Expression of laccase IIIb from the white-rot fungus Trametes versicolor in the yeast Yarrowia lipolytica for environmental applications

Claude Jolivalt, Catherine Madzak, Agathe Brault, Eliane Caminade, Christian Malosse, Christian Mougin

Expression of laccase IIIb from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica* for environmental applications

Claude Jolivalt[1*], Catherine Madzak[2#], Agathe Brault[3], Eliane Caminade[3], Christian Malosse[3] and Christian Mougin[3]

[1] Laboratoire de Synthèse sélective organique et produits naturels, UMR CNRS 7573, ENSCP, 11, rue Pierre et Marie Curie, 75231 Paris Cedex 05, France.

[2] UMR Génétique Moléculaire et Cellulaire, INRA / CNRS / INA-PG, CBAI, 78850 Thiverval-Grignon, France.

[3] Unité de Phytopharmacie et Médiateurs Chimiques, INRA, route de Saint-Cyr, 78026 Versailles Cedex, France.

Abstract

Improvement of the catalytic properties of fungal laccases is a current challenge for an efficient bioremediation of natural media polluted by xenobiotics. We developed the heterologous expression of a laccase from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica*, as a first step for enzyme evolution. The full-length cDNA consisted of a 1561 bp open reading frame encoding lacIIIb, a 499 amino acids protein and a 21 amino acids signal peptide. Native and yeast secretion signals were used to direct the secretion of the enzyme, with the native signal yielding higher enzyme activity in the culture medium. Level of laccase activity secreted by the transformed yeast was similar to that observed for the non-induced wild-type strain of *T. versicolor*. The identity of the recombinant enzyme was checked by Western blot and MALDI-TOF analysis. Electrophoresis separation in native conditions indicates a molecular mass of the recombinant protein slightly higher (5 kDa) than that of the mature *T. versicolor* laccase IIIb, suggesting a limited excess of glycosylation. The laccase production level reached 2.5 mg/l (0.23U/ml), which is suitable for engineering purpose.

Keywords: multicopper oxidase, laccase, *Trametes versicolor*, recombinant enzyme, *Yarrowia lipolytica*

Introduction

Among the enzymatic systems potentially useful in biotransformation of some environmental organic pollutants, fungal laccases (EC 1.10.3.2) are of particular interest. They form a group of multicopper oxidoreductive enzymes involved in lignin transformation, morphogenesis, pathogenesis and fungal virulence, as well as in the oxidation of numerous xenobiotics. They are widely used in several processes of the dye, paper (pulp bleaching) and textile industries. A wide range of their substrates are recognized as pollutants of the environment. For example, we have shown that agrochemicals (herbicides and their transformation products: Jolivalt *et al*. 1999; Mougin *et al*. 2000) and polycyclic aromatic compounds (Mougin 2002) are substrates for purified laccases.

One objective of our research program is the design of laccases with: (1) a wider variety of substrates, (2) a pH activity profile optimized for natural media, and (3) a high efficiency in catalysis (higher redox potential), thus avoiding the need of redox mediators.

To benefit from an efficient system for heterologous laccase expression would be of special interest for generation and screening of site-directed mutants. However, ligninolytic enzymes are known to be generally difficult to over-express heterologously in active form (Jönsson *et al.* 1997). Fungal laccases are glycosylated enzymes whose sugar moieties are involved in the stabilisation process against proteolysis (Yoshitake *et al.* 1993). Consequently, it is necessary to express these enzymes in eukaryotic micro-organisms able to perform such post-translational modifications. Laccases genes have been expressed in fungi. For example, Hatamoto *et al.* (1999) described the cloning of a laccase from *Schizophyllum commune* and its expression in *Aspergillus sojae*. Alternatively, *Aspergillus oryzae* was used to express laccases from *Trametes villosa* (Yaver *et al.* 1996) and from *Coprinus cinereus* (Ducros *et al.* 1997). Recently, expression of fungal laccases in *Trichoderma reesei* (Kiiskinen *et al.* 2004) and in *Aspergillus niger* (Record *et al*. 2002) was reported with high laccase production levels. In *A. niger*, the characteristics of the recombinant enzyme, including glycosylation, were similar to those of the native laccase. However, the low transformation efficiency and

the ectopic integration mechanism from the *A. niger* expression system impair its use for the genetic engineering of recombinant laccases.

Yeasts are available as hosts for heterologous protein production because they combine a high capacity for growth, the easy manipulation of unicellular organisms, and a eukaryotic organization enabling post-translational modifications. *Coriolus hirsutus* laccase was the first laccase expressed in *Saccharomyces cerevisiae* (Kojima *et al.* 1990). The well studied *Pichia pastoris* was shown to be able to secrete active recombinant laccases from *Pycnoporus cinnabarinus* (Otterbein *et al.* 2000) and from *T. versicolor* (Jönsson *et al.* 1997); but, in both cases, the production remained low and the enzymes were hyper-glycosylated. Recently, Brown *et al*. (2002) reported the successful expression of a previously uncharacterized laccase isoform from *T. versicolor* in *P. pastoris*. In *S. cerevisiae*, Cassland and Jönsson (1999), and more recently Larsson *et al.* (2001), reported interesting works on the improvement of *T. versicolor* laccase expression by changing cultivation conditions, especially lowering the temperature.

Among a number of "non-conventional" yeasts available as hosts for heterologous protein production, a comparative study found *Yarrowia lipolytica* to be one of the most attractive one (Müller *et al.* 1998). This yeast is a dimorphic hemiascomycetous yeast with high secreting capacities (Barth and Gaillardin, 1996). A large range of genetic markers and molecular tools are now available in this yeast (reviewed in Madzak *et al.* 2004). We choose to use, for production of *T. versicolor* laccase, a *Y. lipolytica* strain designed to improve heterologous production, together with an expression/secretion integrative vector based on a strong hybrid promoter (hp4d promoter - Madzak *et al.* 2000). Two different secretion signals were used and compared: (1) the native laccase IIIb signal peptide and (2) the pre region from *Y. lipolytica XPR2* gene.

The present paper is the first report on heterologous expression of a *T. versicolor* laccase in *Y. lipolytica* as the preliminary step towards enzyme evolution for environmental applications.

Materials and Methods

Strains and transformation procedures

Escherichia coli DH5αF' (Life Technology) was used for construction and routine propagation of laccase expression vectors. *Yarrowia lipolytica* Po1g strain (Madzak *et al.* 2000) was used as host for heterologous laccase expression. Transformation procedure of

Po1g with the laccase-expressing vector was performed according to Xuan *et al*. (1988). The *Y. lipolytica* Po1t strain (Madzak, 2003), used as a non-producing control, corresponds to the Po1g strain transformed with pINA1269 vector (see hereafter).

Laccase cDNA cloning and sequencing

RNA was isolated from *T. versicolor* ATCC 32745 grown in conditions described in Jolivalt *et al*. (1999). Five days after addition of 2,5-xylidine, the mycelium was harvested, washed twice in sterile water, quickly frozen in liquid N_2 and stored at –80°C. Total RNA was extracted from frozen, powdered mycelia using the RNeasy plant Mini kit from Qiagen. cDNA was synthesized by reverse transcription from 1µg of *T. versicolor* total RNA using 5 mM oligo(dT) anchor primers, following the instructions of the manufacturer (Advantage RT kit , Clontech). The laccase ORF, with added bordering restriction sites, was produced by a subsequent PCR experiment using the obtained cDNA as a template according to the following procedure: 94°C 3 min + 94°C 30 s, 55°C 30 s, 72°C 1 min (10 cycles) + 94°C 30 s, 65°C 30 s, 72°C 1 min (20 cycles) + 72°C 7 min. PCR primers for gene amplification were designed from the sequence from *lcc2* gene (accession number Y18012, Cassland and Jönsson, 1999) encoding a *T. versicolor* laccase A with the same N-terminal sequence as lacIIIb from *T. versicolor* ATCC 32745 targeted in this work, with replacement of the nineteenth base after the ATG start codon by a thymine instead of a cytosine. The oligonucleotides were the following: 5'- TCTAGA**ATG**TCGAGGTTTCACTCTCTTTTCGC -3' (*Xba*I restriction site underligned, ATG codon from laccase ORF in bold type), and 5'- GGTACCGGATCC**TTA**CTGGTCGCTCGGGTCGCGCGCGTC -3' (*Kpn*I and *Bam*HI restriction sites underlined, stop codon from laccase ORF in bold type). The lacIIIb cDNA was sequenced in both directions by primer walking. The PCR fragment was inserted into the pGEM vector using a pGEM-T cloning kit (Promega), according to the specifications of the supplier. The construction was checked by sequencing (Genaxis).

Construction of laccase-expressing vectors and of yeast transformants

A blunt-*Bam*HI restriction fragment, carrying the laccase ORF, was obtained from the pGEM-laccase construction, purified by gel electrophoresis and inserted into the *Y. lipolytica* mono-integrative vector pINA1269 (Madzak *et al.* 2000), digested with the restriction endonucleases *Pml*I (blunt) and *Bam*HI. This allows transcriptional in frame fusion of the inserted laccase ORF with hp4d promoter. This construct constitutes the laccase-expressing vector p69TVLB. This plasmid uses the native laccase IIIb signal peptide to direct the

secretion of the recombinant protein. We took advantage of the construction of p69TVLB to perform more easily the insertion of the mature laccase sequence into the *Y. lipolytica* mono-integrative vector pINA1296, which carry the pre sequence from *Y. lipolytica XPR2* gene as a secretion signal. A DNA fragment carrying the 3' end of the mature laccase sequence, with an added bordering restriction site, was produced by PCR using the pGEM-laccase vector as a template, according to the procedure described above. The oligonucleotides used for the amplification were:

5'- CTTACGGCGGCCGTTCTGGCC**GGTATCGGTCCCGTCGCCGACC**-3' (*Sfi*I restriction site – compatible with that in *XPR2* pre sequence – underlined, mature laccase sequence – corresponding to amino acids GIGP… – in bold type), and 5'-GGCGCGAATCCAGTAGTTGTCGACGG -3' (located downstream from the unique *Sfi*I restriction site in the laccase sequence). The resulting PCR fragment was digested with *Sfi*I and ligated to (1) the 4373 bp *Sfi*I–*Pst*I DNA fragment from p69TVLB (carrying the 5' end of the laccase sequence, the terminator, the *LEU2* marker gene, and part of pBR322 sequence) and to (2) the 3657 bp *Pst*I–*Sfi*I DNA fragment from pINA1296 (carrying part of pBR322 sequence, the hp4d promoter, and the *XPR2* pre sequence), both purified by gel electrophoresis. The resulting construct is the laccase-expression vector p96preTVLB. Prior to yeast transformation, p69TVLB and p96preTVLB plasmids were linearized with *Not*I restriction endonuclease, in order to direct their integration at the pBR322 docking platform (integrated pBR322 sequence) of the *Y. lipolytica* Po1g recipient strain. The transformants were selected on YNB minimal medium plates, as previously described (Sherman *et al.* 1986).

Analysis of yeast transformants, **media and culture conditions**

The transformants obtained from Po1g strain using either p69TVLB or p96preTVLB vector were checked for active laccase production on modified PPB medium (Madzak *et al.* 2000): glucose (20 g/L), yeast extract (1.32 g/L), NH_4Cl (1.32 g/L), KH_2PO_4 (0.32 g/L), $MgSO_4$-$7H_2O$ (0.24 g/L), thiamine (0.33 mg/L), agar (15 g/L), supplemented with $CuSO_4$ (0.1 mM) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) (0.2 mM). Plates were incubated in the dark at 28°C for 3 days and checked for the development of a green colour.

For assaying laccase activity, selected positive clones were cultivated on modified PPB liquid medium described above, under vigorous shaking (180 rpm), in baffled 2 L Erlenmeyer flasks containing 200 mL medium, at 28°C. Modified PPB medium was buffered using 20 mM

Na_2HPO_4-citric acid buffer (pH 6.0). Inoculation was performed using one-day cultures to give an initial OD_{600} of 0.1. Yeasts were cultivated for at most 10-12 days.

Analysis of recombinant laccase from yeast culture supernatant

Western Blot analysis

Samples of crude supernatants were separated by SDS-PAGE (Mini protean II, Biorad). Detection of active laccase was performed by *in situ* oxidation of 1 mM guaïacol into a brown product in a phosphate/citrate buffer 20 mM, pH 5.

The second method was a classical immunodetection procedure. The proteins were electro-transferred from the gel to a nylon membrane using a Trans-blot semi-dry cell (Biorad). Detection of laccase on the membrane was performed using rabbit antibodies raised against purified laccase IIIb from *T. versicolor*, in combination with donkey anti-rabbit Ig conjugated with peroxidase.

MALDI-TOF analysis

Bands of interest were excised from the gel and destained by sequential washing with 25 mM ammonium bicarbonate and water/acetonitrile 1:1 (v/v). The sample was then reduced with dithiothreitol and alkyled with iodoacetamide according to the procedure described by Jensen *et al* (1999). Maldi-mass spectra were recorded with a Bruker Reflex III MALDI-TOF instrument in reflectron mode with 4-hydroxy-α-cyanocinnamic acid (10 mg/ml in 0.1% TFA/acetonitrile 2:1, (v/v)) as the matrix. An internal calibration using peptides from trypsin autoproteolysis was performed for each measurement.

Other analytical methods

Assays for laccase activity were assessed by measurement of enzymatic oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic)acid (ABTS) at 420 nm ($\varepsilon = 3.6 \times 10^4$ cm^{-1} M^{-1} for the oxidation product) or 2,6-dimethoxyphenol (2,6-DMP) at 468 nm ($\varepsilon = 4.96 \times 10^4$ cm^{-1} M^{-1} for the oxidation product). The reaction mixture contained 100 µL extracellular fluid and 900 µL 1 mM ABTS or 2mM 2,6-DMP in 0.1 M Na_2HPO_4/citric acid buffer (pH 3.0) at 30°C. The buffer solution was saturated with air by bubbling prior to the experiment. One unit of enzyme activity is defined as the amount of enzyme that oxidizes one µmole ABTS in one min.

Protein concentrations were calculated from absorbance measurements at 280 nm ($\varepsilon = 5.903 \times 10^4$ cm^{-1} M^{-1}) in purified laccase solutions or determined using the bicinchoninic acid (BCA)

assay (Pierce) according to the protocol of the manufacturer and using bovine serum albumin as a standard.

Dry weight of fungal and yeast biomass was determined by filtering a 10 mL broth sample through a 0.22µm Millipore filter, rinsing twice with 25 mL distilled water and drying at 100°C overnight.

Nucleotide sequence data

The sequence data for *T. versicolor* LacIIIb have been submitted to the NCBI Nucleotide Sequence Database under the accession number AF414109.

Results

A cDNA fragment was generated by reverse PCR using RNA extracted from *T. versicolor* ATCC 32745 mycelium cultivated in the presence of 2,5-xylidine. This 1561 open reading frame encodes a laccase of 499 amino-acid residues and a signal peptide of 21 amino-acids. The translation of the sequence allows the identification of a laccase A isoenzyme and shows total identity with lac IIIb, a laccase produced with the same *T. versicolor* strain and which structure was resolved from X-Ray diffraction data (Bertrand *et al*. 2002). The deduced protein has a predicted molecular mass of 53331 Da and an isoelectrical point of 4.6. The protein displays high homology to several laccase enzymes, with the highest degree of similarity (99.2% identity) with a *T. villosa* laccase (Yaver *et al.* 1996).

The laccase-expressing vectors p69TVLB and p96preTVLB were used to generate laccase-producing transformants of *Y. lipolytica*, which were detected by the development of a green colouration on ABTS-containing agar plates. For each vector, eighteen transformant clones have been tested. All p96preTVLB-derived transformants exhibited low laccase activity: these colonies appeared only very slightly coloured on ABTS-containing plates. Two p69TVLB-derived transformants exhibited no or very low laccase activity. These clones were probably due to marker gene conversion events and did not integrate correctly the laccase-expressing cassette: this kind of events is known to occur at a frequency around 10% during transformation of *Y. lipolytica* (Barth and Gaillardin, 1996). In contrast, sixteen p69TVLB-derived transformants exhibited an intense green colouration on ABTS-containing plates. Spectrophotometric assays performed with the liquid culture medium showed that laccase activities of the positive p69TVLB-derived transformants were in the same range, from 0.12 U/mL to 0.18 U/mL, after a seven day old culture.

Among the sixteen positive p69TVLB-derived transformants, the most efficient was retained for further studies. The integration of one single copy of p69TVLB plasmid, at the pBR322 docking platform of this transformant clone, was checked using PCR analysis (data not shown). In order to study the relationship between the production of laccase and the growth of the yeast culture, both extracellular laccase activity and cell mass were studied as a function of time course. Twenty hours after the beginning of the culture, the observed cells concentration was maximal whereas the pH was lowered from 6 to 2.5. In order to compensate for this pH lowering, pH was adjusted to 6 by adding NaOH or H_3PO_4 at 1.5 mol/L after each daily sampling. Extracellular activity was detected only from the third day and increased to reach 0.23 U/mL after six days of cultivation while cell density declined continuously during the same period (Figure 1). The time course variations of laccase extracellular activity indicate that enzyme production is detectable only at end of the yeast growth phase and then increases continuously for the next three days. This observation appears consistent with the growth-phase-dependence of hp4d promoter, which was shown to begin to be fully active at the end of the growing phase (Nicaud *et al*. 2002). However, measurements of laccase activity alone do not allow concluding clearly about protein expression pattern. Indeed, because of the absence of continuous pH regulation during the experimental course, pH decreased to 2.5, possibly leading to denaturation of the enzyme and consecutively to an underestimation of the laccase production levels.

The expression of laccase by transformed *Y. lipolytica* was further investigated by native SDS-PAGE (no heating of the protein sample prior to its loading on the gel) followed by an *in situ* detection of laccase activity by colouration with guaïacol (Figure 2, lane 7). A unique band around 52 kDa was detected. For comparison, *T. versicolor* laccase IIIb migrates in the same conditions (lanes 3 and 6) at around 47 kDa, which is not representative of its molecular mass (around 59 kDa, as determined by MALDI-TOF spectroscopy). Thus, in non-denaturing conditions, electrophoresis separation results indicate that the molecular mass of the recombinant protein is only slightly higher than that of the mature *T. versicolor* laccase IIIb. This slight difference, in the range of 5 kDa, can possibly be ascribed to a limited excess of glycosylation. Under denaturation conditions, electrophoretic separation restored the correlation between the mobility of the enzyme and its molecular weight, as indicated by a strong hybridization of the supernatant of p69TVLB-transformed *Y. lipolytica* with rabbit antibodies raised against *T. versicolor* laccase, revealing a single band at around 60 kDa (data not shown). The protein bands co-migrating, at around 60 kDa, with the antibody-hybridizing band, were analyzed using MALDI-TOF spectroscopy. This analysis revealed the presence of

6 peptides which can be assigned to the proteolytic digestion of laccase IIIb, strongly suggesting the presence of the target protein (Figure 3).

The recombinant laccase was partly purified (85-fold, 10 % yield) by a protocol similar to that used for the native enzyme (Bertrand *et al.* 2002) and some kinetic data were acquired. The pH activity profiles on 2,6-DMP (figure 4) were found to be very similar for both native lacIIIb and the recombinant protein. Bell shaped profile were observed, with a maximum at pH 3.6. Kinetic parameters were measured at this optimum pH (table 1) for 2,6-DMP and at pH 3 for ABTS. For both substrates, comparison between the two laccases showed that K_m values are in the same order of magnitude, K_m being 10 fold higher for 2,6-DMP than for ABTS. k_{cat} values are also in the same range for the native and the recombinant enzyme. However, due to a partial purification, the measured concentration of recombinant lacIIIb is likely to be overestimated, leading to an underestimation of corresponding k_{cat} values.

Discussion

The knowledge of the three-dimensional structure of lacIIIb from *T. versicolor* complexed with 2,5-xylidine highlighted some amino-acid residues potentially involved in interactions between the enzyme and its reducing substrate (Bertrand *et al.* 2002). Heterologous expression of laccase IIIb in a host suitable to perform directed mutagenesis experiments would thus be interesting to assess such information and tune these interactions. The preliminary step towards this aim was to get the gene encoding the targeted protein. This gene was cloned into *Y. lipolytica* using two different secretion signals: the native *T. versicolor* laccase signal peptide and the pre sequence from *Y. lipolytica XPR2* gene. Both secretion signals tested were able to direct the secretion of active recombinant laccase, the *T. versicolor* native signal peptide being however more efficient than the *Y. lipolytica* one.

Significant levels (0.23 U/ml) of laccase activity were measured in six-day old liquid cultures of p69TVLB transformed *Y. lipolytica* strain, showing that this yeast is actually able to express this complex metalloprotein in an active form. MALDI-TOF analysis was performed and established unambiguously the presence of laccase IIIb in the culture medium of the transformed yeast.

pH activity profiles and kinetic parameter measurements on both ABTS and 2,6-DMP showed that both native *T. versicolor* lacIIIb and recombinant lacIIIb exhibited rather comparable enzymatic properties, which makes *Y. lipolytica* recombinant laccase a relevant tool for further protein engineering studies.

Assuming that the specific activity of the laccase purified from the crude culture supernatant remained unchanged, the laccase production level from p69TVLB transformed *Y. lipolytica* can be estimated to 2.5 mg/l, which is suitable for protein engineering purpose. An interesting feature is that this laccase production level (0.23 U/ml) is very close and even slightly higher than that observed in control cultures of *T. versicolor* (Mougin *et al.* 2002), when no xenobiotics were added to induce the expression of laccases. In those non-inducing conditions, *T. versicolor* produces at least two constitutive families of laccases. However, it is likely that the expression level in *Y. lipolytica* can be enhanced further. In particular, growth of the recombinant yeast in a bioreactor with pH regulation facilities is expected to be an efficient mean to improve active laccase yield. Indeed, it is likely that growing the yeast to high cell density would enhance laccase production, while the concomitant lowering of the pH in the culture medium could be carefully controlled in order to prevent any enzyme denaturation. Several examples of heterologous proteins production in *Y. lipolytica* showed that the scale-up to batch or fed-batch cultivation could allow a ten-fold increase in production (reviewed in Madzak *et al.* 2004). Another mean to increase heterologous protein yield would be the use of multicopy vectors, generally enabling a ten-fold increase in production (Madzak *et al.* 2004). Thus, we consider that it should be possible to increase the expression level of recombinant lacIIIb**.** However, our main objective is not to maximise protein production but to design a heterologous expression system to enable directed evolution studies. From that point of view, *Y. lipolytica* expression system is of peculiar interest because it offers (1) a very high transformation efficiency (up to 10^6 transformants per µg – Xuan *et al.* 1988), and (2) a precise targeting of monocopy integration events (at the pBR322 docking platform) allowing to obtain transformants comparable in terms of copy number and integration locus. Moreover, yeasts also have the potential advantage, compared to filamentous fungi, that they produce laccases free from any contamination by other enzymes involved in lignocellular biodegradation.

In addition to its characteristics permitting the genetic engineering of laccases, *Y. lipolytica* also presents the interesting potential of being able to grow in soils. This yeast was shown to be able to overgrow bacteria and several rival yeast species during competition assays in sandy soil (Schmitz *et al.* 2000). Thus, the ecological characteristics of *Y. lipolytica* would contingently make possible the direct *in situ* use of strains producing engineered laccases for the bioremediation of polluted environments.

The results we obtained with the *Y. lipolytica* expression system open the way to the use of directed mutagenesis or *in vitro* evolution for the design of laccases adapted to fundamental mechanistic studies or environmental applications.

Acknowledgements

The authors wish to express their gratitude to Dr. Ludovic Otterbein for helpful discussions.

References

Barth G, Gaillardin C (1996) *Yarrowia lipolytica*. In: Wolf K (ed) Nonconventional yeasts in biotechnology, a handbook. Springer-Verlag, Berlin-Heidelberg, pp 313-388

Bertrand T, Jolivalt C, Briozzo P, Caminade E, Joly N, Madzak C, Mougin C (2002) Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. Biochemistry 41: 7325-7333

Brown MA, Zhao Z, Mauk AG (2002) Expression and characterization of a recombinant multi-copper oxidase: laccase IV from *Trametes versicolor*. Inorganica Chimica Acta 331: 232-238

Cassland P, Jönsson LJ (1999) Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. Appl Microbiol Biotechnol 52(3): 393-400

Ducros V, Davis GJ, Lawson DM, Wilson KS, Brow, SH, Ostergaard P, Pedersen AH, Schneider P, Yaver DS, Brzozowski AM (1997) Crystallisation and preliminary X-ray analysis of the laccase from *Coprinus cinereus*. Acta Cryst D D53: 605-607

Hatamoto O, Sekine H, Nakano E, Abe K (1999) Cloning and expression of a cDNA encoding the laccase from *Schizophyllum commune*. Biosci Biotechnol Biochem 63(1): 58-64

Jensen O, Wilm M, Schevschenko A, Mann M (1999). In: Link AJ (ed) Methods in Molecular Biology, volume 112, 2-D Proteome Analysis Protocols, Humana Press Inc. Totowa, NJ

Jolivalt C, Raynal A, Caminade E, Kokel B, Le Goffic F, Mougin C (1999) Transformation of *N',N'*-dimethyl-*N*-(hydroxyphenyl)ureas by laccases from the white rot fungus *Trametes versicolor*. Appl Microbiol Biotechnol 51: 676-681

Jönsson LJ, Saloheimo M, Penttilä M. (1997) Laccase from white–rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris*. Curr Genet 32: 425-430

Kojima Y, Tsukuda Y, Kawai Y, Tsukamoto A, Sugiura J, Sakaino M, Kita Y (1990) Cloning, sequence analysis and expression of ligninolytic polyphenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J Biol Chem 265: 15224-15230

Kiiskinen LL, Kruus K, Saloheimo M (2004) Proceedings of Oxizyme in Naples, 2nd european meeting, 3-5 June 2004

Larsson S, Cassland P, Jönsson LJ (2001) Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. Appl Environ Microbio 67(3): 1163-1170

Madzak C, Tréton B, Blanchin-Roland S (2000) Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. J Mol Microbiol Biotechnol 2: 207-216

Madzak C (2003) New tools for heterologous protein production in the yeast *Yarrowia lipolytica*. In: Pandalai SG (ed) Recent Research Developments in Microbiology, volume 7, Research Signpost, Trivandrum, pp 453-479

Madzak C, Gaillardin, C, Beckerich J-M (2004) Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. J Biotechnol 109/1-2: 63-81

Mougin C, Boyer F-D, Caminade E, Rama R (2000) Cleavage of the diketonitrile derivative of the herbicide isoxaflutole by extracellular fungal oxidases. J Agric Food Chem 48: 4529-4534

Mougin C (2002) Bioremediation and phytoremediation of industrial PAH-polluted soils. Polycycl Aromat Comp 22: 1-33

Mougin C, Kollmann A, Jolivalt C (2002) Enhanced production of laccase in the fungus *Trametes versicolor* by the addition of xenobiotics. Biotechnol Lett 24, 139-142

Müller S, Sandal T, Kamp-Hansen P, Dalboge H (1998) Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. Yeast 14: 1267-1283

Nicaud, J.-M., Madzak, C., van den Broek, P., Gysler, C., Duboc, P., Niederberger, P. and Gaillardin, C. (2002) Protein expression and secretion in the yeast *Yarrowia lipolytica*. *FEMS Yeast Research* **2**, 371-379.

Otterbein L, Record E, Longhi S, Asther M, Moukha S (2000) Molecular cloning of the cDNA encoding laccase from *Pycnoporus cinnabarinus* I-937 and expression in *Pichia pastoris*. Eur J Biochem 267: 1619-1625

Record E, Punt PJ, Chamkha M, Labat M, van den Hondel CAMJJ, Asther M (2002) Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme. Eur J Biochem 269(2): 602-609

Sherman F, Fink GR, Hicks J (1986) Methods in yeast genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schmitz C, Goebel I, Wagner S, Vomberg A, Klinner U (2000) Competition between n-alkane-assimilating yeasts and bacteria during colonization of sandy soil microcosms. Appl Microbiol Biotechnol 54(1): 126-132

Xuan JW, Fournier P, Gaillardin C (1988) Cloning of the *LYS5* gene encoding saccharopine dehydrogenase from the yeast *Yarrowia lipolytica* by target integration. Curr Genet 14: 15-21

Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K, Dalboge H (1996) Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl Environ Microbiol 62(3): 834-841

Yoshitake A, Katayama Y, Nakamura M, Iimura Y, Kawai S, Morohoshi N (1993) N-linked carbohydrate chains protect lacase III from proteolysis in *Coriolus versicolor*. J Gen Microbiol 139: 179-185

Figure 1. Biomass and levels of laccase activity (ABTS 1 mM as the substrate) produced by p69TVLB transformed *Y. lipolytica* expressing laccase IIIb. The transformant was grown in 250 mL of modified PPB medium at 28°C and aliquots of the culture supernatant were assayed for laccase activity on the indicated days. Empty circles: dry weight; black diamonds: laccase activity in the supernatant.

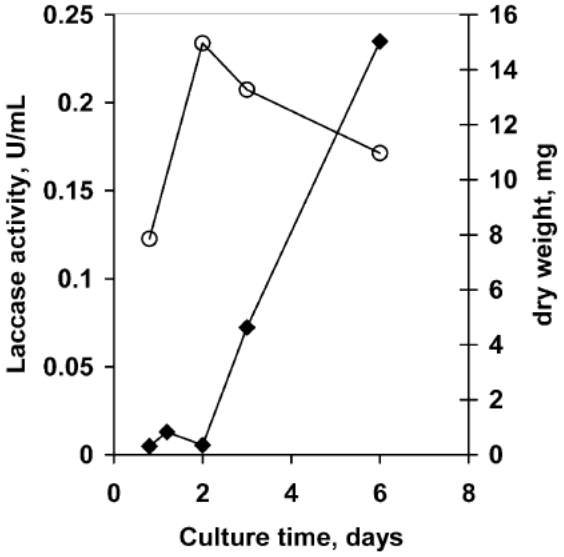

Figure 2. Electrophoresis analysis of recombinant laccase IIIb. Comparison with wild-type laccase IIIb from *T. versicolor*.

[A] Samples were boiled before loading on the gel. Lane 1 - Laccase IIIb from *T. versicolor*. Lane 2 - Concentrated crude cell free extract from p69TVLB transformed *Y. lipolytica* expressing laccase IIIb.

[B] Samples were not boiled before loading on the gel. Lanes 3 and 6 - Laccase IIIb from *T. versicolor*. Lanes 4 and 7 - Concentrated crude cell free extract from p69TVLB transformed *Y. lipolytica* expressing laccase IIIb. Lane 5 - Molecular weight markers.

Mass of molecular weight markers is indicated on the right side. The electrophoresis was carried out on an 11.5% gel. Lanes 1 to 5 were stained with Coomassie bleu (R250). Laccase activity was detected in lanes 6 and 7 by coloration with guaïacol.

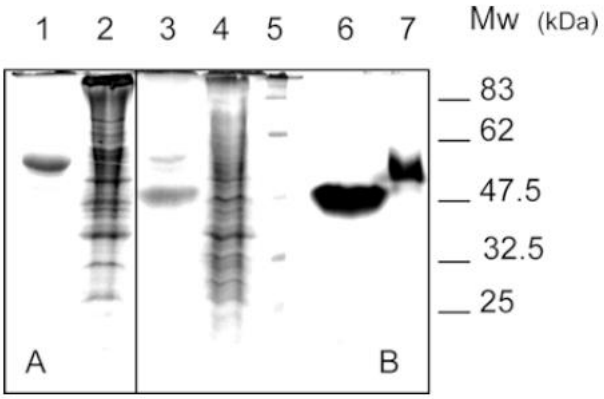

Figure 3. MALDI-TOF spectra

[A] Laccase IIIb from *T. versicolor*.

[B] Analysis of the band corresponding to the activity detected after electrophoresis of the crude supernatant from p69TVLB transformed *Y. lipolytica* expressing laccase IIIb.

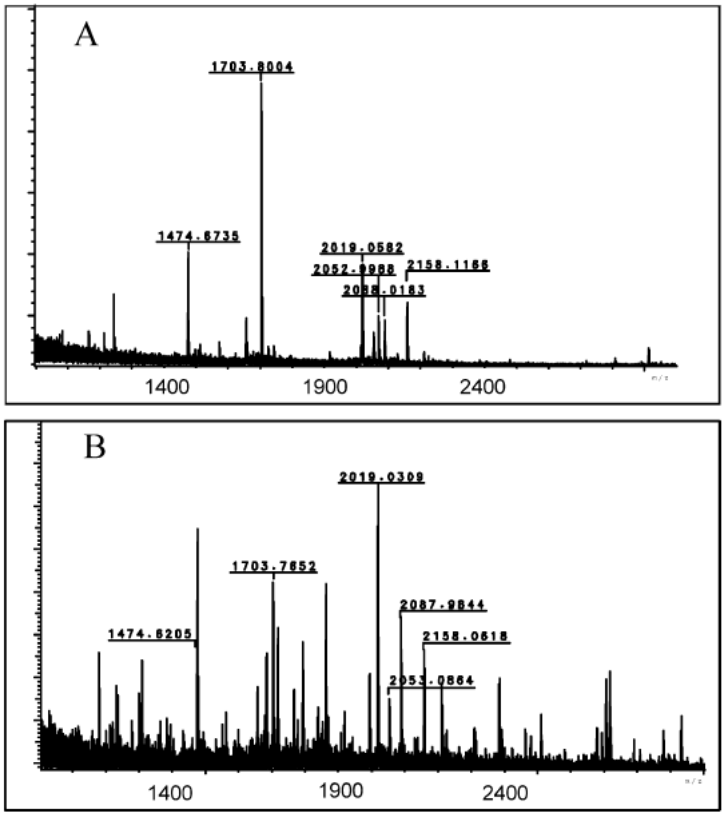

Figure 4. pH activity profiles of *T. versicolor* lacIIIb (empty circle) and recombinant *Y. lipolytica* laccase (empty square) in the presence of 2,6-dimethoxyphenol as the substrate (2mM in citrate/phosphate buffer 0.1 M) at 30°C.

Y-axis: activity, exprimed as the slope of the absorbance variation at 468 nm as a function of time

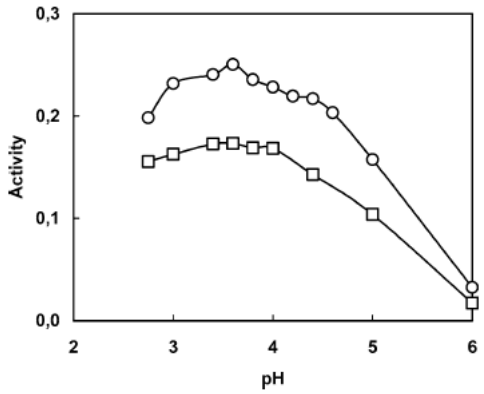

Table 1. Comparison of kinetic parameters of the native and the recombinant lacIIIb

	Substrate: ABTS pH 3		Substrate: 2,6-DMP pH 3.6	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
Native lacIIIb	50	107	275	54
Recombinant lacIIIb	26	64	235	17