Fungal Laccases
(Review)

V. K. GOCHEV[1] and A. I. KRASTANOV[2]
[1]Dep. "Biochemistry and Microbiology", "Paisiy Hilendarsky" University of Plovdiv,BG - 4000 Plovdiv, Bulgaria
[2] Dep. "Biotechnology", University of Food Technologies, BG - 4000 Plovdiv, Bulgaria

Abstract

GOCHEV, V. K. and A. I. KRASTANOV, 2007. Fungal laccases. *Bulg. J. Agric. Sci.,* 13: 75-83

The present review summarized the information for structure and mode of action of fungal laccases. A special attention was paid to distribution and physiological role of fungal laccases. Biotechnological producing of fungal laccases, especially influence of carbon and nitrogen sources, inductors, cultivation conditions, pH and temperature were discussed.

Key words: fungal laccases, structure, production

Introduction

Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes that catalyze one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water. Fungi are the main producers of laccase, especially Basidiomycetes. Laccases play an important role in lignification and delignification in nature. Due to their broad substrate specificity, which can be further extended to various recalcitrant non-phenolic compounds in the presence of mediators, laccases are widely used in many industrial processes and environmental bioremediations. Their commercial applications are found in the pulp and paper industry, biobleaching, biosensing and beverage refining. Laccases have been applied in removal of a large number of environmental pollutants, such as alkenes, chlorophenols, dyes, herbicides, polycyclic aromatic hydrocarbons and benzopyrene (Cuoto and Herrera, 2006; Gianfreda et al., 1999). For these reasons for the last decades laccases focused scientific attention. Laccase's structure, mode of action, mediator systems and enzyme properties have been reviewed many times (Duran et al., 2002, Messerschmidt, 1993; Solomon et al., 1996). The review of Cuoto

e-mail vgochev2000@yahoo.com

and Herrera (2006) summarized in a brilliant way the information of laccase applications.

The aim of present review is to gather the scientific information for structure, mode of action and mainly biotechnological producing of fungal laccases.

Structure and Classification

Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes that catalyze one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water, and whose active site is similar to that of ascorbatoxidase, ceruloplasmin and bilirubin oxidase. All fungal laccases are glycoproteins (Mayer & Staples, 2002). The catalytic site of laccase is quite conserved among different species, but the rest of the enzyme structure shows high diversity. Fungal laccases are mostly inducible, extracellular, monomeric glycoproteins with carbohydrate contents of 8%- 50% (Heinzkill et al., 1996) and are multinuclear enzymes (Gayazov and Rodakiewicz-Nowak, 1996). Typically the molecular mass of fungal laccases is from 60 to 80 kDa (Heinzkill et al., 1998; Leontievsky et al., 1997 and Thurston, 1994). However, atypical fungal laccases have also been reported; for example, a 390 kDa laccase of *Podospora anserine* (Thruston, 1994) while laccase of *Botrytis cinerea* was previously reported as small as 38 and 36 kDa. Laccases from the same fungal species were reported to contain 49-91 % reducing-sugar content (Slomczynski et al., 1995). The tetriery structure of laccase has been determined by X-rays crystallography (Bertrand et al., 2002 and Piontek et al., 2002). Typically the active site of laccase comprises four

Cu atoms in three groups. Cu atoms differ from each other in their electron paramagnetic resonance (EPR) signals (Gianfreda et al., 1991). Type I Cu exists in its oxidized form and is responsible for the blue colour of the enzyme at 600nm and is EPR detectible (Bertrand et al., 2002 and Piontek et al., 2002). Type II Cu is colourless, but EPR detectible. Type III Cu exhibits a weak absorption at 600 nm but has no EPR signal (Palmieri et al., 1998). The type II and type III Cu site are closely together and form a trinuclear centre (Leontievsky et al., 1999) that is involved in the catalytic mechanism of the enzyme action. Almost all fungi that have been examined produce more than one isoform of laccase (Hoshida et al., 2002).

Redox potential (RP) of fungal laccases range from 0.4 V to 0.8 V. Fungal laccases with RP between 0,4 and 0,6 V belong to the "Low RP" group such as Coprinus cinereus whereas those with a RP between 0,6 and 0,8 V are referred as "High RP" laccase such Trametes versicolor (Klonowska et al., 2005). Except blue laccase yellow laccase have also been purified from several fungi (Leontievsky et al., 1997 and Morohoshi et al., 1997). More interestingly white laccase was found to be produced by *Pleurotus ostearus*.

Mode of Action and Mediators

Laccase only attacks the phenolic compounds leading to $C\alpha$ oxidation, $C\alpha$-$C\beta$ cleavage and aryl-alkyl cleavage. Laccases are able to reduce one molecule of dioxygen to two molecules of water while performing one electron oxidation of a wide range of aromatic compounds which includes polyphenols (Bourbonnais and Paice, 1996), methoxy-substituted

monophenols and aromatic amines (Bourbonnais et al., 1995). This oxidation results in an oxygen-centered free radical, which can then be converted in a second enzyme-catalyzed reaction to quinine. Laccase catalysis is agreed to take place in three steps: 1. Type I Cu reduction by substrate. 2. Electron transfer from type I Cu to the type II Cu and type III Cu trinuclear cluster. 3. Reduction of oxygen to water at the trinuclear cluster (Gianfreda et al., 1999).

The substrate range of laccase can be extended to non-phenolic subunits by the inclusion of mediators. Mediators are a group of low molecular-weight organic compounds that can be oxidized by laccase first and from highly active cation radicals to reach with carious chemicals including non-phenolic compounds that laccase alone can not oxidase (Figure 1). The most common synthetic mediators are 1-hydroxybenzotriazole (HOBT), N-hydroxyphthalimide (NHPI) and 2,2'-azinobis-3-ethylthiazoline-6-sulfonat (ABTS) (Figure 2).

Distribution and Physiological Role of Fungal laccases

Laccases are widely distributed in fungi, higher plants, bacteria and insects. More than 60 fungal strains, belonging to various classes such as *Ascomycetes, Basidiomycetes* and *Deuteromycetes*, have been demonstrated to produce laccase (Gianfreda et al., 1999). The majority of laccases characterized so far have been derived from fungi, especially white-rot *Basidiomycetes* that are efficient lignin degraders. Well-known laccase producers include fungi such as *Agaricus bisporus*, *Botrytis cinerea* (Marbach et al., 1984), *Coprinus cinereus* (Schneider et al., 1999), *Neurispora crassa* (Froehner and Eriksson, 1974), *Phlebia radiate* (Niku-Paavola et al., 1990), *Pleurotus ostreatus* (Sannia et al., 1986),
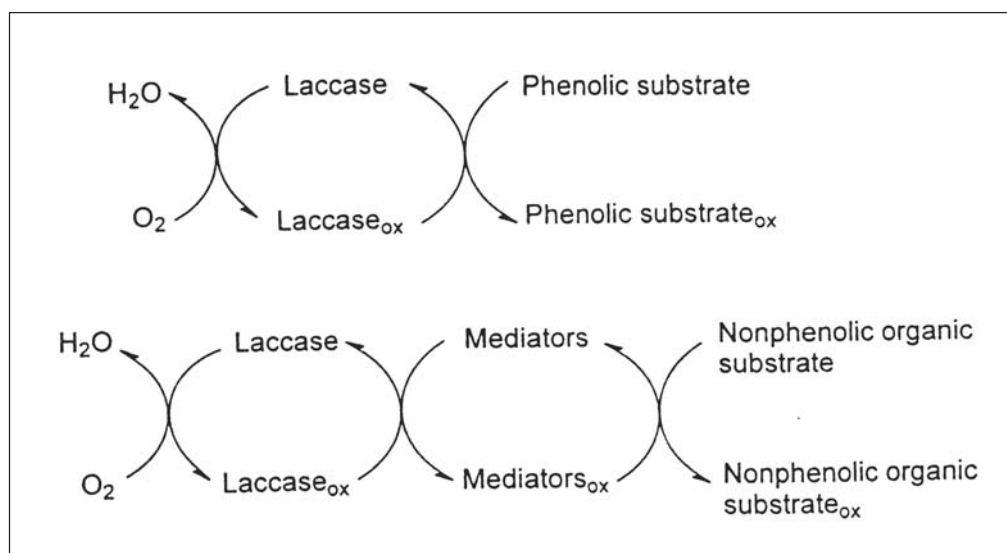

Fig. 1. A comparison of catalytic mechanism of laccase between with and without mediators
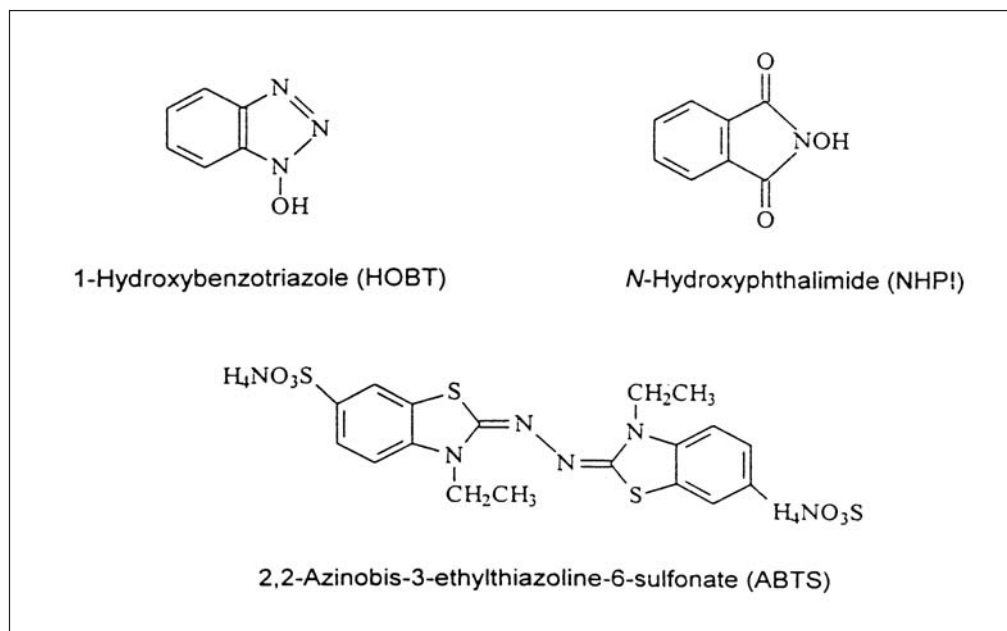
Fig. 2. Structures of three common synthetic mediators

Picnoporus cinnabarius (Eggert et al., 1996) and *Trametes* (Coriolus, Polyporus) versicolor (Rogalski et al., 1991). Extra-cellular laccases are produced by *Trichoderma atroviride* and *T. harzianum* (Holker et al., 2002).

The physiological roles of fungal laccases are various. Laccases from white-rot fungi such as *T. versicolor* and *P. cinnabarius*, participate in lignin bio-degradation, where they mainly oxydaze the phenolic subunit of lignin (Bourbonnais and Paice, 1996; Thurston, 1994). In plant-pathogenic fungi, laccases are important virulence factors. The grapevine grey mould *B. cinerea*, produces a laccase that necessary for pathogenesis, and the role of that laccase is presumably related to detoxification of toxic defense metabolites produced by the plant (Bar-Nun et al., 1988). Laccase have also been shown to be important for pathogenesis in the chest-

nut blight fungus *Cryphonectria parasitica* (Mayer and Staples, 2002) and in the human pathogen *Cryptococcus neoformans* (Williamson, 1994). In *Aspergillus nidulans*, laccase activity is re-lated to pigment production.

The role of laccases recently has been reevaluated because new information on their biodegradative mechanisms has been obtained in several fungal species (Bourbonnais and Paice 1992, Archibald and Roy 1992, Leonowicz et al., 2001). Moreover, some genera of basidiomycetes, such as *Pleurotus* spp., were found to lack lignin peroxidases (Fukushima and Kirk, 1995; Galliano et al., 1988; 1991), indicat-ing that different enzymes are probably involved in lignin biodegradation and that, among these enzymes, laccases could play a key role. Studies on the enzymes se-creted by the basidiomycete fungus *Pleurotus ostreatus* have shown that the

concerted action of laccase and aryl-alcohol oxidase, produces significant reduction in the molecular mass of soluble lignosulphonates (Marzullo et al., 1995).

Production of Fungal Laccases

Cultivation

Laccases ere generally produced during the secondary metabolism of different fungi growing on natural substrate or in submerged culture (Gayazov and Rodakiewicz-Nowak, 1996). Various cultivation parameters such as carbon limitation, nitrogen source and concentration and microelements influence laccase production. Gayazov and Rodakiewicz-Nowak (1996) reported faster laccase production under semi-continuous production with high aeration and culture mixing compared to static conditions. It was found that the production of high titers of laccase was not dependent on high biomass yields. Some fungal strains were successfully cultivated in stirred tank reactors (Nuske et al., 2001). Fed-batch cultivation was used, too but cultivation conditions were not described.

Influence of carbon and nitrogen source on laccase production

The use of excessive concentrations of glucose as carbon source in cultivation of laccase producing fungal strains has an inhibitory effect on laccase production (Eggert et al., 1996). It was found that increase in the amount of glucose in the media resulted in a delay of the laccase production. An excess of sucrose or glucose reduce the production of laccase, as these components allow constitutive production of the enzyme, but repress its induction. A simple but effective way to overcome this problem is the use of cellu-lose as carbon source during cultivation (Egger et al., 1996). Fungal laccases are often triggered by nitrogen depletion (Keyser et al., 1974) but it was also found that in some strains nitrogen concentration had no effect on enzyme activity (Leathman and Kirk, 1983). Some authors reported high laccase activity using low carbon to nitrogen ratio (Montiero et al., 1998) but other stated that high er laccase activity was achieved at high carbon to nitrogen ratio (Buswell et al., 1995). Laccase was also produced earlier when the fungus was cultivated in nitrogen rich media rather than the nitrogen limited media (Heinzkill et al., 1998).

Induction of laccase production

Laccase production has been found to be highly dependent on the conditions for cultivation and nutritive media composition (Heinzkill et al., 1998; Xavier et al., 2001). Laccases were generally produced al low concentrations but higher yealds were achieved with addition of various supplements to media (Lee et al., 1999). The addition of xenobiotic compounds such as xylidene, lignin, veratryl alcohol is known to increase and induce laccase activity (Xavier et al., 2001). Some of these compounds affect the metabolism or growth rate while others, such as ethanol, indirectly trigger laccase production (Lee et al., 1999). Lu et al. (1996) found that the addition of cellobiose can induce profuse branching in certain *Trametes* spp. and consequently increase laccase activity. The addition of low concentrations of copper to the cultivation media of laccase producing fungi stimulates laccase production (Palmieri et al., 2000). Various basidiomycetes, ascomycetes, and deuteromycetes, grown in a sugar-rich liquid medium, were compared for laccase-produc-

ing ability and for the inducing effect of 2,5-xylidine on laccase production. Clear stimulation of the extracellular enzyme formation by xylidine was obtained in the cultures of *Fomes annosus*, *Pholiota mutabilis*, *Pleurotus ostreatus,* and *Trametes versicolor*, whereas *Rhizoctonia praticola* and *Botrytis cinerea* were not affected by the xylidine, and in the case of *Podospora anserina* a decrease in laccase activity was observed (Bollag and Leonowicz, 1984).

Influence of pH and temperature on laccase production

There is not much information published on the influence of pH on laccase production, but most reports indicated initial pH levels set between pH 4.5 and pH 6.0 prior to inoculation (Thurston, 1994). The levels are not controlled during most cultivation. It has been found that the optimal temperature for laccase production is between 25 °C and 30 °C (Pointing et al., 2000). When cultivated fungi at temperatures higher than 30 °C the activity of lignolytic enzymes was reduced (Zadrazil et al., 1999).

Commersial applications of laccase

The scientific interest to fungal laccases is influenced by wide spectrum of laccase applications. Fungal laccases find applications within food industry, pulp and paper industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation, biodegradation of xenobiotics and removal of endocrine disruptors (Cuoto and Herrera, 2006). Laccases are attractive, industrially relevant enzymes that can be used for a number of diverse applications, e.g. for biocatalytic purposes such as delignifcation of lignocellulosics and cross-linking of polysaccharides, for bioremediation such

as waste detoxifcation and textile dye transformation (Gianfreda et al., 1999), for use in food technological, for personal and medical care applications (Xu, 1999) and for biosensor and analytical purposes (Yaropolov et al., 1994).

Conclusion

Laccases are a group of multi-copper enzymes that catalyze the one electron oxidation of phenols and are produced mainly by fungi. The broad commercial applications of laccases concern a great scientific interest on this group of enzymes. Many factors such as producer strain, cultivation conditions, composition of nutritive media, pH and temperature affect fungal laccases production.

The effective use of industrial laccases may be hindered by their non-reusability, and high sensitivity to denaturing agents. These undesirable restraints can be removed by use of modified or immobilized laccase. Many attempts have been made to improve the storage and operational stability of laccase, leading to lower cost and expansion of its biotechnological and environmental applications (Duran et al., 2002).

For this reason the nowadays researches concerned on laccase are focused on the isolation of new fungal laccase producers, isolation and expression of laccase genes, genetic construction of super producers strains and development of low cost and effective biotechnological processes for laccase production.

References

Archibald, F. and B. Roy, 1992. Production of manganic chelates by laccase from the lignin-degrading fungus *Trametes versicolo*r.

Appl Environ Microbiol., **58**:1496-1499.

Bertrand, T., C. Jolivalt, P. Briozzo, J. N. Caminade, C. Madzak and C. Mougin, 2002. Crystal structure of four-copper laccase complexed with an arylamide: insights into substrate recognition and correlation with kinetics. *Biochem*., **41**:7325-7333.

Bollag, L-M and A. Leonowicz, 1984. Comparative Studies of Extracellular Fungal Laccases, *Appl. Environ. Microbiol.*, **48** (4): 849-854.

Bourbonnais, R. and M. G. Paice, 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Let., t 267: 99-102.

Bourbonnais, R., M.G. Paice, I. D. Reid, P. Lanthier and M. Yaguchi, 1995. Lignin oxidation by laccase isozymes from T.versicolor and role of the mediator ABTS in kraft lignin depolymerization. *Appl. Environ. Microbiol,*. **61**: 1876-1880.

Buswell, J. A., Y. Cai and S. Chang, 1995. Effect of nutrient nitrogen and manganese on laccase production by *L.edodes*, *FEMS Microbiol. Lett*., **128**: 81-88.

Cuoto, S. and J. L. Herrera, 2006. Fungal laccases:Biotechnol. Application., *Biotechnol. Adv.*, **24**: 500-513.

Duran, N., , M.A. Rosa, A. D'Annibale and L. Gianfreda, 2002. Application of laccase and tyrosinase immobilized on different supports, a review. *Enzyme. Microb. Technol*., **3**1: 907-931.

Eggert, C., U. Temp, J.F.D. Dean. and K. E. L. Eriksson, 1996. A fungal metabolite mediates degradationof non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett*., **391**: 144-148.

Froehner, S. T., and K.E. Eriksson, 1974. Induction of N. crassa laccase with protein synthesis inhibition. *J. Bacteriol*., **120**: 450-457.

Fukushima, Y. and K. T. Kirk, 1995. Laccase component of the *Ceriporiopsis subver-*

*mispo*ra lignin-degrading system. *Appl Environ Microbiol*., **61**: 872-876.

Galliano, H., G. Gas and A. M. .Boudet, 1988. Biodegradation of *Hevea brasiliensis* ligno celluloses by *Rigidosporus lignosus*: influence of culture conditions and involvement of oxidising enzymes. P*lant Physiol Biochem*.,**26**:612-627.

Galliano, H., J. L. Seris and A. M. Boudet, 1991. Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. *Enzyme Microb Technol*., **13**: 478-482.

Gayazov, R. and J. Rodakiewicz-Nowak, 1996. Semi-continuous production of laccase by P.radiata in different culture media. *Folia Microbiol.Lett*., **41**: 480-484.

Gianfreda, L., F. Xu and J. M. Bollag, 1999. Laccases: A useful group of oxidore ductive enzymes. *Bioremed. J*., **3**: 1-26.

Heinzkill, M., Bech, L., halkier, T., Schneider, P., and Anke, T. 1998. Characterization of laccase and peroxidase from wood-rotting fungi. *Appl.Environ. Microbiol*. **64**: 1601-1606.

Holker, V., J. Dohse and M. Hofer, 2002. Extracellular laccases in ascomycetes T.atroviride and T.harzianum, *Folia Microbiol*., **47** (4): 423-427.

Hoshida, H., M. Nakao, H. Kanazawa, K. Kubo, T. Hakukawa, K. Morimasa, R. Akada and Y. Nishizawa, 2001. Isolation of five laccase gene sequences from white-rot fungus T.versicolor by PCR and cloning, characterization and expression of the laccase cDNA in yeasts. *J. Biosci. Bioeng*., **92**: 372-380.

Klonowska, A., C. Gaudin, A. Fournel, M. Asso, J. Le Petit, M. Giorgi and T. Tron, 2002. Characterization of low redox potential laccase from the basidiomycete C30. *Eur.J. Biochem*., **269**: 6119-6125.

Leatham, G. F., and T. K. Kirk, 1983. Regula-

tion of lignolityc activity by nutrition nitrogen in white-rot basidiomycetes. *FEMS Microbiol. Lett.*, **16**: 65-67.

Lee, I.-Y., Jung, K.-H., Lee, C.-H and Park, T.-H. 1999. Enhanced production of laccase in T.versicolor by addition of ethanol., *Biotechnol.Lett.* **21**:9 65-968.

Leonowicz A, Cho NS, Luterek J, Wilkolazka A, Wojtas-Wasilewska M, Matuszewska A, Hofrichter M, Wesenberg D, Leontiewsdky, A.A., Myasodoeva, N.M., Baskunov, B.P., Pozdnyakova, N.N., Vares, T., Kalkkinene and Goloveva, L.A. 1999. Reactions of blue and yellow fungal laccases with lignin model compounds. *Biochim.*, **64**:1150-1156.

Lu, S.X.F., Jones, C.L., and Lonergan, G.T. 1996. Correlation between fungal morphology and laccase expression under the influence of cellobiose. Proceedings of the 10th Int. Biotech. Symp. Sydney, Australia.

Marbach, L., Harel, E. and Mayer, A.M. 1985. Pectin,a second inducer for laccase production by B.*cinerea, Phytochem.* **24**: 2559-2561.

Marzullo L, Cannio R, Giardina P, Santini MT, Sannia G. 1995. Veratryl alcohol oxidase from Pleurotus ostreatus participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. *J Biol Chem* **270**: 3823-3827.

Mayer, A.M., and Staples, R.C. 2002. Laccase:new functions for an old enzyme. *Phytochemistry*, **60**: 551-5.

Messerschmidt, A., 1993. Blue copper oxidases, *Adv. Inorg. Chem.* **40**: 121-185.

Montiero, M.C., and De Carvacho, M.E.A. 1998. Pulp bleaching using laccase from T.versicolor under high temperature and alkaline conditions, *Appl. Biochem. Biotechnol.*, **70**: 983-993.

Morohoshi, N., Wariishi, H., Muraso, C.,

Nagai, T. and Haraguchi, T., 1987. Degradation of lignin by the extracellulare enzyme of C.versicolor IV Properties of three laccase fractions fractionated from the extracellular enzymes. *Mokuzai Gakkaishi*, **33**: 218-225.

Niku-Paavola, M.L., E. Karhunen, A. Kantelinen, L. Viikari, T. Lundell and A. Hatakka. 1990. The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus Phlebia radiate. *Journal of Biotechnology*, **13**: 211-221.

Nuske, J., Scheibner, K., Dornberger, U., and Hafrichter, M. 2001. Large scale production of manganese peroxidase using white-rot fungi. Proceedings 8th Int. Conf. Biotechnol , Helsinki, Finland

Palmieri, G., Giardina, P., Bianco, A., Capasso, A., and Sannia, G. 2000. A novel white laccase from P.ostreasus. *J.Biol.Chem.*, **272**:31301-31307.

Piontek, K., Antorini, M. and Choinowski, T., 2002. Crystal structure of a laccase from the fungus T.versicolor at 190 A Resolution containing a full complement of coppers. *J. Biol.Chem.* **277**:633-669.

Pointing, S.B., Jones, E.B.G. and Vrumed, L.L.P. 2000. Optimization of laccase production by P.sanguineus in submerged liquid culture. *Mycologia.* **92**:139-144.

Rodakiewicz-Nowak, J., Haber, J., Pozdnyakova, N., Leontievsky, A., and Goloveva, L.A. 1999. Effect of ethanol on enzymatic activity of fungal laccases. *Biosci.Rep.*, **19**:589-600.

Rogalski J. 2001. Fungal laccase: properties and activity on lignin. J Basic Microbiol 41:185-227.

Sannia, G., Faraco, V., Giardina, P. and Palmieri, G. 2001. Metal-activated laccase promoters. Proceedings 8th Int. Conf. Biotechnol , Helsinki, Finland

Slomczynski, D., Nakas, J.P., and

Tanenbaum, S.W. 1995. Production and characterization of laccase from B.cinerea 61-34. *Appl.Environ.Microbiol.* **61**: 907-912.

Schneider, P., Caspersen, M.B., Mondorf, K., Halkier, T., Skov, L.K., Ostergaard, P.R., Brown, K.M., and Xu, F. 1999. Characterization of *C.cinereus* laccase. Enzyme Microb. Technol., **25**:502-508.

Solomon, E. I. 1998. Site-directed mutations in fungal lacccase: effect on redox potential, activity and pH profile. *Biochem.J.*, **334**: 63-70.

Thurston, C.F. 1994. The structure and function of fungal laccases. *Microbiology*, **140**: 19-26.

Williamson, P.R. 1997. Laccase and melanin in the pathogenesis of *C. neoformans*, *F. Biosci.*, **199**: 99-107.

Xavier , A.M.R.B., Evtuguin, D.V., Ferreira, R.M.P. and Amado, F.L. 2001. Laccase production for lignin oxidase activity. Proceedings 8th Int. Conf. Biotechnol , Helsinki, Finland.

Xu, F., 1997. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *J. Biol. Chem.*, **272:** 924-928.

Xu, F., 1999. Laccase. In Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation, pp. 1545-1554. Edited by M. C. Flickinger & S. W. Drew. New York: Wiley.

Yaropolov, A. I., Skorobogat'ko, O. V., Vartanov, S. S. & Varfolomeyev, S. D. (1994). Laccase: properties, catalytic mechanism, and applicability. *Appl Biochem Biotechnol.,* **49**: 257-280.

Zadrazil, F., Gonser, A. and Lang, E. 1999. Influence of incubation temperature on the secretion of extracellulare lignolytic enzymes of Pleurotus and Dichomitus squalus into soil. Proceedings of the Conf. on Enzymes in the environment, Granada, Spain.