315–322
- Xu, F., W. Shin, S. H. Brown, J. A. Wahleithner, U. M. Sundaram and E. I. Solomon 1996 A study of a series of recombinant fungal laccase and bilirubin oxidase that exhibit significant differences in redoxpotential, substrate specificity, and stability. *Bioch. Biophys. Acta*, **1292**: 303–311