Chapter 13
Copper-Containing Oxidases: Occurrence in Soil Microorganisms, Properties, and Applications

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13.1 Introduction

Copper is an essential trace element in living systems, where it serves as a cofactor in many enzymatic redox reactions and oxygen transport (Fig. 13.1). The physiological oxidation states of copper are Cu^{1+} and Cu^{2+}, whereas Cu^{3+} is not a biologically relevant species because of the high redox potential of the Cu^{3+}/Cu^{2+} couple (Shleev et al. 2005). The copper at the active sites of redox proteins has been divided into three main classes (Table 13.1): type 1 (T1), blue copper; type 2 (T2), normal copper, and; type 3 (T3), a binuclear copper center (Malkin and Malmström 1970; Reinhammar 1984; Solomon et al. 1996, 2004; Kaim and Rall 1996).

T1 copper confers a typical blue color on the protein, which results from an intense electronic absorption band (around 600 nm) due to the covalent copper–cysteine bond. These sites are found in mononuclear copper proteins involved in intermolecular electron transfer pathways (azurin, plastocyanin, amicyanin, stellacyanin, rusticyanin), multicopper proteins (ascorbate oxidase, bilirubin oxidase, laccase, ceruloplasmin), and in a subclass of nitrite reductases, where they function in intramolecular electron transfer.

T2 copper in proteins yields positive EPR signals and only weak absorption in the visible spectrum. Type 2 sites are present in all blue multicopper oxidases, as well as in galactose oxidase, prokaryotic and eukaryotic copper amine oxidases, copper-containing superoxide dismutase, and cytochrome *c* oxidase.

The T3 binuclear copper center contains two ligand-bridged spin-coupled copper ions (Cu_A and Cu_B). T3 sites are diamagnetic and display a distinctive absorption band near 330 nm as well as a characteristic luminescence spectrum (Wynn et al. 1983; Solomon et al. 1996; Shin and Lee 2000; Shleev et al. 2005). This site is present in tyrosinase and in hemocyanin, the oxygen carrier found in molluscs and arthropods. In blue multicopper oxidases, the T2 and T3 sites form a trinuclear

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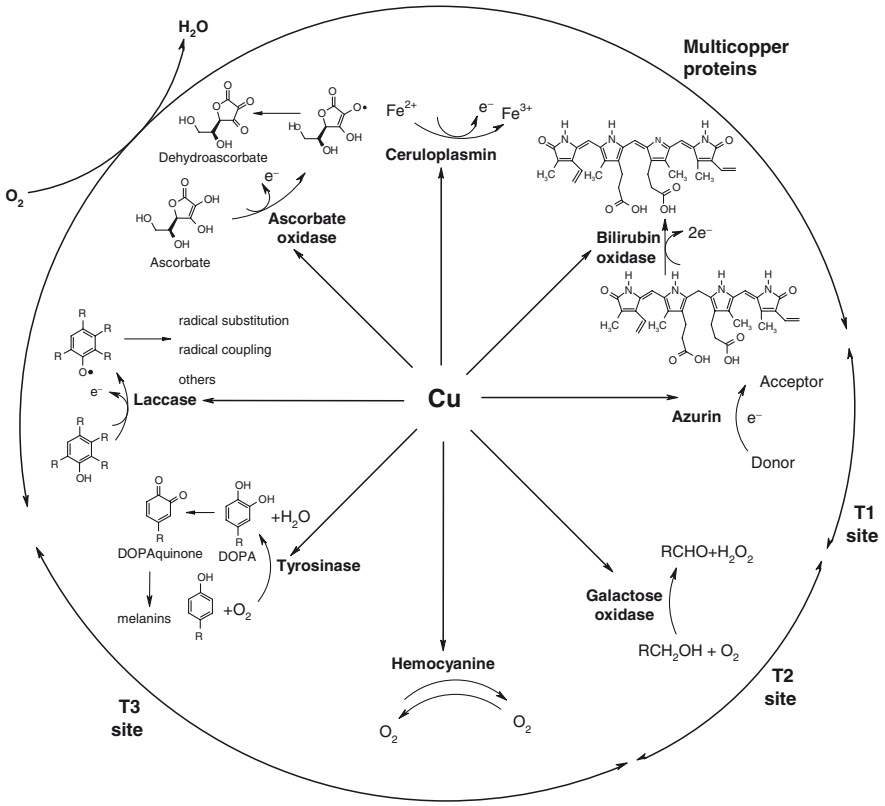

Fig. 13.1 Copper enzymes and their reactions (adapted from Shleev et al. 2005)

copper cluster (the T2/T3 cluster) (Allendorf et al. 1985; Messerschmidt and Huber 1990; Messerschmidt et al. 1992).

Tyrosinases and laccases are ubiquitously distributed in nature, and their corresponding activities can be observed intra- and/or extracellularly in soil microorganisms. A common feature is the existence of a T3 copper center, and both enzyme classes use molecular oxygen for substrate oxidation with the formation of water (Fig. 13.1).

Tyrosinases are involved in the initial steps of melanin synthesis. They catalyze the *ortho*-hydroxylation of monophenols to *ortho*-diphenols, and the latter into reactive *ortho*-quinones, which are then polymerized into dark pigments. Laccases oxidize various aromatic and nonaromatic compounds through a radical mechanism. They contribute to host defense mechanisms and the metabolic turnover of complex organic substances such as lignin and humic matter.

Both of the copper oxidases have been proposed for various biotechnological applications, such as the treatment of wastewaters or polluted soils, the removal of

Table 13.1 Some features of copper in proteins (modified from Lewis and Tolman 2004; Shleev et al. 2005)

Features	Type 1 copper	Type 2 copper	Type 3 copper
Cu atoms/ protein	1 (mononuclear)	1 (mononuclear)	2 (binuclear, spin-coupled CuA/CuB pair)
EPR signal	Paramagnetic	Paramagnetic	Diamagnetic
Light adsorption	High at 610 nm in ox. state: blue color	Low	High at 330 nm in ox. state
Coordination	Cys, 2 His, Met or Leu or Phe in multicopper proteins	3 His (2 His and 1 H_20 in the T2/T3 cluster of multicopper proteins)	6 His
Function	Electron transfer, catalysis	Electron transfer, catalysis	Binding of O_2 for transport and/or catalysis
Examples	• Multicopper proteins • Nitrite reductase • Small blue Cu proteins: Azurin Pseudoazurin Amicyanin Plastocyanin Stellacyanin Rusticyanin	• Multicopper proteins • Nitrite reductase • Amine oxidase • Cytochrome *c* oxidase (+Fe) • Galactose oxidase • Glyoxal oxidase • Quercetin 2,3-dioxygenase • Superoxide dismutase	• Multicopper proteins:Ascorbate oxidase Billirubin oxidase Ceruloplasmin[a] Fet3 protein (*Saccharomyces*)[a] Laccase • Laccase-like proteins (bacteria): Metallo oxidases (Mn, Cu, Fe) Phenoxazinone synthase • Tyrosinase • Hemocyanin[b] • Dopamine β-monooxygenase[c] • Peptidylglycine α-amidating monooxygenase[c]

[a] Also exhibits cuprous oxidase activity (Stoj and Kosman 2003)
[b] Displays tryosinase activity after specific activation (Decker et al. 2007)
[c] Contains two uncoupled Cu ions; it is not known if one or both activate oxygen

polyphenols from breweries, the synthesis of pharmaceutical drugs and new biopolymers, or for use as additives in food and cosmetic products (Couto and Herrera 2006; Halaouli et al. 2006).

This contribution provides an overview of the general biochemical and structural properties of tyrosinases and laccases, focusing on their occurrence and relevance in soil microorganisms and giving some examples of biotechnological applications of them.

13.2 Tyrosinases

13.2.1 Occurrence

The first biochemical investigations of tyrosinases were carried out with the mushroom *Russula nigricans*, the cut flesh of which turned red and then black upon exposure to air (Bourquelot and Bertrand 1895). The catalyst responsible was later found to be a copper enzyme that is widely distributed throughout the phylogenetic scale from lower to higher lifeforms, e.g., in the soil bacterium *Streptomyces*, in the common mushroom (*Agaricus bisporus*), and in human melanocytes or malignant melanoma cells (Nishioka 1978; van Gelder et al. 1997; Claus and Decker 2006; Halaouli et al. 2006). In higher plants and fungi, tyrosinases can occur in various immature, mature but latent, and active isoforms (Sánchez-Ferrer et al. 1989, 1990). Tyrosinase-like activities have been identified in the hemolymphs of insects (Lu and Jiang 2007) and as an inducible catalytic property of the hemocyanins (Decker and Tuczek 2000, Decker and Jaenicke 2004, Decker et al. 2001, 2007).

13.2.2 Relation to Melanin

Melanins are a diverse group of polymeric pigments that are widespread in a variety of organisms ranging from bacteria to humans (Plonka and Grabacka 2006). Three main types can be distinguished:

(1) *Eumelanins* (black or brown) are produced during the course of the enzymatic oxidation of tyrosine to *o*-dihydroxyphenylalanine (DOPA) and dopaquinone (Fig. 13.2). The latter spontaneously converts via the unstable leucodopachrome to red dopachrome, which can be used for the photometric determination of tyrosinase activity. Especially under alkaline conditions, dopachrome undergoes decarboxylation and further nonenzymatic polymerization reactions to become high-molecular eumelanins (Raper 1928, Mason 1948; Lerner et al. 1949). Melanogenesis in mammals is controlled by additional tyrosinase-related proteins: dopachrome tautomerase (TRP-2), which converts dopachrome into 5,6-dihydroxyindole-2-carboxylic acid, and TRP-1, which oxidizes this compound to indole-5,6-diquinone carboxylic acid. The subsequent reactions to form the dark polymers occur nonenzymatically (García-Borrón and Solano 2002). In invertebrates, additional enzymes besides tyrosinase are involved in melanogenesis, and dopamine is the preferred precursor.
(2) *Pheomelanins* (yellow-red), which initially are synthesized like eumelanins, but the DOPA undergoes the addition of cysteine or glutathione.
(3) *Allomelanins*, a heterogeneous group of polymers that arise from the oxidative polymerization of di- or tetrahydroxynaphthalene via the pentaketide pathway (*DHN-melanins*), homogentisic acid (*pyomelanins*), as well as from

γ-gluaminyl-4-hydroxybenzene, catechols and 4-hydroxyphenylacetic acid. In eu- and prokaryotes, melanins fulfill various functions such as photoprotection, photoconductivity, thermoregulation, immune defense, and chelation of metal ions (Plonka and Grabacka 2006; Wan et al. 2007).

Tyrosinase (monophenol, *o*-diphenol: oxygen oxidoreductase, EC 1.14.18.1) is the key enzyme involved in the formation of eumelanins. It catalyzes two distinct reactions: (a) the hydroxylation of monophenols to *o*-diphenols (cresolase or monophenolase activity), and (b) the (subsequent or separate) oxidation of *o*-diphenols to *o*-quinones (catechol oxidase or diphenolase activity) (Figs. 13.1 and 13.2).

The catecholoxidases (EC 1.10.3.1) frequently found in chloroplasts and fruits of higher plants (Mayer and Harel 1979, 1981; Mayer 1987, 2006) exhibit only the diphenolase activity, not the monophenolase activity, and will not be discussed further here.

It should also be pointed out that laccases (see below) can catalyze melanization when a diphenol is used as the precursor (Fig. 13.2).

Despite decades of intensive biochemical investigation, only limited information on the protein structure and the exact reaction mechanism of tyrosinase exists. Reasons for this include difficulties in purifying sufficient amounts of the enzyme from eukaryotic sources due to their intracellular localization, low enzyme concentrations, contamination with pigments, the occurrence of isoenzymes, and post-translational modifications. However, significant progress has recently been made with tyrosinases from the soil bacterium *Streptomyces*.

13.2.3 Copper Sites

The copper binding sites of tyrosinases share a high sequence homology with those of the hemocyanins, the oxygen carrier proteins of the molluscs and arthropods (Schoot-Uiterkamp and Mason 1973; van Gelder et al. 1997; Decker et al. 2007; Decker and Tuczek 2000; van Holde et al. 2001). A functional change in this protein family is proposed to have occurred during the course of evolution, from enzymatic oxygen detoxification towards oxygen transport (Jaenicke and Decker 2004).

The common feature of tyrosinases is a "type 3 copper center," a diamagnetic spin-coupled copper pair (Lerch 1995; Sánchez-Ferrer et al. 1995; García-Borrón and Solano 2002) (Table 13.1). Sequence alignments of many pro- and eukaryotic tyrosinases have shown that the copper binding regions are highly conserved. The signatures of Cu_A and Cu_B are H-x(*n*)-H-x(8)-H and H-x(3)-H-x(*n*)-H, respectively.

Each of the two metal atoms, Cu_A and Cu_B, at the active site are coordinated by three conserved histidines located in a "four α-helix bundle." During the catalytic cycle, the type 3 copper center can adopt different functional forms: the *oxy*-state [Cu(II)-O_2^{2-}-Cu(II)], the *deoxy*-state [Cu(I) Cu(I)], the *half-met* state [Cu(I) Cu(II)], and the *met* state [Cu(II)-OH^--Cu(II)]. In the latter case, the two copper atoms are bridged by hydroxo ions. The valences of the two copper atoms change from Cu(I)

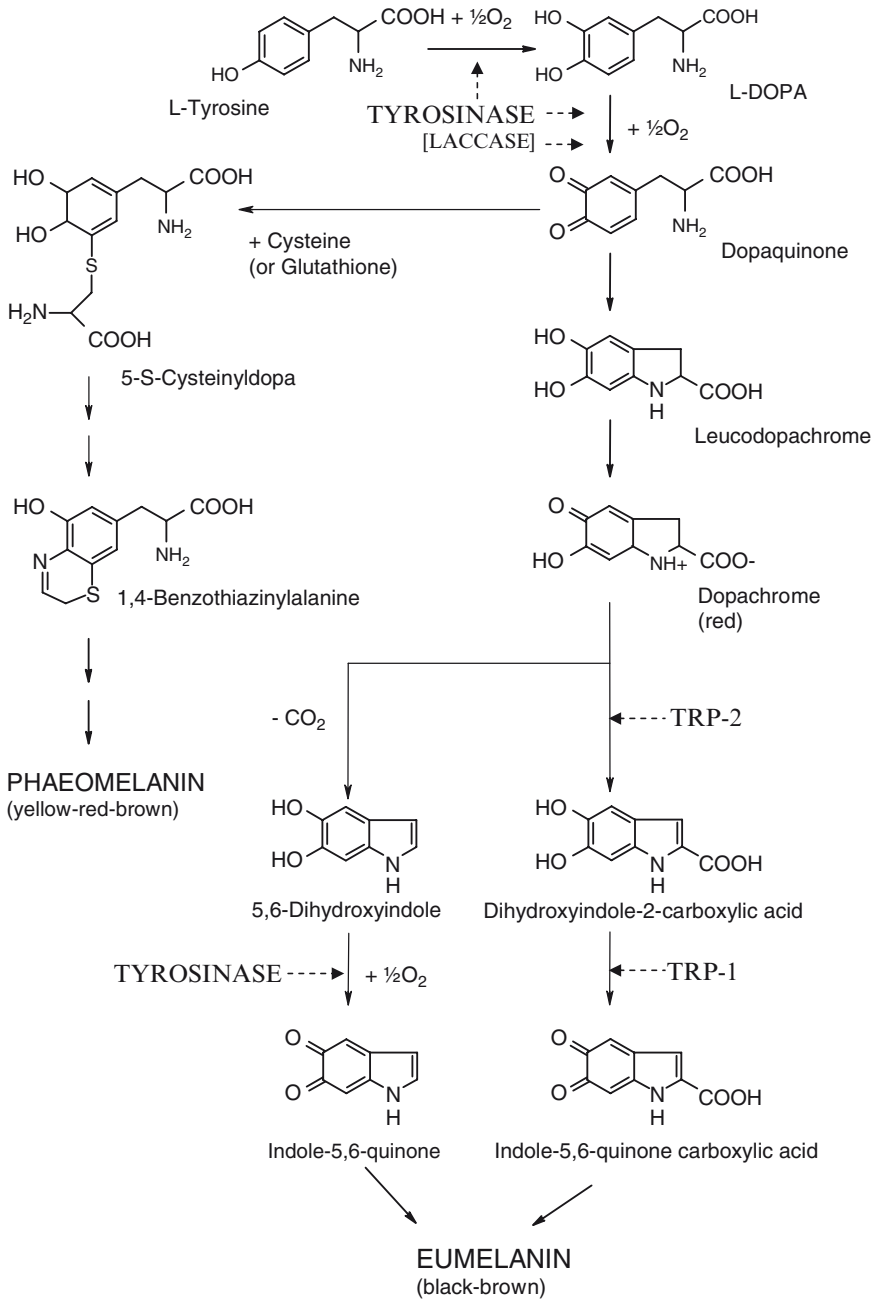

Fig. 13.2 Biosynthesis of melanin from tyrosine (modified from Kobayashi et al. 1995; Sanchez-Ferrer et al. 1995; Seo et al. 2003)

to Cu(II), which can be followed spectroscopically. In the *oxy* state, the molecular oxygen is reversibly bound as a peroxide between the two copper atoms in a "side-on" conformation. In the absence of any substrate, more than 85% of the enzyme is in the *met* state, which can be regarded as the resting form of tyrosinase. The current view is that both the *met* and the *oxy* states of tyrosinases enable diphenoloxidase activity, whereas the monohydroxylase reaction requires the *oxy* state.

13.2.4 Streptomyces Tyrosinases

Actinomycetes are Gram-positive soil bacteria with mycelial growth. Members of the genus *Streptomyces* are involved in the formation and/or degradation of complex biopolymers like lignin, melanins, and humic substances (Kutzner 1968). In addition, they are important industrial sources of bioactive compounds such as antibiotics, antitumor agents, antiparasites, immunosuppressant agents, and enzymes (Anzai et al. 2008).

About 40% of *Streptomyces* species produce melanin-like exopigments on tyrosine-containing agar media (Fig. 13.3), which usually (but not always) correlate with the appearance of tyrosinase activity (Arai and Mikami 1972; Claus and Kutzner 1985).

Fig. 13.3 Formation of melanin by a *Streptomyces* strain on a tyrosine-containing agar medium

Unlike most other tyrosinase-producing organisms, these bacteria secrete the enzyme into the environment, which facilitates isolation and biochemical characterization. Natural and recombinant tyrosinases have been purified from *Streptomyces glaucescens* (Lerch and Ettlinger 1972), *Streptomyces michiganensis* (Philipp et al. 1991), *Streptomyces castaneoglobisporus* (Kohashi et al. 2004), and *Streptomyces antibioticus* (Bernan et al. 1985). The enzyme from the latter species was the first tyrosinase for which a crystallographic structure could be elucidated (Matoba et al. 2006).

Tyrosinase genes from various *Streptomyces* species have been sequenced and translated into a protein sequence (Claus and Decker 2006). Interestingly, putative tyrosinase genes have been found in *Streptomyces* species that are phenotypically melanin negative (e.g., *Streptomyces coelicolor*), and several tyrosinase genes have been identified in some genomes (*Streptomyces avermitilis).*

Other bacterial tyrosinases have been detected and/or purified from the genera *Vibrio* (Pomerantz and Murthy 1974), *Rhizobium* (Mercado-Blanco et al. 1993; Piñero et al. 2007), *Bacillus* (Liu et al. 2004), *Thermomicrobium (*Kong et al. 2000), *Marinomonas* (López-Serrano et al. 2002, 2004), *Pseudomonas* (Wang et al. 2000), and *Ralstonia* (Hernandez-Romero et al. 2005). The presently documented molecular masses of bacterial tyrosinases range from 14 to 75 kDa; those of *Streptomyces* are about 30 kDa (Claus and Decker 2006).

13.2.4.1 Biochemical Properties

The typical double-enzymatic activity of tyrosinases has been demonstrated in melanin-positive *Streptomyces* species, whereas melanin-negative mutants lose the cresolase activity but sometimes retain some catecholase activity (Claus and Kutzner 1985). Tyrosine methylester and caffeic acid have been shown to be the best substrates for measuring both of the enzymatic activities of *Streptomyces* tyrosinase.

Electrophoretic characterizations have suggested that the intra- and extracellular tyrosinases from each *Streptomyces* species are identical, but that enzymes from different species are not (Claus and Kutzner 1985). Isoelectric focusing revealed the presence of several tyrosinase isoenzymes in some species, with their isoelectric points lying between 5.0 and 8.0. The heterogeneity of *Streptomyces* tyrosinases is also reflected in their different K_m constants and temperature stabilities.

Apart from the essential conserved copper-binding regions, significant sequence variations among bacterial tyrosinases have been detected. Among streptomycetes, the overall relationship varies between 36 and 86% (Claus and Decker 2006).

13.2.4.2 Incorporation of Copper

The melanin operons of *S. antibioticus* (Katz et al. 1983; Bernan et al. 1985; Betancourt et al. 1992), *S. glaucescens* (Hintermann et al. 1985; Huber et al. 1985),

Streptomyces lavendulae (Kawamoto et al. 1993), and *S. castaneoglobisporus* (Ikeda et al. 1996) consist of two components: *melC1*, which encodes upstream for a small chaperon-like ("caddy") protein, and the tyrosinase structure gene *melC2*. Genetic and biochemical studies, predominantly with *S. antibioticus*, have shown that the MelC1 protein is responsible for the incorporation of copper and thus the activation of the apotyrosinase (Lee et al. 1988; Chen et al. 1992). The histidine residues of the caddy protein may serve as the copper ligands: mutational exchanges of specific histidines in the MelC1 protein resulted in significant losses of tyrosinase activity (Chen et al. 1993). The MelC1 and MelC2 proteins form stable binary complexes which can be purified by chromatographic methods. Addition of copper to the binary complexes resulted in the incorporation of two copper molecules and the release of the activated tyrosinase (Chen et al. 1992).

13.2.4.3 Induction and Secretion

Tyrosinase synthesis by *S. glaucescens* is surprisingly not induced by tyrosine, but by different amino acids like phenylalanine, methionine and leucine (Baumann et al. 1976). Methionine also induces the tyrosinase from *S. antibioticus* (Katz and Betancourt 1988; Betancourt et al. 1992). The expression of the *S. castaneoglobisporus* tyrosinase is favored by methionine and copper (Ikeda et al. 1996). On the other hand, the transcription of the *S. michiganensis* tyrosinase is induced by copper and repressed by ammonium (Held and Kutzner 1990). In chemostat experiments, oxygen was found to be a negative regulator of the tyrosinase of *S. glaucescens* (Wyss and Ettlinger 1981).

Although *Streptomyces* tyrosinases are found intra- and extracellularly, they contain no signal sequences for secretion, like all bacterial tyrosinases studied so far. The TAT pathway (twin-arginine translocation pathway) allows the transport of (metallo)proteins in their native folded conformation. Proteins secreted in this way display a characteristic twin-arginine motif between the charged N-terminus and the hydrophobic core of the leader peptide. The MelC1 "caddy" proteins have this recognition signature and are most likely transported by the TAT route, which is widely used by streptomycetes (Schaerlaekens et al. 2004). A mechanism has been proposed in which the apotyrosinase forms a binary complex with the "caddy" protein, copper is incorporated, and it is then transported across the cytoplasmic membrane (Leu et al. 1992).

13.2.5 Role in Nature

Mammalian tyrosinases are located in specialized melanocytes and are responsible for the photoprotective pigmentation of hair, skin, and retina (García-Borrón and Solano 2002). Disorders in tyrosinase-catalyzed melanin synthesis are not only an aesthetic problem; they are linked with serious skin diseases, such as the well-known malignant

melanoma. Vitiligo is another such disease, characterized by hypopigmentation and total melanocyte depletion in the basal layer of the epidermis. Immunological studies of vitiligo show the generation and presence of autoantibodies directed against tyrosinase antigens in patient sera. This indicates that tyrosinase acts as an autoantigen and can serve as a marker for vitiligo (Parvez et al. 2007). Albinism, the total loss of pigmentation, is caused by different gene defects that do not primarily affect tyrosinase activity but rather transport of the enzyme into the melanosomes (Kushimoto et al. 2003).

Plant tyrosinases may be involved in biosynthetic processes and in defense against herbivores. During browning reactions, the injured tissues build up a melanin layer as protection against microbial pathogens (Mayer and Harel 1979; Mayer 2006).

In sponges and many invertebrates, tyrosinases are important components of wound healing and the primary immune response (Cerenius and Söderhäll 2004). In arthropods they are involved in sclerotization of the cuticle after molting or injury (Anderson et al. 1996). After their activation from inactive proenzymes by a cascade of serine proteases, insect phenoloxidases generate cytotoxic quinones and other reactive intermediates to immobilize and kill invading pathogens and parasites. Bacterial cell wall components are effective activators of these systems (Jiang et al 1998; Söderhäll and Cerenius 1998; Sugumaran 2002).

Fungal tyrosinases are generally associated with spore pigmentation, formation, and stability, as well as with defense and virulence mechanisms, or wound healing by melanin production (Seo et al. 2003; Halaouli et al. 2006; Mayer 2006).

The biological roles of bacterial tyrosinases are rather diverse. In soil environments, extracellular *Streptomyces* tyrosinases are probably involved in the polymerization and detoxification of plant phenolic compounds and the formation of humic matter (Kutzner 1968; Sjoblad and Bollag 1981).

Bacteria of the genus *Rhizobium* living in the root nodules of *Papillionaceae* plants carry tyrosinase genes in plasmids required for symbiosis (Mercado-Blanco et al. 1993). It was recently shown that the tyrosinase from *Rhizobium etli* plays a role in nodulation efficiency and symbiosis-associated stress resistance. Tyrosinase probably protects symbiotic microorganisms against toxic phenolic compounds in the soil environment and phytoalexins produced by plants (Piñero et al. 2007). The same mechanism is expected to be present in other plant-associated bacteria, like *Ralstonia solanacearum.*

The best-documented function of the enzyme is restricted to the formation of eumelanins. The dark pigments protect cells and spores against UV radiation, heat, enzymatic hydrolysis, antimicrobial compounds, heavy metals, or phagocytosis (Butler and Day 1998; Ruan et al. 2004; Wan et al. 2007), and contribute to microbial pathogenesis (Nosanchuk and Casadevall 2003; Plonka and Grabacka 2006)**.**

An attractive theory suggests that bacteria may use melanin as a redox polymer for adaptating to different oxygen concentrations:

- The aerobic soil bacterium *Azotobacter chroococcum* contains an active polyphenol oxidase (tyrosinase?) and forms melanin from catechol (Shivprasad and

Page 1989). This microorganism produces particularly large amounts of melanin when cultured under aerobic conditions. Although the intensity of melanogenesis does not seem to be directly correlated with the activity of nitrogenase (the key enzyme of atmospheric nitrogen fixation), it is possible that *Azotobacter* employs melanogenesis to enhance the utilization of oxygen and to maintain the reducing conditions necessary to bind atmospheric nitrogen.

- Soil bacteria can use humic acids as an electron acceptor for anaerobic respiration (Coates et al. 2002). A similar function can be assumed for the melanins.
- In *Proteus mirabilis,* an important cause of infections of the urinary tract, tyrosinase was identified as the enzyme responsible for pigmentation. The melanin decreases the level of reactive oxygen species, which probably makes the pathogen more resistant to the oxygen burst connected with the immunological response of the host (Agodi et al. 1996).

13.2.6 Applications

Tyrosinase is widely distributed in microorganisms, animals, and plants, and is a key enzyme in melanin biosynthesis and pigmentation of mammalian skin and hair. Its oxidative activities have a positive impact on the organoleptic properties of some fermentation products (raisins, cocoa, tea, coffee), but are also responsible for the undesirable enzymatic browning of fruits and vegetables, thereby causing a decrease in their nutritional quality and an inability to sell foods that have turned brown (Mayer and Harel 1979; Martinez and Whitaker 1995). Current conventional techniques of avoiding browning include heat inactivation of tyrosinase, but these processes cause undesirable losses to the quality of the product. Various chemicals such as halide salts and aromatic carboxylic acids as well as reducing compounds such as sulfite, citric acid, ascorbic acid, and cysteine are known to inhibit tyrosinase. The benefit of ascorbic acid is the focus of some discussion, and the use of sulfites is being restricted due to potential health hazards (Taylor and Bush 1986).

Widely used tyrosinase inhibitors for in vitro studies include L-mimosine, kojic acid, tropolone, phenylthiourea, and azide. However, as safety is paramount in the food industry, there is a constant search for better inhibitors from natural sources that are largely free of any harmful side effects. A number of tyrosinase inhibitors from natural sources (plants, fungi) that inhibit monophenolase and/or diphenolase have been already identified (e.g., arbutin, oxyresveratrol). Presently, 4-hexylresorcinol is considered to be safe for use in the food industry for browning control (Mayer 2006; Parvez et al. 2007).

A search for new tyrosinase inhibitors has also been launched by the cosmetic and pharmaceutical industries. Although melanin plays a crucial protective role against UV radiation and as an antioxidant, abnormal melanin pigmentation is a serious aesthetic problem in humans. Thus, tyrosinase inhibitors are important in the cosmetic industry due to their skin whitening and preventive effects (Parvez et al. 2007).

A number of tyrosinase inhibitors from natural sources have been reported, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns. For example, linoleic acid, hinokitiol, kojic acid, arbutin, naturally occurring hydroquinones, and catechols were reported to inhibit enzyme activity but have also exhibited side effects (Maeda and Fukuda 1991). Currently, arbutin (a hydroquinone glycoside) and aloesin (a glycosylated chromone) are used in the cosmetic industry as whitening agents because they are strong inhibitors of tyrosinase (Kahn 1995; Parvez et al. 2007).

Malignant melanoma is an increasingly serious clinical problem, with a high mortality rate among humans due to the failure of melanoma cells to respond to cytotoxic treatment in the form of radiation and chemotherapy. A selective strategy toward the treatment of malignant melanoma is called melanocyte-directed enzyme prodrug therapy (Jordan et al. 2001). Instead of tyrosine itself, a derivate coupled with an inactive prodrug serves as substrate in the biosynthetic pathway that converts tyrosine into melanin (Prota et al. 1994). This would allow selective conversion of inactive prodrugs into cytotoxic drugs in melanoma cells.

The substrate stereospecificity of the monophenolhydroxylase and diphenoloxidase activities of tyrosinase are the basis for many industrial applications (Halaouli et al. 2006): as biosensors for the monitoring of phenols; in the pharmaceutical industry for the production of *o*-diphenols (e.g., L-dopa, dopamine for the treatment of Parkinson's disease), and for the synthesis of biopolymers. Synthetic melanins find application as protective agents against radiation (UV, X-rays, γ-rays), cation exchangers, drug carriers, antioxidants, antiviral agents, and immunogens (Nosanchuk and Casadevall 2003; Wang et al. 2000). Their ability to crosslink proteins has opened up new application markets for tyrosinases in food industries (Thalman and Lötzbeyer 2002; Halaouli et al. 2005).

Tyrosinase has been suggested as an environmental tool for the detoxification of phenol-contaminated sites (Durán and Esposito 2000; Gianfreda and Rao 2004). However, due to their broad substrate spectrum and higher stabilities and activities under environmental conditions (such as variations in pH and temperature; presence of soil constituents), laccases appear to be much more suitable for bioremediation purposes (Claus and Filip 1988a,b, 1990a,b, 1991, Filip and Claus 1995).

13.3 Laccases

13.3.1 Distribution

Laccase [EC 1.10.3.2] belongs to the family of blue multicopper oxidases, including the eukaryotic proteins ceruloplasmin, ascorbate oxidase and bilirubin oxidase (Nakamura and Go 2005; Hoegger et al. 2006; Table 13.1). Laccase was first discovered by Yoshida (1883) in plants, based on the observation that the latex of the Japanese lacquer tree (*Rhus* sp.) hardened rapidly in the presence of air.

Subsequently, laccase enzymes have been discovered in numerous other plants (Lehman et al. 1974; Bligny and Douce 1983; de Marco and Roubelakis-Angelakis 1997; Ranocha et al. 1999). Many fungal species, including yeasts and ectomycorrhizal fungi, exhibit laccase activities (Baldrian 2006). Some laccase-like enzymes have been purified from larval and adult cuticles of insects (Kramer et al. 2001; Dittmer et al. 2004; Suderman et al. 2006). Prokaryotic laccases have been purified and investigated from the soil-inhabiting genera *Streptomyces* and *Bacillus* (Alexandre and Zhulin 2000; Claus and Filip 1997; Claus 2003, 2004; Sharma et al. 2007).

13.3.2 Properties of Fungal Laccases

Ligninolytic white-rot fungi produce high amounts of laccases and usually excrete several isoforms of the enzyme (Blaich and Esser 1975; Bollag and Leonowicz 1984; Baldrian 2006). Depending on the species, the addition of copper (Palmieri et al. 2000; Galhaup and Haltrich 2001), sugars and amino acids (Sandhu and Arora 1985), ethanol (Lomascolo et al. 2003), and phenolic compounds such as 2,5-xylidine (Sandhu and Arora 1985; Fåhreus and Reinhammar 1967) increase the production of extracellular laccases or induce the secretion of additional isoenzymes into the culture medium. Fungal laccases are glycosylated, usually in the range between 10 and 25 mol%. The glucans consist of arabinose, xylose, mannose, galactose and glucose units, which are N-linked to the polypeptide. Glycosylation may protect laccases from proteolytic degradation in the environment.

The mean optimum reaction temperature is around 55°C, although the thermostability of fungal laccases varies considerably. The half-life at 50°C ranges from minutes in *Botrytis cinerea* to over 3 h in *Lentinus edodes* and *Agaricus bisporus* and up to 70 h in *Trametes* sp. Typical fungal laccases have a molecular mass of 60–70 kDa and an acidic isoelectric point around pH 4.0. The amino acid chain contains about 520–500 amino acids, starting with an N-terminal secretion peptide (Gianfreda et al. 1999; Baldrian 2006).

Laccase is a prominent member of the blue multicopper oxidase family, which have four copper ions in their polypeptide chains (Table 13.1). The T1 copper has a trigonal coordination, with two histidines and a cysteine as conserved ligands, while one position is usually variable. It is the site of substrate oxidation and it has been widely argued that this axial ligand strongly influences the oxidation potential of the enzyme, which varies between E^0 +400 and +800 mV, depending on the individual laccase (Xu et al. 1996; Shleev et al. 2005). The T2 and T3 copper atoms form a trinuclear cluster, where the reduction of molecular oxygen to water takes place. The T2 copper is coordinated by two histidines and one water molecule, and each of the two T3 copper atoms by three histidines. Some laccase variants lack the T1 copper and are often referred to as the "yellow laccases," as they show no characteristic absorption band around 600 nm (Leontievsky et al. 1997).

The crystal structures of the fungal laccases from *Coprinus cinerius* (Ducros et al. 1998), *Melanocarpus albomyces* (Hakulinen et al. 2002), *Trametes versicolor*

(Antorini et al. 2002; Bertrand et al. 2002a, b), *Pycnoporus cinnabarinus* (Antorini et al. 2002), and *Rigidoporus lignosus* (Garavaglia et al. 2004) have been resolved. They show that the protein monomer is organized into three sequentially arranged cupredoxin domains. All three domains display a similar β-barrel type architecture that is related to those of smaller blue copper proteins such as azurin or plastocyanin. Disulfide bonds link domain one with domains two and three, while the trinuclear cluster bridges the first and third domains. The T1 copper located in domain three is the primary substrate electron acceptor site and is connected to the oxygen-reducing T2/T3 trinuclear cluster by a His–Cys–His tripeptide. Although usually active as monomeric proteins, some laccases consist of several subunits, forming hetero- (Yaver et al. 1996) or homodimers (de Souza and Peralta 2003).

Prokaryotic laccase enzymes have a similar structure (Enguita et al. 2003), although only two cupredoxin domains have been found for the bacterial laccases of *Streptomyces griseus* (Endo et al. 2003), which is active as a homotrimer, and *S. coelicolor* (Machczynski et al. 2004; Skálová et al. 2007).

13.3.3 Reaction Mechanism

Although still a matter of discussion, the following general catalytic cycle can be assumed (Messerschmidt and Huber 1990; Messerschmidt et al. 1992; Solomon et al. 1996, 2004; Tadesse et al. 2008). The reducing substrate is bound in a cleft at the enzyme surface and is oxidized by the T1 copper site in domain three, which is in close proximity. Electrons donated by four equivalents of the reducing substrate are transferred via a strongly conserved His–Cys–His tripeptide, which progressively leads to the reduction of all four Cu(II) ions in the polypeptide to the Cu(I) state. Reoxidation of the cuprous ions occurs at the trinuclear T2/T3 cluster with the concomitant reduction of molecular oxygen, resulting in the formation of two water molecules. Reduction of oxygen by laccase appears to occur in two $2e^-$ steps involving an intermediate peroxide bridging the trinuclear copper site.

The free radicals generated from laccase oxidation are very reactive and undergo further nonenzymatic reactions.

13.3.3.1 Crosslinking

The enzymatic oxidation of phenolic compounds and anilines generates radicals that react with each other to form dimers, oligomers or polymers that are covalently coupled by C–C, C–O, and C–N bonds. It should be noted that the nature of the crosslinked product is strongly influenced by the environmental pH (Leonowicz et al. 1984). In soils, natural and xenobiotic phenolics or aromatic amines can be bound to the organic humic matrix by this mechanism (Sjoblad and Bollag 1981). The oxidation of substituted compounds is accompanied by partial decarboxylations, demethylations, and dehalogenations (Dec et al. 2003). In higher plants, the

crosslinking of phenolic precursors by laccases forms part of the lignification process (O'Malley et al. 1993; Dean and Eriksson 1994). In insects, the laccase-catalyzed oxidative coupling of catechols with proteins may be involved in cuticle sclerotization (Suderman et al. 2006). In bacteria, it has been proposed that the crosslinking of protein residues (e.g., tyrosine to dityrosine) during the assembly of heat- and UV-resistant *Bacillus* spores is a function of laccases (Hullo et al. 2001; Martins et al. 2002).

13.3.3.2 Polymer Degradation

Laccase is involved in the degradation of complex natural polymers, such as lignin (Leonowicz et al. 2001) and humic acids (Claus and Filip 1998). The intermediate reactive radicals lead to the cleavage of covalent bonds and the subsequent release of monomers. The enzyme itself may not come into direct contact with the bulky polymers, and the reaction has to occur via low-molecular redox mediators.

13.3.3.3 Ring Cleavage of Aromatics

A few studies have reported laccase-catalyzed ring cleavages of aromatic compounds (Kawai et al. 1988), which are of biotechnological interest in relation to the degradation of xenobiotic compounds.

13.3.4 Substrates and Inhibitors

Laccases have low specificities for their reducing substrates but strong affinities for oxygen. Basically, any compound with characteristics similar to a diphenol will be oxidized by laccase as long as its redox potential is not too high ($E < +1{,}000$ mV). Classical substrates of laccases include various lignin-derived phenols and aromatic amines. *Ortho*-substituted compounds (e.g., guaiacol, caffeic acid, gallic acid, dihydroxyphenylalanine, pyrogallol, *o*-phenylenediamine) tend to be better laccase substrates than *para*-substituted compounds (e.g., *p*-cresol, *p*-phenylenediamine), while the lowest oxidation rates are obtained with *meta*-substituted compounds (e.g., *m*-phenylenediamine, orcinol, resorcinol). About 100 natural and artificial compounds are currently known to be oxidized by laccases, including unexpected substrates such as Mn^{2+} (Muñoz et al. 1997; Höfer and Schlosser 1999; Ridge et al. 2007) and certain lipids (Zhang et al. 2002).

Compounds commonly used for the photometric detection and measurement of laccase activity include 4-hydroxy-3,5-dimethoxy-benzaldehyde azine (syringaldazine) (Harkin and Obst 1973), 2,2′-azino-di-(-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Johannes and Majcherczyk 2000a), and 2,6-dimethoxyphenol (Solano et al. 2001). A more reliable method of measuring laccase activity is to

determine oxygen consumption, which is directly related to substrate oxidation (Claus and Filip 1990a,b, 1991; Filip and Claus 1995).

Laccases from various origins differ markedly in their substrate specificities. For ABTS, K_m ranges from 4 to 770 μM (Baldrian 2006). The optimal pH for the oxidation of ABTS is generally <4.0, while phenolic compounds like 2,6-dimethoxyphenol, guaiacol, and syringaldazine all exhibit higher values of between 4.0 and 7.0. Although a higher pH favors the phenol–phenolate interconversion of the substrate, the enzyme activity actually decreases due to the binding of OH^- to the T2/T3 copper (Muñoz et al. 1997; Xu et al. 1998).

Laccases can oxidize some low molecular compounds which in turn attack molecules that would not otherwise be appropriate substrates, such as nonphenolic chemicals (with E^0>+1,000 mV), or bulky polymers such as lignin and humic acids. Synthetic redox mediators have dramatically increased the potential use of laccases in industrial processes. Typical compounds include TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical), HBT (1-hydroxybenzotriazole), and ABTS (Bourbonnais et al. 1997; Xu et al. 2000; Baiocco et al. 2003; Riva 2006; Wells et al. 2006). There are also natural lignin-derived compounds (vanillin, acetovanillone, methylvanillate, acetosyringone, syringaldehyde, 2,4,6-trimethylphenol, *p*-coumaric acid, ferulic acid, sinapic acid, 3-hydroxyanthranilic acid) that can function as redox mediators (Cañas et al. 2007).

Laccase per se, or mediated by redox mediators, oxidizes numerous hazardous compounds, such as halogenated phenols (Bollag et al. 1988; Claus and Filip 1990a,b; Roy-Arcand and Archibald 1991; Canfora et al. 2008), aromatic amines (Claus and Filip 1990a,b), hydroxyindoles (Cai et al. 1993), the herbicide dymron (Marayuma et al. 2006), organophosphorus compounds (Amitai et al. 1998), polycyclic aromatic hydrocarbons (PAHs) (Johannes et al. 1996; Johannes and Majcherczyk 2000b; Cañas et al. 2007), chlorinated hydroxybiphenyls (Schultz et al. 2001), bisphenol A and nonylphenol (Uchida et al. 2001; Saito et al. 2004; Junghans et al. 2005), and hydroxyphenylureas (Jolivalt et al. 2006).

Fungal laccases are rather resistant to detergents like SDS, but high concentrations of heavy metals (like Fe) and NaCl can inhibit their activities. So far, no specific inhibitor has been described in addition to general inhibitors of metal-containing oxidases, like cyanide, sodium azide or fluoride. Johannes and Majcherczyk (2000a) tested a number of sulfhydryl organic compounds (dithiothreitol, thioglycolic acid, cysteine, diethyldithiocarbamic acid) that are thought to exert an inhibitory effect by interacting with the copper at the catalytic center of laccase. Only sodium azide was found to be a true laccase inhibitor and it showed no significant interference with the photometric test.

13.3.5 Role in Nature

Due to the abundance of laccase and laccase-like enzymes, there are numerous and diverse natural functions for these oxidoreductases. Although laccase is able to

polymerize lignin precursors, and its presence has been identified in xylem tissue of higher plants, there is still discussion about their involvement in lignification (Dean and Eriksson 1994; Thurston 1994; Mayer and Staples 2002). Peroxidases are regarded as the main biocatalysts in that process, but laccases operate in the absence of toxic peroxide and could play a role in the early stages of lignification in living cells (Sterjiades et al. 1992).

Evidence of laccase activity in the cuticles of larval and adult insects suggests their involvement in sclerotization (Dittmer et al. 2004; Suderman et al. 2006).

Physiological functions of laccase-like activities in bacteria include melanin production, spore coat resistance, morphogenesis, and detoxification of copper (Sharma et al. 2007). Laccase-like genes have been identified in important human pathogens such as *Escherichia coli*, *Bordetella pertusis*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Yersinia pestis*, and *Mycobacterium leprae* (Alexandre and Zhulin 2000). In all of these pathogens, the potential mechanism of virulence is suspected to be the production of melanin and laccase activity. *M. leprae* has an ability, unique among mycobacteria, to oxidize diphenols to *o*-quinones, and so the oxidation of L-DOPA has become a diagnostic feature for *M. leprae* (Prabhakaran and Harris 1985).

Fungal laccases probably play diverse roles in spore pigmentation and morphogenesis (Leatham and Stahmann 1981), fungal plant–pathogen/host interactions and stress defense (Mayer and Staples 2002), degradation of lignin (Thurston 1994; Leonowicz et al. 2001; Baldrian 2006), and turnover of humic matter (Claus and Filip 1998; Filip et al. 1998).

Similar to bacteria, laccase has been identified as a virulence factor in several human-pathogenic fungi such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Filobasidiella neoformans* due to the synthesis of melanin or the involvement of laccase in polysaccharide capsule formation (Mayer and Staples 2002).

Bollag et al. (1988) showed that the addition of laccase reversed the inhibitory effects of a number of phenolic compounds upon the growth of *Rhizoctonia praticola* inocula. They attributed the detoxification of the original phenolic compound to an ability of the laccase to transform it or cross-couple it with another phenol. This allows phytopathogenic fungi such as *B. cinerea* to detoxify phytoalexins and tannins, thereby increasing fungal virulence (Mayer and Staples 2002). It has been also demonstrated that interactions of different microorganisms, including soil fungi and bacteria, can be accompanied by strong laccase induction (Freitag and Morrell 1992; Savoie et al. 1998; Savoie 2001; Velazquez-Cedeno et al. 2004). This has been shown for laccase-producing basidiomycetes (Iakovlev and Stenlid 2000; Baldrian 2004), and also for the plant-pathogenic soil fungus *Rhizoctonia solani* when exposed to *Pseudomonas* strains producing antifungal compounds (Crowe and Olsson 2001). Laccase can probably also contribute to the degradation of phenolic antibiotics that inhibit fungal growth, like 2,4-diacetylphloroglucinol. The role of laccases in defense against heavy metals has been attributed to the production of melanins (Galhaup and Haltrich 2001; Baldrian et al. 2000; Baldrian 2003).

White-rot fungi secrete laccases and other oxidative enzymes in order to degrade complex natural polymers such as lignin (O'Malley et al. 1993; Dean and Eriksson

1994; Leonowicz et al. 2001). Laccase activity also plays an important role during composting processes, and it was isolated from both compost-specific fungi and the compost itself (Chefetz et al. 1998a,b; Chamuris et al. 2000).

Soil humic substances are considered to be the most stable part of decomposing organic matter in nature, and there is evidence that they are in a steady-state equilibrium of formation and degradation. Laccases have been shown to participate in the transformation of humic substances (Dehorter and Blondeau 1992; Chefetz et al. 1998a; Filip et al. 1998; Fakoussa and Frost 1999; Kluczek-Turpeinen et al. 2003, 2005). Laccase activity was also positively correlated with the degradation and synthesis of humic matter in experiments with *Cladosporium cladosporiodis* (Claus and Filip 1998). In vitro studies have demonstrated a 50% decolorization of humic acids by a laccase preparation from *T. versicolor* in the presence of a redox mediator (Claus and Filip 1998).

Ectomycorrhizal (EM) symbiotic fungi play a central role in the nutrition of trees by mobilizing and transporting nutrients to the roots (Smith and Read 1997). Phosphorus- and nitrogen-delivering compounds are entrapped in the complex organic macromolecules of litter and humic matter of forest soils (Ponge 2003). Acid phosphatases, proteases, and laccases are important exoenzymes that help to release matrix-bound nutrients and make them accessible to plant roots (Courty et al. 2006).

Laccase gene sequences have been identified in several EM fungi (Chen et al. 2003) and the enzymes have been purified from *Cantharellus cibarius*, *Lactarius piperatus*, *Russula delica*, *Thelephora terrestris*, and *Armillaria mellea* (Baldrian 2006). Other researchers have pointed out that tyrosinase appears to be the major phenoloxidase of EM because the oxidation of the laccase-specific substrate syringaldazine has scarcely been reported (Burke and Cairney 2002).

The seasonal dynamics of the laccase and acid phosphatase activities of EM were monitored in an oak forest. Among the most frequent and abundant EM morphotypes, those of *Lactarius quietus* and *Cortinarius anomalus* showed a peak in laccase activity in spring, while those of *Xerocomus chrysenteron* displayed their highest laccase activities in summer and fall (Courty et al. 2006).

Several authors have investigated the production of enzymes by fungi introduced into soils, and a number of protocols for laccase extraction have been proposed to optimize the extraction yield (Lang et al. 1997, 1998; Criquet et al. 1999; Baldrian et al. 2000). Laccase activities in soil extracts have been repeatedly demonstrated (Suflita and Bollag 1980; McClaughtery and Linkins 1990). An enzyme purified from a soil sample exhibited a high similarity to a laccase from *Polyporus versicolor* (Mayaudon and Sarkar 1975). A thermostable humic acid–laccase complex was isolated by Ruggiero and Radogna (1984).

Relatively high activities of laccase – compared to agricultural or meadow soils – can be detected in forest litter and soils (Rosenbrock et al. 1995; Criquet et al. 2000; Carreiro et al. 2000; Ghosh et al. 2003). The laccase activities reflect the temporal course of organic substance degradation (Fioretto et al. 2000), and their isoenzyme patterns vary during the succession (Nardo et al. 2004). Laccase activities in soil correlate with fungal biomass, which in turn is influenced by factors like temperature (Criquet et al. 2000) or nitrogen fertilization (Carreiro et al. 2000; Gallo et al. 2004).

Laccase activity in water-saturated environments (peatlands) is low due to poor oxygen availability, but increases dramatically when the oxygen concentration increases (Pind et al. 1994; Williams et al. 2000). The burst of laccase activity can lead to the depletion of phenolic compounds that inhibit organic matter degradation by oxidative and hydrolytic enzymes (Freeman et al. 2004), and it can be assumed that oxygen-regulated laccase activity plays an important role in carbon cycling in such environments (Baldrian 2006).

13.4 Applications

Due to its broad substrate spectrum, high oxidation potential (especially when combined with redox mediators), its thermal and pH stability, and its activity in organic solvents, laccase has become a powerful biocatalyst for numerous industrial applications, including delignification in the pulp and paper industries, ethanol production, solubilization of low-rank coal, textile bleaching and dyeing, bioremediation of wastewaters, and removal of polyphenols from breweries. It is also used as antioxidant and crosslinking agents in the food industry, as a catalyst in synthetic chemistry, and as a component of biosensors. These applications are not within the scope of this chapter, but they are addressed elsewhere (e.g., Yaropolov et al. 1994; Call and Mücke 1997; Smith et al. 1997; Xu 1999, 2005; Leonowicz et al. 2001; Durán et al. 2002; Minussi et al. 2002; Burton 2003; Claus et al. 2002; Wesenberg et al. 2003; Sigoillot et al. 2004; Couto and Herrera 2006; Riva 2006; Wells et al. 2006; Alcalde 2007; Camarero et al. 2005, 2007; Morozova et al. 2007; Strong and Burgess 2007; Xu et al. 2007; Kunamneni et al. 2008). One promising new application is the use of laccases in biofuel cells. Vincent et al. (2006) generated electricity from just 3% H_2 using an open fuel cell comprising an anode modified with a aerotolerant hydrogenase from *Ralstonia metallidurans* CH34, which oxidizes trace H_2 in atmospheric O_2, connected via a film of electrolyte to a cathode coupled with an O_2-reducing fungal laccase from *T. versicolor*.

The high biotechnological potential of laccases has triggered the research into new enzyme variants with appropriate features for use in industrial processes (Zumarraga et al. 2007; Festa et al. 2008) and their large-scale production in reactors (Couto and Herrera 2007).

Detoxification and bioremediation of contaminated soil environments is one of the earliest proposed and most intensively studied applications of laccases, and this will be discussed below.

13.4.1 Treatment of Polluted Soils

Many agricultural and industrial activities produce numerous xenobiotics that affect both soil and aquatic environments. Recalcitrant xenobiotics can accumulate and become harmful to these environments and their inhabitants. Enzymatic treatment

is considered an alternative method for the detoxification of contaminated aquatic and terrestrial environments (Sjoblad and Bollag 1981; Durán and Esposito 2000; Chiacchierini et al. 2004; Wesenberg et al. 2003; Claus and Filip 1991; Filip and Claus 1995; Nannipieri and Bollag 1991; Bollag 1992; Ahn et al. 2002; Gianfreda and Rao 2004; Gianfreda et al. 1999; Bollag et al. 2003). Numerous studies have shown that white-rot fungi and their ligninolytic enzymes are capable of the in vitro transformation or degradation of several xenobiotics. The underlying mechanism for laccase-induced detoxification involves oxidation of the pollutants to free radicals or quinones that subsequently undergo polymerization and partial precipitation. The pollutants are less toxic in their insoluble form and can be removed from waters by physical procedures (Bollag et al. 1988; Claus and Filip 1991; Dec and Bollag 1990; Nannipieri and Bollag 1991). In soils, detoxification occurs through the covalent coupling of the enzymatic oxidation products to the humus in the soil organic matrix (Bollag 1992). The enzymatic oxidation of substituted compounds is often accompanied by their partial dehalogenation (Claus and Filip 1990a, Dec et al. 2003; Roy-Arcand and Archibald 1991; Schultz et al. 2001), which contributes to the overall detoxification effect.

Concentrations of 2,4-dichlorophenol (2,4-DCP), a pesticide, in soils can reach up to 3,100 mg kg^{-1} (Ahn et al. 2002). Laccase enzymes from *T. versicolor* and *R. praticola* have been shown able to bind up to 65% of 2,4-DCP to humic materials in contaminated soils (Sarkar and Bollag 1987; Sarkar et al. 1988, 1989), and the transformation of phenolic derivatives has occurred after applying free and immobilized laccase (Shannon and Bartha 1988). The irreversible binding of these pollutants by laccases was shown to prevent further spread through the soil or leaching through to the water table. Dec et al. (2003) and Bollag (1988) have shown through isotope labeling that the humic–xenobiotic complexes resulting from the oxidative coupling are rather stable. Only small amounts of the xenobiotics were released over time, but these were further mineralized by soil microflora and abiotic factors. Ahn et al. (2002) compared the potential of montmorillonite-immobilized laccase and unbound laccase from *Trametes villosa* to remediate 2,4-DCP-contaminated soil. In general, immobilized laccase performed better than unbound laccase.

The presence of 2,4,6-trinitrotoluene (TNT) in soils, groundwaters, and surface waters at sites where this explosive was manufactured, loaded or demilitarized represents a serious ecological problem worldwide. Typical explosive-contaminated sites may contain up to 10,000 mg kg^{-1} of 2,4,6-trinitrotoluene (TNT) in soils and up to 100 mg L^{-1} in water. Trinitrotoluene itself is not a substrate for oxidative enzymes like laccase. However, its partial degradation results in the accumulation of reduced metabolites such as aminodinitrotoluenes (ADNT), azoxy compounds, and diaminonitrotoluenes (DANT) (Claus et al. 2007), which can be oxidized by laccase.

A number of researchers have reported the immobilization of TNT and its metabolites in complex soil organic matter and clay during composting or during anaerobic and aerobic slurry treatments. The potential of laccase for immobilizing TNT degradation metabolites in a humic matrix was recently demonstrated (Dawel et al. 1997; Thiele et al. 2002; Wang et al. 2002). During reductive transformation of TNT by *Trametes modesta* (in the presence of 200 mM ferulic acid and guaiacol),

the addition of humic monomers suppressed the accumulation of all major stable TNT metabolites by at least 92% (Nyanhongo et al. 2006).

Despite the abundance of promising experimental data in vitro, a number of limitations still restrict the use of enzymes to detoxify xenobiotics in the environment. Firstly, many cell-free enzymes are short-lived in soil environments. Enzymatic activity may be reduced or entirely eliminated through both nonbiological and biological deactivation factors, such as heavy metals, extreme acidity/alkalinity, protease degradation, and adsorption to soil constituents (Bollag 1992; Sarkar and Bollag 1987; Baldrian 2003; Gianfreda and Rao 2004). To evaluate the possible use of phenoloxidases as a tool in the remediation of chemically polluted soil and underground sites, Claus and Filip (1988a, b) investigated the behavior of laccase, tyrosinase, and peroxidase towards the most prevalent soil constituents, such as clays and humic substances. They demonstrated that laccase was strongly adsorbed to clay minerals at pH values near the isoelectric points of the enzymes. The amount of adsorbed protein correlated with the cation exchange capacities of the clays. In the presence of bentonites, laccase activity in solution was reduced at pH values below 5.0 and disappeared completely at pH 3.0. However, in the presence of kaolinites, some free laccase activity remained at pH 2.0. Adsorption of laccases to soil humic substances or inorganic soil constituents changes their temperature and activity profiles (Criquet et al. 2000). Keum and Li (2004) demonstrated that humic substances do not strongly bind laccase, and so the inactivation is not due to binding but the dissociation of copper chelated by humic substances.

Kaolinite was found to stimulate laccase production when cultures of *P. versicolor* and *Pleurotus ostreatus* were used to inoculate soil in order to replace enzyme preparations (Claus and Filip 1990b). However, in the study, the growth and enzyme production of the inocula were severely inhibited by competitive microorganisms under the nonsterile conditions used. These results indicated limitations on the in situ production of phenoloxidases.

13.5 Conclusion

The copper-containing oxidases tyrosinase and laccase have been intensively investigated for decades. For a long time, research into eukaryotic tyrosinases was hampered by low purification yields, but significant progress was recently made with those from soil microorganisms. New insights were obtained, especially in relation to structural and catalytic data. The formation of protective melanins is one well-established task of tyrosinases in eukaryotes. The physiological importance of the extracellular tyrosinases produced by the bacterium *Streptomyces* is not well understood. Tyrosine is neither an inducer of enzyme production nor a probable substrate in soil.

More assured information exists on the structural and biochemical properties of laccases from fungal sources. Many studies demonstrate their physiological role in the detoxification of phenolic compounds. Their occurrence in soils underlines their ecological importance in the metabolic turnover of complex organic polymers

such as lignin and humic matter. In ectomycorrhizal symbiotic fungi, they contribute to the nutrition of trees by mobilizing and transporting nutrients to the plant roots. The specific monohydroxylase activities of tyrosinases and the high nonspecific oxidation capacities of laccases can be exploited for numerous biotechnological processes. The screening of natural sources and genetic engineering will further expand our knowledge and applications of these old-fashioned metalloenzymes.

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