Appl Microbiol Biotechnol (2005) 69: 178–183
DOI 10.1007/s00253-005-1985-3

Mei Guo · Fuping Lu · Jun Pu · Dongqing Bai · Lianxiang Du

Molecular cloning of the cDNA encoding laccase from *Trametes versicolor* and heterologous expression in *Pichia methanolica*

Received: 18 March 2005 / Revised: 18 March 2005 / Accepted: 31 March 2005 / Published online: 16 April 2005

Abstract A cDNA encoding for laccase was isolated from the ligninolytic fungus *Trametes versicolor* by RNA-PCR. The cDNA corresponds to the gene *Lcc1*, which encodes a laccase isoenzyme of 498 amino acid residues preceded by a 22-residue signal peptide. The Lcc1 cDNA was cloned into the vectors pMETA and pMETαA and expressed in *Pichia methanolica*. The laccase activity obtained with the *Saccharomyces cerevisiae* α-factor signal peptide was found to be twofold higher than that obtained with the native secretion signal peptide. The extracellular laccase activity in recombinants with the α-factor signal peptide was 9.79 U ml^{-1}. The presence of 0.2 mM copper was necessary for optimal activity of laccase. The expression level was favoured by lower cultivation temperature. The identity of the recombinant protein was further confirmed by immunodetection using Western blot analysis. As expected, the molecular mass of the mature laccase was 64.0 kDa, similar to that of the native form.

Introduction

Lignocellulose is used as a renewable energy source worldwide. White rot fungi are generally good producers of laccases, which are considered a part of their ligninlytic system (Hatakka 1994). Laccases belong to the group of enzymes called the blue copper proteins or blue copper oxidases (Messerschmidt 1993). Laccases are glycoproteins, usually monomeric, although some multimeric structures were described in *Podospora anserina* (Durrens 1981), *Agaricus bisporus* (Wood 1998), and *Trametes villosa* (Yaver et al. 1996). Laccase are heterogeneous in their biochemical properties and molecular structures. Generally, laccases can be characterized by a molecular mass around 60–80 kDa, a p*I* of 3–6, a glycosylation corresponding to 10–20% of the protein molecular mass, and the existence of 1–4 isozymes (Thurston 1994).

Trametes (*Coriolus, Polyporus*) *versicolor* is one of the best studied white rot fungi (Jönsson et al. 1997). Various strains of *T. versicolor* are known to produce laccase, MnP and LiP (Archibald 1992; Paice et al. 1993; Hatakka 1994). The most active of the enzymes from *T. versicolor* is laccase (Roy-Arecand and Archibald 1991). The predominant isomers in liquid cultures of *T. versicolor* are Lcc1 and Lcc2 (Fahraeus and Reinhammar 1967). Laccases are very interesting tools for industrial applications, i.e., for biological bleaching in pulp and paper industries, for detoxification of recalcitrant biochemicals, for bioconversion of chemicals, or for treatment of beverages in the agrochemical industry (Gianfreda et al. 1999).

The expression of active recombinant laccases has been reported in the filamentous fungus *Aspergillus oryzae* (Yaver et al. 1996), and the yeasts *Saccharomyces cerevisiae* (Kojima et al. 1990) and *Pichia pastoris* (Jönsson et al. 1997). In this paper, we describe the isolation and characterization of a cDNA corresponding to the *Lcc1* gene isolated from *T. versicolor* and its expression in *Pichia methanolica*. The effects of secretion signal peptide type and the activity of the heterologous laccase as a function of the copper concentration and culture temperature on the expression were investigated. An important feature in the suitability of *P. methanolica* as a host system for heterologous expression lies in the use of the highly efficient AUG1 gene promoter. The filamentous fungi are generally good hosts for protein secretion but are more time-consuming to work with than *P. methanolica*. Heterologous expression of laccases in

M. Guo
Department of Food Science,
Tianjin Agriculture College,
300384, People's Republic of China

F. Lu (✉) · J. Pu · L. Du
Key Laboratory of Industrial Microbiology,
College of Biotechnology,
Tianjin University of Science and Technology,
300222, People's Republic of China
e-mail: lu_fuping@yahoo.com.cn
Tel.: +86-22-60273281

D. Bai
Key Laboratory of Ecology and Aquaculture,
Department of Fishery Science,
Tianjin Agriculture College,
300384, People's Republic of China

the yeast *P. methanolica* would be of special interest in generating large amounts of enzyme.

Materials and methods

Strains, media and vectors

T. versicolor was from Chinese Academy of Sciences.

Escherichia coli strain JM109 and DH5α were used in all DNA manipulations. *E. coli* was grown in Luria–Bertani medium (tryptone 10 g l^{-1}, NaCl 10 g l^{-1}, yeast extract 5 g l^{-1}, agar 16 g l^{-1}).

The *P. methanolica* strain used for heterologous expression was pMAD16 (ade2–11 pep4▲; prb1▲). *P. methanolica* was grown in YPAD medium (yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, dextrose 20 g l^{-1}, adenine 0.1 g l^{-1}, agar 20 g l^{-1}).

The vectors pMETA and pMETαA (Invitrogen) were used for expression in *P. methanolica*. The expression of inserts in both vectors is controlled by the methanol-inducible AUG1 promoter. pMETαA possesses the α-secretion factor from *S. cerevisiae*, while pMETA does not contain a secretion signal.

Preparation of total RNA

RNA was isolated from *T. versicolor*. The fungus was grown in 250 ml baffled flasks containing 25 ml medium suitable for laccase (Lcc1) production at 25°C in a shaking incubator (150 rpm). After 13 days, mycelia were harvested. Isolation of total RNA was performed using TRIzol Reagent according to the manufacturer's instructions.

RNA-PCR

The first strand cDNA was synthesized from total RNA by RNA-PCR using the Bcabest RNA-PCR kit with oligo dT primers. The Lcc1 cDNA, including signal peptide of *T. versicolor*, was PCR-amplified using the upstream primer 5′-GCGAATTCATGGGCAGGTTCTCATCTCTCTG-3′ and the downstream primer 5′-TTGGATCCTTAGAGGTCGG ATGAGTCAAGAGC-3′. The PCR conditions employed were: 94°C 1 min (once) + 94°C 30 s, 51°C 30 s, 72°C 2 min (30 cycles) + 72°C 5 min (once). The Lcc1 cDNA without laccase signal peptide was PCR-amplified using the upstream primer 5′-GCGAATTCATGGGCAGGTTCTCATC TCTCTG-3′ and the downstream primer 5′-TTGGATCCT TAGAGGTCGGATGAGTCAAGAGC-3′. PCR was carried out under the same conditions as those described above. The PCR products were digested with *Eco*RI and *Bam*HI, cloned into the pUC19 vectors, and sequenced.

Cloning into *P. methanolica* expression vector

The vector pMETA, without the native *S. cerevisiae* α-factor secretion signal, was used for cloning the entire Lcc1 cDNA including its secretion signal sequence. The vector was digested with *Eco*RI and *Bam*HI. The resulting 7.8-kb fragment was purified and recovered from agarose gel electrophoresis using the Gel Extraction Kit (Shanghai Sangon). The recombinant pUC19 vector containing the entire Lcc1 cDNA was digested with *Eco*RI and *Bam*HI, and the 1.56-kb fragment obtained was purified as described above. This digested fragment was ligated with the 7.8-kb *Eco*RI/*Bam*HI fragment from pMETA using T4 DNA ligase overnight at 4°C.

E. coli strain JM109 was transformed with the ligation mixture and transformants were selectcted in Luria–Bertani medium plates containing ampicillin (100 μg ml^{-1}). Plasmids were extracted from randomly choosen transformant colonies, and the final construct pMETA/*Lcc1* was verified by restriction enzyme analysis and PCR.

The vector pMETαA with the native *S. cerevisiae* α-factor secretion signal was used for cloning Lcc1 cDNA without its native signal peptide. As in the case of pMETA/*Lcc1*, the vector pMETαA/*Lcc1* construction was carried out as described above. The laccase expression plasmids are displayed in Fig. 1. Sequence analysis was performed on pMETA/*Lcc1* and pMETαA/*Lcc1* to ensure the integrity of the constructs.

Yeast transformation and identification

Yeast transformation was performed as described by Choi and Kim (2002). pMETA/*Lcc1* and pMETαA/*Lcc1* plasmid DNA were digested with *Pst*I and *Asc*I, respectively, prior to transformation into electrocompetent cells of *P. methanolica*. The vectors pMETA and pMETαA without inserts were simultaneously transformed into *P. methanolica* and used as controls. Transformant colonies were picked from minimal glucose (MD) plates and transferred to plates with either MD or minimal methanol (MM) as the carbon source for examination of methanol utilization. Some integrants were checked for the presence of the expression cassette in their genome by PCR using primer specific for the Lcc1 cDNA.

Assay of laccase activity

Culture aliquots (1 ml) were collected daily and cells were removed by centrifugation (10,000*g* for 15 min). The laccase activity assay system contained 100 mM sodium acetate (pH 4.5), 0.5 mM ABTS (Sigma), and 100 μl culture supernatant appropriately diluted in a total volume of 1.0 ml. The reaction mixture was kept at 25°C for 5 min, then the change in absorbance at 420 nm was recorded with a spectrophotometer. One unit was defined as the amount of enzyme which oxidized 1 μmol ABTS per minute. The molar extinction coefficient for ABTS at 420 nm was taken to be 36,000 M^{-1} cm^{-1}.

The agar plate assay, which allowed the selection of transformants secreting laccase, was carried out on minimal methanol histidine plates supplemented with 0.2 mM

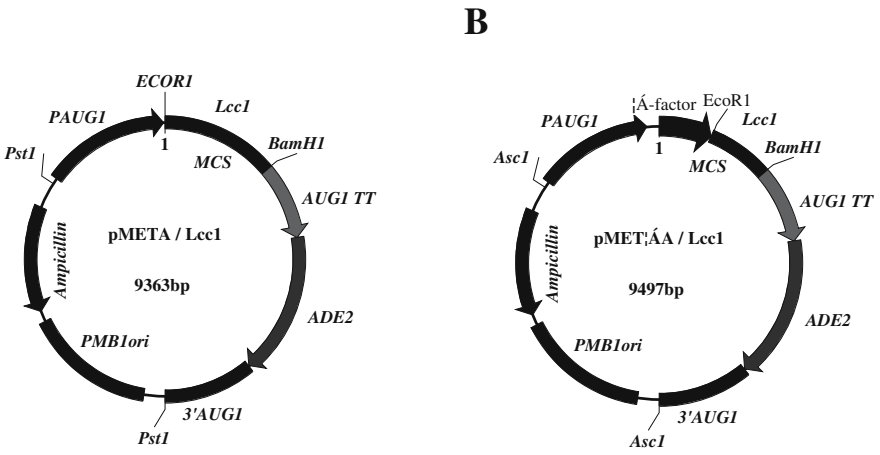

Fig. 1 Plasmids constructed for the expression of laccase in *P. methanolica*. The Lcc1 cDNA was cloned into either pMETαA (**a**) downstream of the native *S. cerevisiae* α-factor secretion signal or into pMETA (**b**) together with its own signal sequence

ABTS. The plates were incubated for 3 days at 30°C and checked for the development of a green colour.

Induction and expression of the foreign gene

The control and laccase-secreting transformants were inoculated into buffered dextrose complex medium (BMDY) (yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, pH 6.0, 100 mM potassium phosphate buffer, YNB 13.4 g l^{-1}, biotin 400 μg l^{-1}, dextrose 20 g l^{-1}) and incubated at 30°C in a shaking incubator (250 rpm). When the turbidity of the culture reached an optical density at 600 nm of ~4.0, the cells were harvested by centrifugation at 3,000*g* for 5 min. The cells were resuspended in buffered methanol complex medium (BMMY) (yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, pH 6.0, 100 mM potassium phosphate buffer, YNB 13.4 g l^{-1}, biotin 400 μg l^{-1}, methanol 5 ml l^{-1}). Flasks were incubated at 30°C, and 0.5% (v/v) methanol was added daily. Samples were taken at intervals for laccase activity. After performing the above experiments, the transformant with the highest laccase-secreting ability was selected for the following experiments. In order to examine the effect of copper on laccase activity, $CuSO_4$ was added at various concentrations. The effect of different cultivation temperatures on laccase expression was also investigated. Polyacrylamide (12%) gel electrophoresis in 0.1% SDS was carried out. Proteins were visualized after staining with Coomassie brilliant blue R-250 (0.1%). The molecular mass of the heterologous laccase was estimated by using the low molecular mass marker.

Western blot

P. methanolica transformants with the pMETαA/*Lcc1* plasmid and with the vector without insert were cultured in BMMY medium supplemented with 0.2 mM $CuSO_4$ at 20°C. After 3 days of growth, the cultures were harvested by centrifugation. The supernatant was collected and dialysed against distilled water for 6 h at 8°C. The samples were subsequently lyophilized, dissolved in loading buffer and separated by SDS-PAGE (Mini-Protean II, Bio-Rad, USA). The proteins were electrotransferred to a poly (vinylidene difluoride) membrane using a Trans-Blot semidry cell (Bio-Rad). Detection of laccase on the membrane was performed using antibodies from a rabbit immunized with laccase from *T. versicolor* in combination with goat anti-rabbit Ig conjugated with alkaline phosphatase (Bio-Rad).

Results

Cloning of the *T. versicolor* Lcc1 cDNA

The RT-PCR product, confirmed by agarose gel electrophoresis analysis, was subjected directly to cDNA sequence analysis. A 1,563-bp nucleotide sequence was obtained. The cDNA corresponds to the gene *Lcc1*, which encodes a laccase isoenzyme of 498 amino acid residues preceded by a 22-residue signal peptide.

Construction of the vectors for the expression of Lcc1 in *P. methanolica*

To study the expression of Lcc1 gene in *P. methanolica*, two distinct expression plasmids were used. Plasmid pMETA/*Lcc1* was composed of the inducible promoter AUG1, the native signal sequence of Lcc1 cDNA upstream of the open-reading frame for the mature laccase, and a termination transcription signal. The second plasmid, pMETαA/*Lcc1*, differs from the first in that it contains the *S. cerevisiae* α-factor secretion signal upstream of the Lcc1 cDNA sequence (Fig. 1). For each construct, ~50–70 transformants were obtained after selection of recombinants on MD plates.

Expression of Lcc1 in *P. methanolica*

Initially, transformants were assayed for activity on agar plates containing ABTS, giving a green zone around transformants secreting laccase. Coloured zones on assay plates

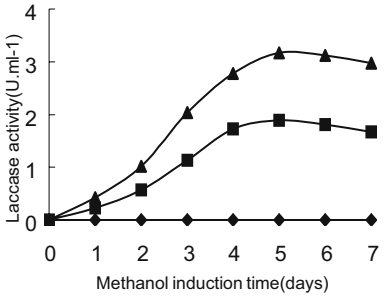

Fig. 2 Comparison of laccase expression using the native and the α-factor secretion signal in *P. methanolica*. Activity is indicated for the following constructions: vectors without laccase gene insertion (♦), vector with α-factor secretion signal sequence (▲), and native secretion signal sequence (■)

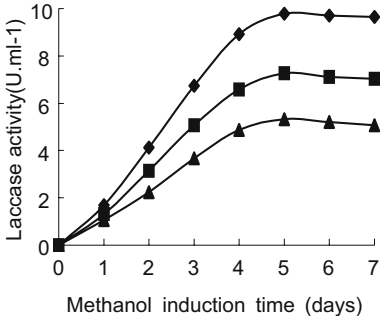

Fig. 4 The effect of temperature on laccase activity of the transformant pMETαA/*Lcc1* in BMMY medium supplemented with 0.2 mM $CuSO_4$. The temperatures evaluated were 20°C (♦), 25°C (■), and 30°C (▲)

were never observed for control transformants without the laccase gene. Subsequent experiments using liquid cultures also included controls with transformants generated using a vector without any insert. Activity was never detected in any of the control cultures. For both pMETA/*Lcc1* and pMETαA/*Lcc1* expression vectors, laccase activity was found in the supernatant of the culture medium, indicating that Lcc1 was secreted from yeast cells. For the pMETA/*Lcc1* construction, the laccase activity reached a maximum of 1.89 U ml^{-1} after 5 days. On the other hand, for the pMETαA/*Lcc1* expression vector, the laccase activity reached a maximum of 3.17 U ml^{-1} after 5 days (Fig. 2). The cell culture density reached an OD_{600} value of 12 after 5 days. The native signal peptide of Lcc1 cDNA was effective for directing both the secretion and proper proteolytic maturation of Lcc1 expressed in *P. methanolica*. In contrast, the use of the *S. cerevisiae* α-factor secretion signal peptide resulted in greater production of active Lcc1.

Effect of copper concentration on laccase activity

The transformant (pMETαA/*Lcc1*) with the highest laccase-secreting ability was selected for examining the effect of copper concentration on laccase activity (Fig. 3). If copper was absent in the medium, the laccase activity level was very low. With increasing copper concentration, the laccase activity was enhanced. A copper concentration of 0.2 mM produced the highest laccase activity (5.32 U ml^{-1}). When the copper concentration increased to 0.3 mM, the activity began to decrease slightly.

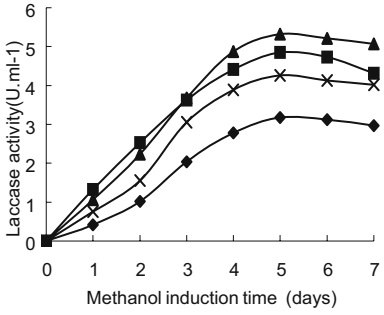

Fig. 3 Laccase activity level of transformant pMETαA/*Lcc1* incubated at 30°C in BMMY medium at different copper concentrations. The $CuSO_4$ concentrations evaluated were 0 mM (♦), 0.1 mM (■), 0.2 mM (▲), and 0.3 mM (×)

Effect of cultivation temperature on laccase expression

The transformant (pMETαA/*Lcc1*) was also used to study the effect of temperature on laccase expression (Fig. 4). Cultures were first grown at 30°C in BMDY medium containing 0.2 mM $CuSO_4$ and then transferred to BMMY medium containing 0.2 mM $CuSO_4$ at 20, 25, and 30°C, respectively. The culture supernatants were assayed daily for 7 consecutive days. The optimal secreting time of laccase was 5 days. The production of active enzyme is favoured by lower cultivation temperatures. Comparing the highest values during this period, the cultures kept at 20°C provided about twofold more activity than those kept at 30°C.

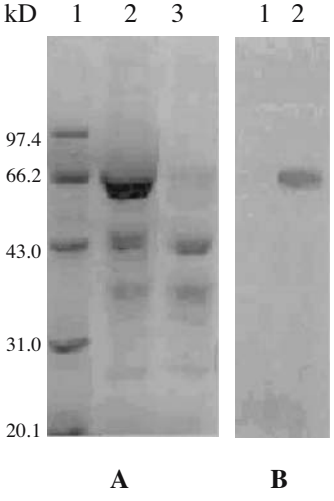

Fig. 5 SDS-PAGE gel (**a**) and Western blot (**b**) analyses of proteins secreted by *P. methanolica*. **a***Lane 1* Low molecular weight markers, *lane 2* pMETαA/*Lcc1*, *lane 3* pMETαA control. **b***Lane 1* pMETαA control, *lane 2* pMETαA/*Lcc1*

Western blot

Production of the recombinant laccase for the pMETαA/*Lcc1* was checked by electrophoresis on an SDS-PAGE (Fig. 5a). A clear band of around 64 kDa was observed, corresponding to the native form. The Western blot (Fig. 5b) analysis showed a unique band corresponding to the 64 kDa protein, demonstrating that this protein is the recombinant laccase.

Discussion

In this study, laccase was expressed using the recently developed yeast expression system *P. methanolica*. This system can utilize methanol as its sole carbon and energy source and has a number of desirable features. The yeast host strain is readily amenable to genetic manipulations. Auxotrophic mutant stains facilitate the introduction of protein expression cassettes and the generation of genetically stable expression host. Stability is maintained by the integration of the cassette into the host chromosome. The cultures readily adapt to high-biomass fermentation in a low-cost, defined medium. In addition, the yeast is able to introduce some eukaryotic-specific posttranslational modifications including glycosylation and disulfide bridge formation. *P. methanolica* secrete very low levels of endogenous proteins, resulting in easier recovery of an active product directly from the medium (Kjeldsen et al. 1999). The *P. methanolica* system employing the AUG1 methanol promoter is capable of producing yields of 2,500 mg of secreted protein per liter. Although the laccase expression level is 9.79 U ml^{-1} with the pMETαA expression vectors used, laccase production could be improved further by additional optimization. Selection for multiple integration events would be another way to enhance expression levels.

The activity of heterologous laccase had also been reported in *S. cerevisiae* and *P. pastoris*. In our study, the cDNA cloned from *T. versicolor* was expressed in *P. methanolica* PMAD16, and we detected the functional form of heterologous laccase in the cultures. In several cases, the native signal peptide of laccase has proved to be very useful for expression in *P. pastoris* (Jönsson et al. 1997; Liu et al. 2003). Unfortunately, replacing the native signal sequence of laccase with the α-factor, secretion signal sequence only resulted in twofold higher activity in our study. This might result, for example, from inefficient processing of the fusion protein or interference of the propeptide with the folding of laccase.

Laccases are copper-containing enzymes and contain two to four copper atoms per enzyme. A previous study in this laboratory demonstrated the importance of copper availability to the activity of laccase enzymes from *T. versicolor*. Copper ions did not influence the degree of protein synthesis, but the activity of the laccase was directly proportional to the concentration of copper in the growth medium (O'Callaghan et al. 2002). The level of laccase expression from the AUG1 promoter should be the same in all cases. The correlation between copper availability and laccase activity is probably due to the enzymes for copper requirement.

The temperature effect is of particular interest, since it has a marked effect on the production of active heterologous laccase. It has been previously reported that a lower cultivation temperature improves the production of heterologous protein in *E. coli* systems, which has been attributed to aggregation problems at higher temperatures (Schein and Noteborn 1988). *P. pastoris* is a widely used system for heterologous protein expression, and the finding that production can be improved by using lower temperatures is of particular interest (Cassland and Mnsson 1999). In the present study, we have thus identified the temperature as an important parameter for the optimization of the production of heterologous laccase in yeast systems, in general.

In conclusion, we have demonstrated several factors affecting the levels of activity obtained, such as signal peptide, copper concentration, and the cultivation temperature. We have also characterized the heterologous laccase by Western blot and found a heterogeneity similar to that expected, thus suggesting no hyperglycosylation. Recombinant laccase from *P. methanolica* will be used to test this enzyme for industrial applications. In this study, the medium has been used for heterologous expression of a laccase enzyme, but it is likely that this system could be used for the expression of other heterologous proteins in *P. methanolica*.

References

Archibald FS (1992) Lignin perixidase activity is not important in biological bleaching and delignification of unbleached kraft pulp by *Trametes versicolor*. Appl Environ Microbiol 58:3101–3109

Cassland P, Mnsson 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 52:393–400

Choi J, Kim EE (2002) Expression of the active human and duck hepatitis B virus polymerases in heterologous system of *Pichia methanolica*. Antivir Res 55:279–290

Durrens P (1981) The phenoloxidases of the ascomycete *Podospora anserina*: the three forms of the major laccase activity. Arch Microbiol 130:121–124

Fahraeus G, Reinhammar B (1967) Large scale production and purification of laccase from the fungus *Polyporus versicolor* and some properties of laccase. Acta Chem Scand 21:2367–2378

Gianfreda L, Xu F, Bollag JM (1999) Laccase: a useful group of oxidoreductive enzymes. Biorem J 3:1–25

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol Rev 13:125–135

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

Kjeldsen T, Pettersson AF, Hach M (1999) Secretory expression and characterization of insulin in *Pichia pastoris*. Biotechnol Appl Biochem 29:79–86

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

Liu W, Chao Y, Liu S, Bao H, Qian S (2003) Molecular cloning and characterization of a laccase gene from the basidiomycete *Fome lignosus* and expression in *Pichia pastoris*. Appl Microbiol Biotechnol 63:174–181

Messerschmidt A (1993) Blue copper oxidases. Adv Inorg Chem 40:121–185

O'Callaghan J, O'Brien MM, Dobson ADW (2002) Optimisation of the expression of *Trametes versicolor* laccase gene in *Pichia pastoris*. J Ind Microbiol Biotechnol 29:55–59

Paice MG, Reid ID, Bourbinnais R, Archibald FS, Jurasek L (1993) Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and delignifies kraft pulp. Appl Environ Microbiol 59:260–265

Roy-Arcand L, Archibald FS (1991) Direct dechlorination of chlorophenolic compounds by laccases from *Trametes* (*Coriolus*) *versicolor*. Enzyme Microb Technol 13:194–203

Schein CH, Noteborn MHM (1988) Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. Biotechnology 6:291–294

Thurston CF (1994) The structure and function of fungal laccase. Microbiologist 140:19–26

Wood DA (1998) Production, purification and properties of extracellular laccase of *Agaricus bisporus*. J Gen Microbiol 333: 2527–2534

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