# OCCURRENCE AND PROPERTIES OF THE MULTICOPPER OXIDASES LACCASE AND TYROSINASE IN LICHENS

by

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Submitted in fulfilment of the academic requirements for the degree of

**PhD**

in the School of Biological and Conservation Sciences,
University of KwaZulu-Natal,
Pietermaritzburg.

January, 2012

# ABSTRACT

The work presented in this thesis describes the occurrence and properties of two multicopper oxidases derived from lichens. Despite numerous data on laccases and tyrosinases in fungi and flowering plants, this is the first report of the occurrence of these enzymes in lichenized ascomycetes. Extracellular laccase and tyrosinase activity was measured in 50 species of lichens from different taxonomic groupings and contrasting habitats. Out of 27 species tested from suborder Peltigerineae, all displayed laccase and tyrosinase activity that correlated to each other, while activity was absent in species tested from other lichen groups. Identification of the enzymes as laccases and tyrosinases was confirmed by the ability of lichen thalli or leachates to readily metabolize substrates such as 2,2'-azino(bis-3-ethylbenzthiazoline-6-sulfonate) (ABTS), syringaldazine and o-tolidine in case of laccase and L-dihydroxyphenylalanine (L-DOPA), L-tyrosine and epinephrine in case of tyrosinase in the absence of hydrogen peroxide. The activities of both enzymes were highly sensitive to cyanide and azide, and tyrosinase activity was sensitive to hexylresorcinol. Laccase activity had typical pH and temperature optima and an absorption spectrum with a peak at 614 nm. Tyrosinases could be activated by sodium dodecyl sulphate (SDS) and had typical tyrosinase molecular masses of approx. 60 kDa. The diversity of laccase isoforms in 20 lichen species from suborder Peltigerineae was investigated. The molecular masses of the active forms of most laccases varied between 135 and 190 kDa, although some lichens within the family Peltigeraceae had laccases with higher masses, typically varying from 200 to over 350 kDa. Most species contained one oligomeric laccase isoform. Desiccation and wounding stimulated laccase activity, while only wounding stimulated tyrosinase activity. The ability of laccases to decolorize dye is a classic attribute of laccases, and one with biotechnological potential. The ability of eight lichen species to decolourize different types of dyes was therefore tested. Interestingly, results showed that not only species belonging to suborder Peltigerineae but also species from other lichen group effectively decolourised dyes after 48 h suggesting that other oxidases appear to have ability to decolorize. Hopefully, our work could contribute to the better knowledge and application of lichen multicopper oxidases.

# PREFACE

The experimental work described in this thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg, from March 2004 to December 2005, under the supervision of Professor Richard P. Beckett.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1: Beckett, R. P., Minibayeva, F. V., **Laufer, Z.** (2005): Extracellular reactive oxygen species production by lichens. *The Lichenologist* **37**: 397-407.

Publication 2: **Laufer, Z.**, Beckett, R. P., Minibayeva, F. V., Lüthje, S., Böttger, M. (2006): Occurence of laccases in lichenized Ascomycetes in the Suborder Peltigerineae. *Mycological Research* **110**: 846-853.

Publication 3: **Laufer, Z.**, Beckett, R. P., Minibayeva, F. V. (2006): Co-occurrence of the multicopper oxidases laccase and tyrosinase in lichens in Suborder Peltigerineae. *Annals of Botany* **98**: 1035-1042.

Publication 4: **Laufer, Z.**, Beckett, R. P., Minibayeva, F. V., Lüthje, S., Böttger, M. (2009): Diversity of laccases from lichens in Suborder Peltigerineae. *The Bryologist* **112**: 418-426.

Signed:……

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# ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Prof. Beckett for giving me an excellent PhD project to work on. His encouragement, valuable discussions and guidance have been extremely useful during my work. Thank you, Prof. Beckett for making time, for your suggestions and assistance, wisdom and also for financial support. Thank you for opportunities and constructive criticism. I have learnt a great deal from you and have grown tremendously in the process.

A special word of thanks to my colleagues all over the World, friends and members of staff at the Department of Botany for all their help and also to the University of KwaZulu-Natal for funding this project financially and Special Doctoral Research Grant (Faculty of Science and Agriculture) for running expenses.

I also wish to thank everyone dear in my life in the Republic of South Africa and in Hungary, especially to my family, my Mom and Dad for understanding scientific frustration and for their emotionally support. Also to Tibor for keeping me sane, for his eternal patience and for believing me.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) |
| CDH | Cellobiose dehydrogenase |
| Con A | Concanavalin A |
| DMP | 2,6-dimethoxyphenol |
| DMSO | Dimethyl sulfoxide |
| dopachrome | 2-carboxy-2,3-dihydroindole-5,6-quinone |
| DyPs | "Dye-decolorizing peroxidases" |
| EDTA | Ethylenediaminetetraacetic acid |
| EPR | Electron paramagnetic resonance |
| G-6-PD | Glucose-6-phosphate-dehydrogenase |
| GUA/ guaiacol | 2-methoxyphenol |
| 1-HBT | 1-hydroxybenzotriazole |
| 4HR | 4-hexylresorcinol |
| HS | Humic substances |
| IEF | Isoelectric focussing |
| KCN | Potassium cyanide |
| kDa | Kilodalton |
| L-DOPA | L-dihydroxyphenylalanine |
| LMS | Laccase mediator system |
| L-tyr/L-tyrosine | 4-hydroxyphenylalanine |
| 4-MCC | 4-methylcatechol |
| MES | 2-morpholino-ethane-sulfonic acid |
| NaF | Sodium fluoride |
| $NaN_3$ | Sodium azide |
| $O_2 \cdot^-$ | Superoxide anion radical |
| OH· | Hydroxyl radical |
| PEG | Polyethylene glycol |
| PPFR | Photosynthetic photon fluence rate |
| PPO | Polyphenol oxidase or tyrosinase |
| RBBR | Remazol Brilliant Blue R |
| RH | Relative humidity |
| ROS | Reactive oxygen species |
| rpm | Revolutions $min^{-1}$ |

| | |
|---|---|
| SEC | Size exclusion chromatography |
| S.D. | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SOD | Superoxide dismutase |
| Syringaldazine | 4,4’-[azinobis(methanylylidene)]bis(2,6-dimethoxyphenol) |
| TMB | 3,3’,5,5’-tetramethylbenzidine |
| *o*-tolidine | 3,3’-Dimethylbenzidine |

# CHAPTER 1

## LITERATURE REVIEW


*Rhus vernicifera* DC.
from which laccases were first described in 1883 by Yoshida.

## 1.1. Lichens

Lichens are symbiotic organisms composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont that may be either a green algae or a cyanobacterium (Nash, 1996). Lichens develop in three different growth forms; the foliose (leaf-like), fruticose (shrubby or hair-like) and crustose (crust-like) (Hale, 1969). The literature has recognized two types of lichen thalli: in homoeomerous lichens the photobiont is evenly distributed throughout the lichen thallus, e.g. in *Collema* and *Leptogium*, while in most lichens the photobiont occurs in a layer close to the surface that are referred to as heteromerous thalli (Figure 1.1; Dahl and Krog, 1973).

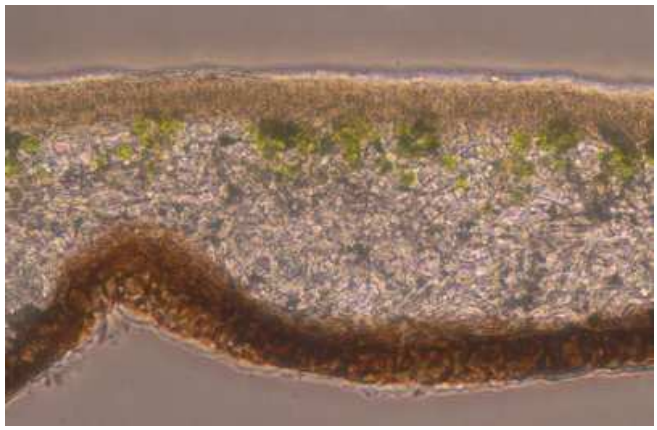


**Figure 1.1.** Cross section of the heteromerous lichen *Alectoria sarmentosa*. The partnership can be seen between green algae cells and fungal hyphae. The alga cells are confined to the upper half, where they intercept light and carry out photosynthesis (taken from Johansson, 2011).

Lichens have two very important features. Firstly, they are poikilohydric organisms and become desiccated relatively rapidly, but after rehydration can resume their metabolic activity (Beckett *et al*., 2008). Secondly, species from stressed habitats contain high concentration of secondary metabolites because they have to protect themselves against the harsh environment and the resulting attack by pathogens following injury and these metabolites have antibiotic and antifungal properties (Lawrey, 1983, 1995; Gietz *et al.*, 1994). Lichen biomolecules belong to two main groups, primary metabolites (mainly intracellular) and secondary metabolites (mainly extracellular). Many lichens can accumulate high concentrations of secondary metabolites such as usnic acid, pulvinic acid and the aliphatic acid depsides and depsidones (Huneck and Yoshimura, 1996). Presumably they have important metabolic functions. Surprisingly, the

members of the widespread genus *Peltigera* contain these metabolites at low concentrations (Hawksworth, 1982), but still can resist pathogen attack.

### 1.1.1. Lichen secretomes

It is well known that lichens can secrete extracellular proteins such as the ice nucleation proteins, hydrophobins, arginases, ureases, phosphatases, cellulases and redox enzymes. Typical secreted enzymes are heavily glycosylated (Varki *et al*., 1999) therefore the characterization of these enzymes is more difficult.

The occurrence of ice nucleation activity in lichens was first described by Kieft (1988), and it was later confirmed that ice nucleation proteins are responsible for this activity (Kieft and Ruscetti, 1990). The proteins were investigated in the genera *Rhizoplaca*, *Xanthoparmelia* and *Xanthoria*. Ice nucleation proteins have a location on the fungal cell wall and are able to nucleate the crystallization of ice (Warren and Wolber, 1991).

Hydrophobins are secreted fungal proteins (Trembley *et al*., 2002) and were first characterized from *Schizophyllum commune* by Schuren and Wessels (1990). The proteins were described from two lichenized ascomycetes, *Xanthoria parietina* and *X. ectaneoides* (Scherrer *et al*., 2000) and from the lichenized basidiocarp *Dictyonema glabratum* (Trembley *et al*., 2002). Hydrophobins are small proteins (approximately 100 amino acids) with eight cysteine residues (Scherrer *et al*., 2002) forming a thin, water-repellent hydrophobic layer around the cell walls of fungal hyphae and the photobionts (Dyer, 2002). The rodlet layer presumedly allows apoplastic transport of water and solutes within the lichens (Wösten and Wessels, 1997) maintaining the symbiotic system (Dyer, 2002). Hydrophobins have important function in fungal water relations (Trembley *et al*., 2002) and fungal development processes (Wessels, 1994).

Arginase is the last enzyme of the urea cycle, hydrolyzes arginine into urea, and contains manganese. At least three forms of arginases were found in the lichen *Evernia prunastri* (Legaz *et al*., 1990), a constitutive, an inducible and a secreted form. The enzymes are fungal proteins and are mostly glycosylated. Legaz *et al*. (2004) purified secreted arginases from *Evernia prunastri* and *Xanthoria parietina* and found that the enzymes have shown lectin function, binding to the cell wall of algae. Therefore, arginases may have significant physiological roles in the relationship between photobiont and mycobiont of symbiotic association (Molina and Vicente, 2000).

Urease was first isolated and crystallized from the jack bean, *Canavalia ensiformis* in 1926 (Sumner, 1926). The enzyme catalyses the hydrolysis of urea to carbon dioxide and ammonia. Urease is widely distributed in lichens as an inducible enzyme (Pérez-Urria *et al*., 1993). Secretion of urease was described for example in *Evernia prunastri* (Vicente and Pérez-Urria, 1989), *Mastodia tesselata*, *Usnea aurantiaco-atra, Ramalina terebrata* (Pérez-Urria *et al*., 1993) and *Xanthoria parietina* (Millanes *et al*., 2004) but the physiological role of the secretion is still unclear. Millanes *et al*. (2004) confirmed that the location of about 80% of total urease is the cell wall.

Phosphatase removes a phosphate group from many types of molecules. Several phosphatases are known but very few have been investigated in lichens. An acid phosphatase was demonstrated in *Peltigera rufescens* by Brown and Kershaw (1986) while Banerjee *et al*. (2000) found phosphatase activity in lichenized *Trebouxia*.

Cellulases catalyze the hydrolysis of cellulose. Yagüe *et al*. (1984) and Yagüe and Estévez (1988) showed that lichen *Evernia prunastri* synthesizes a secretable cellulase that can be induced by cellobiose. Later Guerrero *et al*. (1992) investigated four lichen species from different habitats and found similar results. The cyanolichen *Peltigera canina*, which lacks cellulosic cell walls, was found to produce and secrete several isoforms of cellulase (de los Ríos *et al*., 1997). These enzymes can decompose decaying plant material (Guerrero *et al*., 1992), degrade external cellulosic substrates (de los Ríos *et al*., 1997), breakdown the phycobiont cell and regulate the phycobiont growth (Yagüe and Estévez, 1988).

Redox enzymes catalyse the oxidation or reduction of a substrate. In this study we have focused mainly on laccases and tyrosinases, the two redox enzymes that have many important functions. After the start of the present investigation, Zavarzina and Zavarzin (2006) reported the occurrence of surface laccases and tyrosinases in lichens. To some extent, the first part of the work presented here duplicates this study. However, the work presented in this thesis considerably extends the preliminary report of Zavarzina and Zavarzin (2006) and used different species.

## 1.2. Laccase

### 1.2.1. Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a type of copper-containing polyphenol oxidase, discovered in 1883 by Yoshida from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Thurston, 1994). A few years later, in 1896, it was also demonstrated in fungi by Bertrand (Baldrian, 2006). Laccase is one of a small group of enzymes called blue copper oxidases. Other members of this group are the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin (Thurston, 1994). Laccases are found in some flowering plants and prokaryotes, but mainly in fungi. The enzymes reduce dioxygen to two molecules of water and simultaneously perform one-electron oxidation of many aromatic substrates and generate a free radical (Reinhammar and Malmstrom, 1981). This radical may undergo a second, non-enzymic reaction and/or may partake in a polymerization reaction (Thurston, 1994).

Laccases have an important role in lignin biosynthesis and degradation and they may produce signals that function in fungal developmental processes. Their strong capacity to oxidase aromatic substances means that they have widespread applications in industry. The ability of laccases to oxidise phenolic and non-phenolic compounds, their ability to reduce molecular oxygen to water, their broad applications and the fact that occurrence and role of laccases are still uncertain has led to intensive studies of these enzymes (Bourbonnais and Paice, 1990; Thurston, 1994; Xu, 1996; Jolivalt *et al*., 1999).

### 1.2.2. Occurrence and location

Laccase is the most widely distributed enzyme among the multicopper oxidases, as it has been examined in a wide range of fungi and higher plants (Mayer and Staples, 2002; Baldrian, 2006). Claus and Filip (1997) examined the occurrence and role of the enzyme in prokaryotes (*Bacillus sphaericus*), but many other studies have been published (reviews: Endo *et al*., 2002; Suzuki *et al*., 2003; Claus, 2003). In spite of these reports, it seems to that laccases are not common enzymes from certain prokaryotic groups (Baldrian, 2006). Bacterial laccase-like proteins are intracellular or periplasmic proteins (Claus, 2003).

The occurrence of laccases in higher plants also appears to be far more limited than in fungi. All laccases described to date have been shown to be glycoproteins (Mayer and Staples, 2002). In higher plants, the properties of laccase have been examined in among others *Acer pseudoplatanus* (Driouich *et al*., 1992), *Fagus sylvatica* (Felby *et al*., 1997), *Populus euramericana* (Ranocha *et al*., 1999) and in some gymnosperms such as *Araucaria excelsa* and *Pinus taeda* (Bao *et al*., 1993). Other higher plant species (e.g. vegetables) also appear to contain laccases, although their characterization is less convincing (Levine, 1965; Dean and Eriksson, 1994). The enzyme may be bound to cell walls in some higher plants (Mayer and Staples, 2002).

Laccases have been isolated from Ascomycetes, Deuteromycetes and Basidiomycetes fungi (Assavanig *et al*., 1992). They are probably best known for their occurrence in white-rot and litter decomposing basidiomycete fungi (reviews: Eggert *et al*., 1996; Kerem *et al*., 1999; Guillen *et al*., 2000; Steffen *et al*., 2002; Nagai *et al*., 2003). There are also many records of laccase production by ascomycetes (e.g. Scherer and Fischer, 1998; Edens *et al*., 1999; Kiiskinen *et al*., 2002) but the function of this enzymes in these fungi is unclear. Most fungal laccases are extracellular (Baldrian, 2006) but few reports indicate that they may occur intracellularly (e.g. Nagai *et al*., 2003) or both intra- and extracellularly (e.g. in *Phanerochaete chrysosporium*, Dittmer *et al*., 1997). Baldrian (2006) concluded the possible connection between the localization of laccase and its physiological function: it is possible that the intracellular laccases can participate in the transformation of low molecular weight phenolic compounds. In the cell wall and spores laccases are linked to the possible formation of melanin and other protective cell wall compounds (Eggert *et al*., 1995; Galhaup and Haltrich, 2001). In many fungal species the presence of both constitutive and inducible laccases has been reported (Mayer and Staples, 2002). Interestingly, before the start of this investigation, there were no reports about the occurrence of laccases in an important group of fungi, the lichenized ascomycetes.

### 1.2.3. Structure and reactions of laccase enzyme

*Structural properties*

Our knowledge of the structure and physico-chemical properties of laccases is based on the study of purified proteins, mostly from wood-rotting white-rot basidiomycetes while laccases from other groups have been studied to a much lesser extent. Typical plant laccase has

a molecular weight of approximately 90-110 kDa and is around 40% glycosylated while fungal laccase is a protein of approximately 60-70 kDa with an isoelectric point of around pH 4.0 and is less glycosylated (Table 1.1). Several laccase isoenzymes have been detected in many fungal species (Table 1.1) and in general, more than one is formed in most fungi (Blaich and Esser, 1975). Most fungal laccases are monomeric enzymes but there are many exceptions, however.

**Table 1.1.** Properties of selected purified laccase and laccase-like enzymes. ABTS as substrate was used to determine the optimum pH and temperature and the $K_M$ (modified from Claus, 2003 and Baldrian, 2006).

| Species | MW (kDa) | pI | Number of isozymes | Temperature optimum (°C) | pH optimum | $K_M$ (μM) | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| **Prokaryotes** | | | | | | | | |
| *Azospirillum lipoferum* | 48.9, 97.8, 179.3 | | 3 | 30 | 6.0 | 34.65 | pigmentation, $e^-$ transport | Diamantidis *et al*., 2000 |
| *Bacillus halodurans* C-125 | 56 | | | 45 | 7.5-8.0 | | $Cu^{2+}$ resistance | Ruijssenaars and Hartmans, 2004 |
| *Bacillus sphaericus* | | | | | | | sporulation, pigmentation | Claus and Filip, 1997 |
| *Bacillus subtilis* (*cot*A) | 65 | 7.7 | 1 | 75 | 3 | 106 | UV and $H_2O_2$ resistance | Martins *et al*., 2002 |
| *Escherichia coli* (*yac*K) | 56 | | 1 | 55/70 | 6.5 | 70.5 (DMP) | ferrooxidase activity | Kim *et al*., 2001; Roberts *et al*., 2002 |
| *Oceanobacillus iheyensis* (*cot*A) | 59 | | | | | | sporulation | Takami *et al*., 2002 |
| *Pseudomonas putida* (*cum*A) | 50 | | | | | | Mn oxidation | Brouwers *et al*., 1999 |
| *Sinorhizobium meliloti* CE52G | 45 | 6.2 | 1 | | | | | Rosconi *et al*., 2005 |
| *Streptomyces griseus* (*epo*A) | 100 | | 1 | 40 | 6.5 | 420 | pigmentation, morphogenesis | Endo *et al*., 2002, 2003 |
| *Streptomyces coelicolor* | 69 | 6.2 | 1 | | 9.4 | 400 (DMP) | | Machczynski *et al*., 2004 |
| *Thermus thermophilus* HB27 | 53 | > 7.18 | 1 | 92 | 4.5 | 900 | | Miyazaki, 2005 |

| Species | MW (kDa) | pI | Number of isozymes | Temperature optimum (°C) | pH optimum | $K_M$ (μM) | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| **Fungus** | | | | | | | | |
| **Basidiomycota** | | | | | | | | |
| *Agaricus bisporus* | 65, 96 | | 2 | | | | lignin degradation | Wood, 1980; Perry *et al*., 1993 |
| *Coprinus cinereus* | 58 | 4.0 | 1 | 60-70 | 4.0 | 26 | remove toxic phenolic compounds | Schneider *et al*., 1999 |
| *Coriolopsis rigida* | 66 | 3.9 | 1 | | 2.5 | 12 | lignin degradation | Saparrat *et al*., 2002 |
| *Daedalea quercina* | 69 | 3.0 | 1 | 70, 55 | 2.0 | 38 | decolorize synthetic dyes | Baldrian, 2004 |
| *Lentinula edodes* Lcc1 | 72 | 3.0 | 1 | 40 | 4.0 | 108 | melanization | Nagai *et al*., 2002 |
| *Phellinus ribis* | 152 | | 1 | | 5.0 | 207 | | Min *et al*., 2001 |
| *Pleurotus ostreatus* POXA1w | 61 | 6.7 | 1 | 45-65 | 3.0 | 90 | delignification | Palmieri *et al*., 1997 |
| *Pycnoporus cinnabarinus* | 81 | 3.7 | 1 | | 4.0 (GUA) | | lignin degradation, bioremediation | Eggert *et al*., 1996 |
| *Trametes versicolor* | 68 | | 1 | 55 | 2.5 | 37 | delignification bioremediation | Rogalski *et al*., 1990 |
| *Volvariella volvacea* | 58 | 3.7 | 1 | 45 | 3.0 | 30 | | Chen *et al*., 2004 |
| **Ascomycota** | | | | | | | | |
| *Aspergillus nidulans* | 80 | | 1 | 55 | 6.5 (DMP) | | pigment synthesis | Scherer and Fischer, 1998 |
| *Chalara paradoxa* | 67 | | 1 | | 4.5 | 770 | | Robles *et al*., 2002 |
| *Colletotrichum graminicola* | 85 | | 1 | | 6.0 (SYR) | 214 (SYR) | | Anderson and Nicholson, 1996 |
| *Coniothyrium minitans* | 74 | 4.0 | 1 | 60 | 3.5 (DMP) | 100 (DMP) | | Dahiya *et al*., 1998 |
| *Gaeumannomyces graminis* | 190 | 5.6 | 1 | | 4.5 (DMP) | 26 (DMP) | protection | Edens *et al*., 1999 |

| Species | MW (kDa) | pI | Number of isozymes | Temperature optimum (°C) | pH optimum | $K_M$ (μM) | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| *Magnaporthe grisea* | 60 | | 1 | 30 | 6.0 (SYR) | 118 (SYR) | | Iyer and Chattoo, 2003 |
| *Mauginiella sp.* | 63 | 4.8-6.4 | 1 | | 2.4 | | | Palonen *et al*., 2003 |
| *Melanocarpus albomyces* | 80 | 4.0 | 1 | 65 | 3.5 | | | Kiiskinen *et al*., 2002 |
| *Neurospora crassa* | 64 | | 1 | | | | | Froehner and Eriksson, 1974 |
| *Ophiostoma novo-ulmi* | 79 | 5.1 | 1 | | 2.8 | | protection against pathogens | Binz and Canevascini, 1997 |
| **Plants** | | | | | | | | |
| *Acer pseudoplatanus* | 97 | | 1 | | 6.6 (4-MCC) | 4500 | lignification | Bligny and Douce, 1983 |
| *Camellia sinesis* (tea) | 144 | | 1 | | 5.0 (4-MCC) | | | Gregory and Bendall, 1966 |
| *Liriodendron tulipifera* | 61 | 9.3-9.5 | 1 | | | | lignification | LaFayette *et al*., 1999 |
| *Lolium perenne LAC2-1* | 62 | 5.8 | 1 | | | | | Gavnholt *et al*., 2002 |
| *Nicotiana tabacum* | 62, 59 | 10.0 | 2 | | | | | Kiefer-Meyer *et al*., 1996 |
| *Picea sitchensis* | 48, 120 | | 2 | | | | lignification | McDougall, 2000 |
| *Pinus taeda* | 90 | 9.0 | 1 | | 5.9 | 12000 (coniferyl alc.) | lignin biosynthesis | Bao *et al*., 1993 |
| *Populus euramericana* | 90, 110 | | 2 | | | 26 | lignification | Ranocha *et al*., 1999 |
| *Rhus vernicifera* | 100 | | 1 | 25-30 | 9.0 (coniferyl alcohol) | | lignin biosynthesis | Shiba *et al*., 2000 |

ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); DMP, 2,6-dimethoxyphenol; SYR, 4-hydroxy-3,5-dimethoxybenzaldehyde [(4-hydroxy-3,5-dimethoxyphenyl)methylene]hydrazone (syringaldazine); GUA, 2-methoxyphenol (guaiacol); 4-MCC, 4-methylcatechol.

*Active site*

Laccases contain three different subunits where four copper ions are coordinated at the active site of each enzyme molecule. These copper ions are bound to the interfaces between subunits (domains, Figure 1.2).

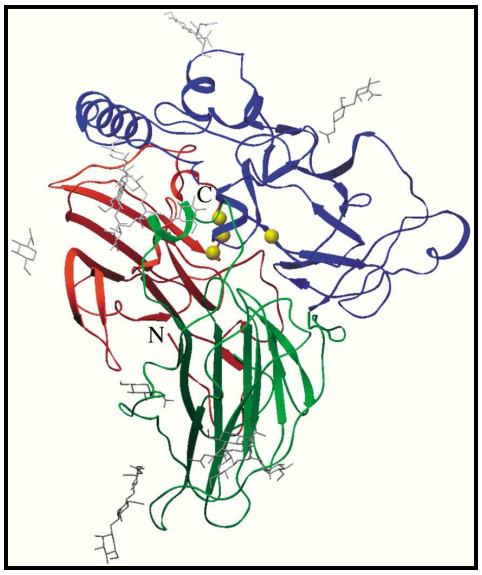



**Figure 1.2.** Three-dimensional structure of *Melanocarpus albomyces* laccase (the three different subunits are in different colours, yellow balls indicate copper atoms) (taken from Hakulinen *et al*., 2002).

For full catalytic activity, laccases need a minimum of four Cu atoms (three types) per monomeric molecule (Claus, 2004). Type 1 copper confers the typical blue colour to multicopper proteins and the substrate oxidation takes place on this site. Type 1 copper has an absorbance of approximately 600 nm, gives a blue colour to concentrated laccase solutions (Leontievsky *et al*., 1997a) and is electron paramagnetic resonance (EPR) detectable. Type 2 copper shows no absorption in the visible spectrum and displays paramagnetic properties in EPR studies. It is rather close to the type 3 copper site that contains a diamagnetic spin-coupled copper-copper pair and can be characterized by first an electron absorption at 330 nm and second by the absence of an EPR signal (Thurston, 1994; Decker and Terwillinger, 2000; Claus, 2004).

However, some laccases do not show these typical characteristics (Bar, 2001). For example not all laccases are reported to possess four copper atoms (Thurston, 1994) per monomeric molecule. Palmieri *et al*. (1997) determined by atomic absorption spectrophotometry that one of

the laccases from *Pleurotus ostreatus* consist of one copper atom, two zinc atoms and one iron atom instead of typical four coppers. Certain laccase enzymes are not “blue” as expected. Leontievsky *et al*. (1997a, b) demonstrated that some laccases have a yellow or yellow-brown rather than blue colour, and supposed that the colour is formed as a result of blue laccase modification by incorporation of aromatic products of lignin degradation.

*Laccase-catalysed reactions*

As discussed above, laccases use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism (Claus, 2004). Oxygen is reduced to water between three coppers whereas phenolic compounds bind near to the mononuclear copper. Substrate oxidation by laccase is a one-electron reaction generating a free radical (Reinhammar and Malmstrom, 1981; Figure 1.3), which is typically unstable and may undergo a second enzyme-catalysed oxidation, what is non-enzymic reactions and/or may partake in a polymerization reaction (Thurston, 1994).

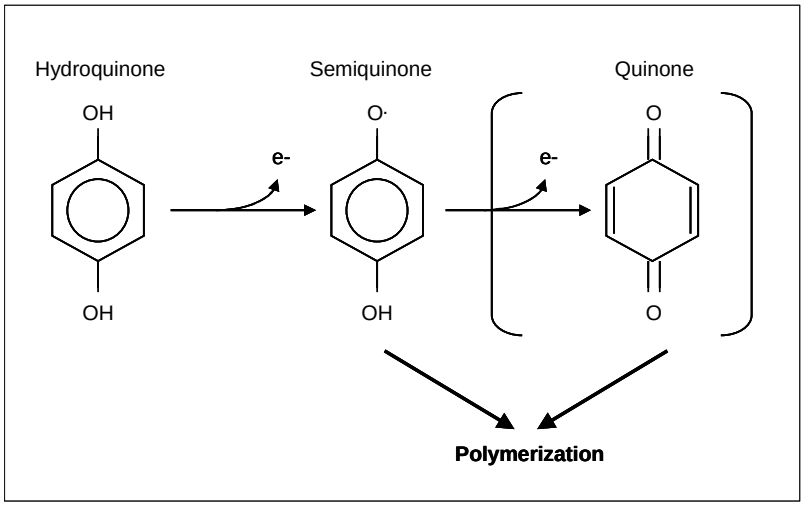



**Figure 1.3.** Typical laccase reaction, where diphenols undergoes a one-electron oxidation to form an oxygen-centred free radical that can be converted to the quinone in a second enzyme-catalysed step or by disproportionation. Quinone and free radical products may undergo polymerization (taken from Thurston, 1994).

### 1.2.4. The possible roles of laccase

Claus (2003) summarizes some possible function of laccase in prokaryotes, and these include sporulation, pigmentation, Mn oxidation and the oxidation of phenolic compounds. Zhu

and Williamson (2004) supposed the role of laccase as a virulence factor for *Cryptococcus neoformans*. In higher plants, laccases participate in the radical-based mechanism of lignin polymer formation (Liu *et al*., 1994), and this is discussed in details below. In addition, McCaig *et al*. (2005) suggested that laccase-like multicopper oxidase gene products have major functions in physiological processes such as wound healing, show ferroxidase activity (Hoopes and Dean, 2004) and may play roles in the metabolism of other essential metals.

*Lignin biosynthesis*

As discussed above, in higher plants laccases probably have a major role in lignin biosynthesis. Lignin is an aromatic biopolymer that has many important properties and function in plants. Lignin is the second most abundant biopolymer after cellulose. It is derived mainly from three alcohol monomers: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 1.4).

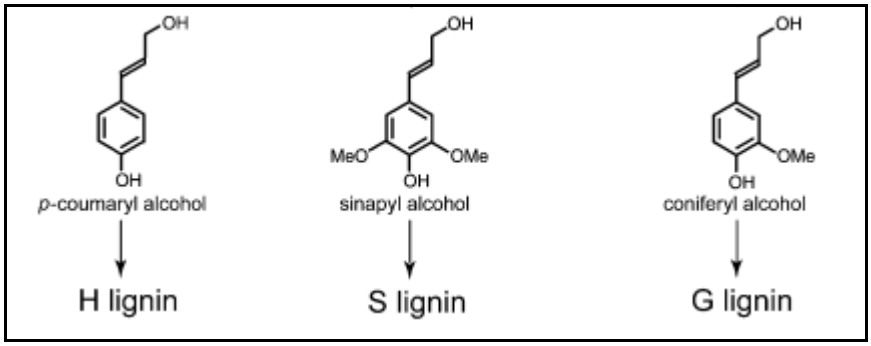



**Figure 1.4.** The structure of monolignols (taken from Vanholme *et al*., 2010).

Monolignols are formed in the cytosol and transported to the apoplast where lignin biosynthesis occurs. The correct mechanism of monolignol polymerization is unclear but probably monolignols are converted into free radicals then polymerize spontaneously. The two enzymes that are catalysing the formation of the monolignol radicals are laccase and peroxidase (Figure 1.5). Laccase activity is more efficient than peroxidase activity because peroxidase activity requires NADH and $H_2O_2$.

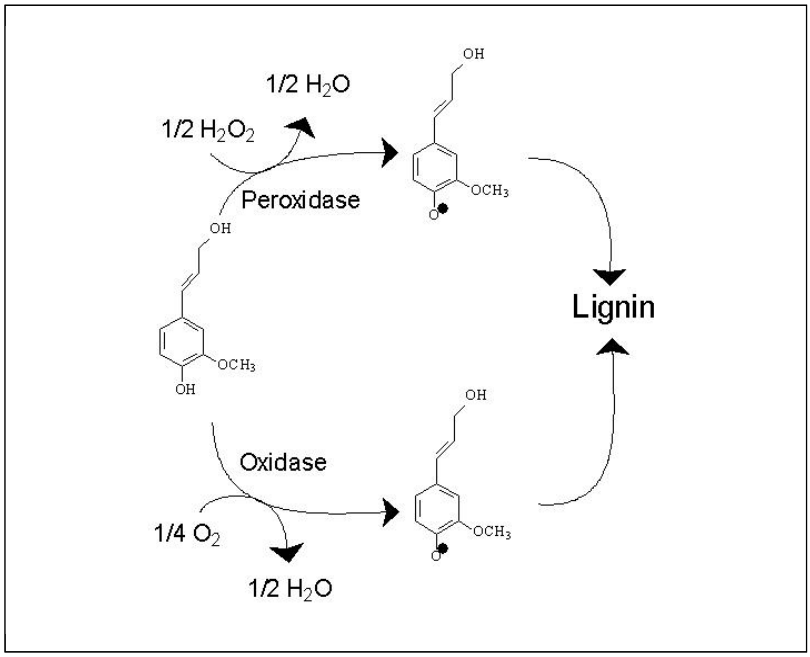



**Figure 1.5.** Lignin biosynthesis. Polymerization of coniferyl alcohol to lignin. The reaction has two alternative routes catalysed by two different oxidative enzymes, peroxidases or oxidases such as laccases (http://en.wikipedia.org/wiki/Lignin).

*Fungal developmental processes and lignin degradation*

Laccases are involved in fungal developmental processes such as sexual morphogenesis and melanization (Zhao and Kwan, 1999; Nagai *et al*., 2003). They are also involved in protection against pathogenic bacteria and fungi (Mayer and Staples, 2002), decolourization of synthetic dyes and probably most importantly in lignin degradation. While many studies have focussed on the role of laccases in fungal growth and development, with the exception of *Aspergillus nidulans* (Aramayo and Timberlake, 1993; Scherer and Fischer, 1998) their role remains unclear (Burke and Cairney, 2002). Aramayo and Timberlake (1993) determined the function of laccase I in *Aspergillus nidulans* as one of the key regulator of asexual development process while Scherer and Fischer (1998) discussed the role of laccase II that is involved in the initiation of early sexual development process such as the yellow pigment formation and hyphal fusion during cleistothecial wall formation. Nagai *et al*. (2003) reported a correlation between gill browning and intracellular laccase activity in *Lentinula edodes*. They supposed that laccase has a role in pigment synthesis in the rind of the cap through typical laccase oxidation reactions (Figure 1.3) that finally form melanin pigments.

However most of the studies have been focussed on the possible role of laccases in lignin degradation. Laccases are involved in the degradation of complex natural polymers, such as

lignin or humic acids (Claus and Filip, 1998) and can also oxidize non-phenolic lignin units in the presence of certain compounds (Bourbonnais and Paice, 1990). While it is clear that lignocellulose-degrading enzymes such as laccase cannot penetrate sound wood (Hammel *et al*., 2002) they can nevertheless use extracellular reactive oxygen species (ROS) to degrade lignin (Koenigs, 1974) through the quinone redox cycling (Figure 1.6).

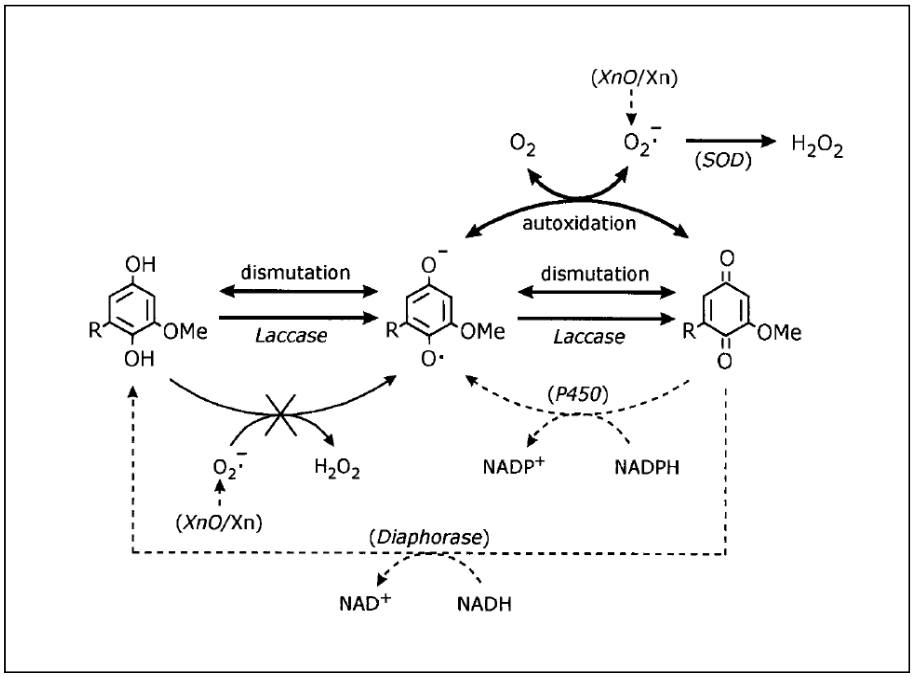



**Figure 1.6.** A possible scheme of the reaction where laccase participate in the production of superoxide anion radical ($O_2\cdot^-$) (ROS) through the quinone redox cycling. The produced $O_2\cdot^-$ can participate in the production of $H_2O_2$ via dismutation by superoxide dismutase (SOD) and able to react indirectly with lignin units. Dashed lines and enzymes in bracket indicate the strategies used to carry out these reactions (taken from Guillen *et al*., 2000).

Guillen *et al*. (1997) demonstrated for the first time in fungi the existence of quinone redox cycling that could generate extracellular superoxide anion radicals ($O_2\cdot^-$). Quinone redox cycling is generally known as an intracellular process that involves the reduction of quinones into semiquinones or hydroquinones. Guillen *et al.* (2000) summarized the possible relation between ROS, quinone redox cycling and lignin degradation (Figure 1.6): fungi are known to extracellularly produce hydrogen-peroxide ($H_2O_2$), hydroxyl radical ($OH\cdot$) and the superoxide anion radical ($O_2\cdot^-$). Autoxidation of semiquinones generated by laccase can generate $O_2\cdot^-$ that can produce $H_2O_2$ via dismutation either spontaneously or in a reaction catalysed by the enzyme superoxide dismutase (SOD) ($2O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2$). $H_2O_2$ can be involved in production

of the hydroxyl radical (OH·) through the iron catalysed Haber-Weiss reaction ($O_2\cdot^- + H_2O_2 \rightarrow OH\cdot + OH^- + O_2$) and/or in biologic systems via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + OH\cdot$). In the latter process the enzyme cellobiose dehydrogenase (CDH), through the hydroquinone/$Fe^{3+}$-oxalate mechanism, generates hydroxyl radicals (Hammel *et al*., 2002). Among reduced oxygen species, OH· is the only one able to react directly with the subunits of lignin (Hammel *et al*., 2002). Consequently laccases may participate in the production of several ROS such as $O_2\cdot^-$, $H_2O_2$ and OH· therefore it may have a role in lignin degradation by fungi.

### 1.2.5. Possible applications of laccase in biotechnology

Laccases offer several advantages of which make them suitable enzymes for biotechnological applications (Baldrian, 2006). These result from their catalytic and electrocatalytic properties (Morozova *et al*., 2007a), and include the possession of a broad substrate specificity and thus the ability to oxidize a great variety of xenobiotic compounds. Industrially, various laccases derived from fungi are used in wine clarification and bioremediation of compounds such as alkenes and herbicides. All approaches and prospects of application of laccases in biotechnology are based on their ability to produce free radicals during the oxidation of different substrates (Guillen *et al*., 2000; Morozova *et al*., 2007a). Morozova *et al*. (2007a) summarize the possible application of laccase enzymes in biotechnology (Figure 1.7).

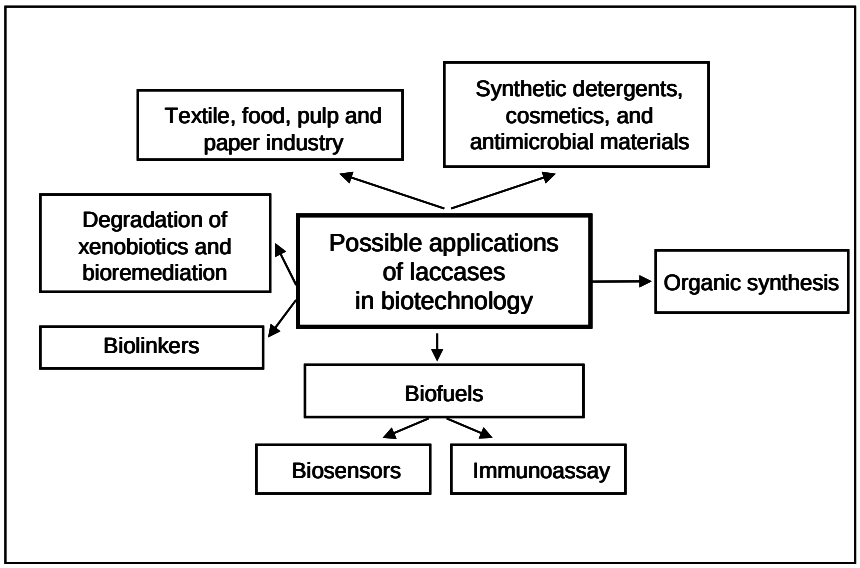



**Figure 1.7.** Possible applications of laccases in biotechnology (taken from Morozova *et al*., 2007a).

During the last decade, many studies have been published on the use of laccases to decolourize dyes (for an overview see: Rodriguez *et al*., 1999; Claus *et al*., 2002; Baldrian, 2004). These authors suggested that decolourization processes are more effective in the presence of redox mediators than without them.

*The Laccase Mediator System*

In many laccase-catalysed reactions the substrate has very high redox potential therefore a redox mediator is required to facilitate the reactions (Burton, 2003). Redox mediators are low molecular weight compounds, that should be good laccase substrates (Morozova *et al*., 2007b), and can be oxidized by laccases to stable radicals (Baldrian, 2006). A laccase mediator system (LMS) was first described by Bourbonnais and Paice (1990). They observed that laccase derived from *Trametes versicolor* in the presence of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) could oxidize non-phenolic lignin compounds with high redox potentials. Since 1990, a wide range of redox mediator compounds have been identified (Morozova *et al*., 2007b) that have biotechnological applications (Bourbonnais *et al*., 1995; Call and Mücke, 1997). For example, they are used for preparative synthesis (Fritz-Langhals and Kunath, 1998) and to degrade environmental chemicals (Majcherczyk *et al*., 1998). Approximately 100 different potential mediator compounds have been described for LMS, including both natural and synthetic mediators. Johannes and Majcherczyk (2000) summarize the natural mediators, such as phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol but the most commonly used are still the synthetic compounds ABTS and 1-hydroxybenzotriazole (HBT).

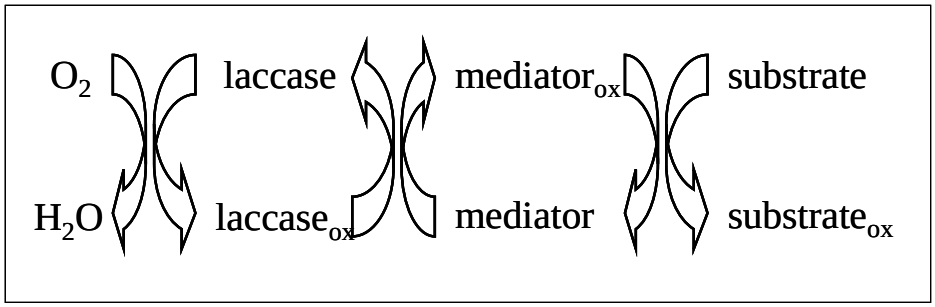



**Figure 1.8.** Schematic illustration of the oxidative catalytic cycle of laccase mediator system (taken from Morozova *et al*., 2007b).

Figure 1.8. demonstrates the laccase-mediator redox cycles. This is a two-step process where the mediator first reacts with the laccase, producing a stable high potential intermediate, and this

oxidized mediator is reduced to the initial form by reacting with the substrate (Burton, 2003; Morozova *et al*., 2007b).

## 1.3. Tyrosinase

### 1.3.1. General

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase enzyme that was first described in 1896 by Bertrand. The enzymes are widely distributed in nature and are essential in the formation of pigments such as melanins and other polyphenolic compounds (Lerch, 1987; van Gelder *et al*., 1997; Claus and Decker, 2006) and may have various other functions. The enzymes occur in bacteria, fungi, plants and in many animals. Two reactions can be catalysed by tyrosinases; hydroxylation of phenols (sometimes called cresolase activity) and oxidation of catechols to *o*-quinones (catecholase activity) (Burton, 2003). The reactive quinones polymerize non-enzymatically to the melanins (Claus and Decker, 2006). However, Claus and Decker (2006) remark that only limited information exists about protein structure and the exact reaction mechanisms of tyrosinases because of the contamination with pigments, occurrence of isoenzymes, post-translational modifications or the low enzyme concentration in eukaryotic cells.

Mayer (2006) summarized the functions and roles of tyrosinases in plants and fungi. The function of polyphenol oxidase (PPO) or tyrosinase in browning reactions and biosynthetic processes is well documented. The role of tyrosinase in plants is under intensive observation. The major focus of research in polyphenol oxidase has been its potential role in defense mechanism such as defense against herbivores and resistance of plants to stress and pathogens. The role of tyrosinase in fungal pathogenicity and fungal defense reactions is much less studied. Tyrosinases have many industrial applications. It is used to detoxify waste waters and soils in environmental technology (Claus and Filip, 1988; 1990). In pharmaceutical industries tyrosinases have very important role in the treatment of Parkinson’s disease while in cosmetic and food industries tyrosinase are utilized in oxidative reactions (Mayer and Harel, 1979).

### 1.3.2. Occurrence and location

In higher plants, the highest activities of tyrosinases occur when the enzyme is upregulated in wounded tissues such as those of apples (Boss *et al*., 1995), potatoes (Thipyapong *et al*., 1995) and tomato leaves (Constabel *et al*., 1995). The enzyme protects the plant against insects and microorganisms by catalysing the formation of melanin. Tyrosinase is

located in plants chloroplast but the synthesis of polyphenol oxidase and its transport to its site in chloroplast is a complex process (Mayer, 2006).

Fungal tyrosinases were firstly characterized from *Agaricus bisporus* (Nakamura *et al*., 1966; Halaouli *et al*., 2006) because of enzymatic browning phenomenon during development and postharvest storage which decreased the commercial value of the product (Jolivet *et al*., 1998). Recently, there has been increasing interest in the biochemical characteristics of mushroom-tyrosinase, and especially the potential of fungal tyrosinases in biotechnological and environmental applications. Further studies on tyrosinases from other fungi have been carried out in *Neurospora crassa* (Kupper *et al*., 1989), *Lentinula edodes* (Kanda *et al*., 1996), *Aspergillus oryzae* (Nakamura *et al*., 2000), *Pycnoporus sanguineus* (Halaouli *et al*., 2005) and *Trichoderma reesei* (Selinheimo *et al*., 2006). Fungal tyrosinases are mostly cytosolic enzymes with a considerable heterogeneity but there are reports that they can be located extracellularly (Rast *et al*., 2003; Halaouli *et al*., 2006). Secretion of tyrosinases was first described by Selinheimo *et al*. (2006) in the culture supernatant of the filamentous fungus *Trichoderma reesei*. Selinheimo *et al*. (2006) inferred that *Gibberella zeae*, *Neurospora crassa* and *Magnaporthe grisea*, as ascomycetes fungi, also have secreted tyrosinases because these fungi have the correct signal sequence.

Tyrosinases have been investigated in many prokaryotes, including both Gram-negative and Gram-positive bacteria. Intensive biochemical investigations have been carried out on the members of the genus *Streptomyces* (see review by Claus and Decker, 2006), for example *Streptomyces nigrifaciens* (Nambudiri and Bhat, 1972), *S. glaucescens* (Lerch and Ettlinger, 1972), *S. antibioticus* (Bernan *et al*., 1985) and *S. michiganensis* (Philipp *et al*., 1991). Secreted tyrosinases have been characterized from the bacterium *Streptomyces.* Here, they were assisted by a second protein and did not have signal sequences (Leu *et al*., 1992; Tsai and Lee, 1998) as were found in *Trichoderma reesei* (Selinheimo *et al*., 2006). Heat-inducible tyrosinases purified from *Bacillus thuringiensis* have the lowest molecular masses of these enzymes that have ever been recorded (Liu *et al*., 2004). Thermostable tyrosinases have been isolated from *Thermomicrobium roseum* (Kong *et al*., 2000) while other tyrosinases were demonstrated in *Vibrio tyrosinaticus* (Pomerantz and Murthy, 1974) and *Pseudomonas melanogenum* (Yoshida *et al*., 1974) as typical example of Gram-negative bacteria. Tyrosinases have been also purified from a marine bacterium, the *Marinomonas mediterranea* (Lopez-Serrano *et al*., 2002). Bacterial tyrosinases are located both in intra- and extracellularly in these species. Interestingly,

tyrosinases from prokaryotes are not glycosylated unlike those observed in eukaryotic organisms (Claus and Decker, 2006).

Tyrosinases have been also found in sponges, many invertebrates such as arthropods and in vertebrates, mainly in mammals. In insects, tyrosinases have major role in sclerotization of the exoskeleton and in protection, while in mammals it is responsible for the development of melanomas and for and it has a role in pigmentation disorders such as albinism and vitiligo (van Gelder *et al*., 1997; Riley, 1997; Halaouli *et al*., 2006).

### 1.3.3. Structure and reactions of tyrosinase enzyme

*Structural properties*

It is well known from the literature that tyrosinases are variable in their properties, distribution and cellular location. Many authors suppose there is no common tyrosinase because the enzymes found in many organisms are different in respect to their sequences, size, glycosylation and activation (Jaenicke and Decker, 2003; Mayer, 2006). The molecular weight of bacterial tyrosinases varies between 14 and 43 kDa (Table 1.2.), and the enzymes may or may not possess post-translational processing such as proteolytic activation of proenzymes or glycosylations (Claus and Decker, 2006). Typical plant tyrosinase has a molecular weight of approximately 42-60 kDa, while fungal tyrosinase is a protein of approximately 30-70 kDa with an acidic isoelectric point. However, the isoelectric point of the filamentous fungus *Trichoderma reesei tyr*2 has been reported to be pH 9.5 (Selinheimo *et al*., 2006; Table 1.2). Plant tyrosinases are mostly glycosylated while in most fungi no N-glycosylation has been found. Several tyrosinase isoenzymes have been detected in fungal and bacterial species but only one in fruit and vegetable bodies (Table 1.2). Tyrosinases found in bacteria, fungi and plants are monomeric proteins with few exceptions.

Many tyrosinases from the investigated organisms exist in both latent and active forms (Whitaker, 1995; van Gelder *et al*., 1997). The inactive form of tyrosinases (about 60-90% of the total enzyme found in mushroom and plants) seems to be very stable but activation results in a greater sensitivity to temperature and pH (Söderhäll, 1995). The latent form can be activated by SDS, methyl jasmonate, proteases such as trypsin or by stresses such as wounding and acid shock (Mayer, 2006).

**Table 1.2.** Properties of selected purified tyrosinase and tyrosinase-like enzymes. L-DOPA as substrate was used to determine the optimum pH and temperature and the $K_M$ (modified from Claus and Decker, 2006 and Halaouli *et al*., 2006).

| Species | MW (kDa) | pI | Number of isozymes | Temperature optimum (°C) | pH optimum | $K_M$ (μM) | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| **Prokaryotes** | | | | | | | | |
| **Gram-negative bacteria** | | | | | | | | |
| *Thermomicrobium roseum* | 43 | 4.9 | 1 | 70 | 9.5 | 180 | | Kong *et al*., 2000 |
| *Vibrio tyrosinaticus* | 41, 38.5 | | 2 | | | 3100 (L-tyr) | | Pomerantz and Murthy, 1974 |
| **Gram-positive bacteria** | | | | | | | | |
| *Bacillus thuringiensis* | 14 | | 1 | 75 | 9.0 | | | Liu *et al*., 2004 |
| *Streptomyces antibioticus* | 29.5 | | 1 | | | | | Bernan *et al*., 1985 |
| *Streptomyces glaucescens* | 29.1 | 6.95 | 1 | | 6.8 | | | Lerch and Ettlinger, 1972 |
| *Streptomyces michiganenis* DSM 40015 | 32, 34.5 | 9.0 | 2 | 33 | 7.0 | | | Philipp *et al*., 1991 |
| *Streptomyces nigrifaciens* | 18 | | 1 | 40 | 7.7 | | | Nambudiri and Bhat, 1972 |
| **Fungus** | | | | | | | | |
| **Basidiomycota** | | | | | | | | |
| *Agaricus bisporus* | 43, 47 | 5.2, 5.1 | | | | 440 | melanin synthesis | Wichers *et al*., 1996 |
| *Amanita muscaria* | 60 | | 1 | | 6.0 | 1200 | betalain biosynthesis | Mueller *et al*., 1996 |
| *Lentinula edodes* | 70-105 | 4.3-4.7 | 6 | | 6.0-6.5 | 74-2201 | | Kanda *et al*., 1996 |
| *Pycnoporus sanguineus* CBS 614.73 | 45 | 4.5-5.0 | 4 | 30 | 6.5-7.0 | 900 | phenolic metabolism | Halaouli *et al*., 2005 |

| Species | MW (kDa) | pI | Number of isozymes | Temperature optimum (°C) | pH optimum | $K_M$ (μM) | Function | Reference |
|---|---|---|---|---|---|---|---|---|
| **Ascomycota** | | | | | | | | |
| *Aspergillus oryzae* | 67 | | 1 | | | 820 (L-tyr) | | Nakamura *et al*., 2000 |
| *Neurospora crassa* | 46 | | 1 | | | | | Kupper *et al*., 1989 |
| *Trichoderma reesei* tyr2 | 43 | 9.5 | 1 | 30 | 9.0 | 3000 | | Selinheimo *et al*., 2006 |
| **Plants** | | | | | | | | |
| *Daucus carota* subsp. s*ativus* (Hoffm.) Arc. | 59 | | 1 | 65 | 8.0 | | | Söderhäll, 1995 |
| *Lycopersicon esculentum* L. | 43 | | | | | | | Sommer *et al*., 1994 |
| *Malus domestica* Borkh. | 45 | | 1 | 30 | 6.5 | | pathogen defence | NiEidhin *et al*., 2006 |
| *Solanum tuberosum* L. | 42 | | 1 | | | | | Thipyapong *et al*., 1995 |
| *Vicia faba* L. | 60 | | | | | | pathogen defence | Robinson and Dry, 1992 |

L-DOPA, 3,4-dihydroxy-L-phenylalanine; L-tyr, 4-hydroxyphenylalanine (L-tyrosine).

*Active site*

Tyrosinases, like laccases, are multicopper oxidase. The enzymes have at least one common feature in that they all contain a binuclear type 3 copper centre in their active site (Mayer, 2006). Therefore, tyrosinase is different to laccase because it contains only two copper ions at one reaction site in each functional unit of the enzyme (Burton, 2003) that are each coordinated with three histidine residue (Figure 1.9).

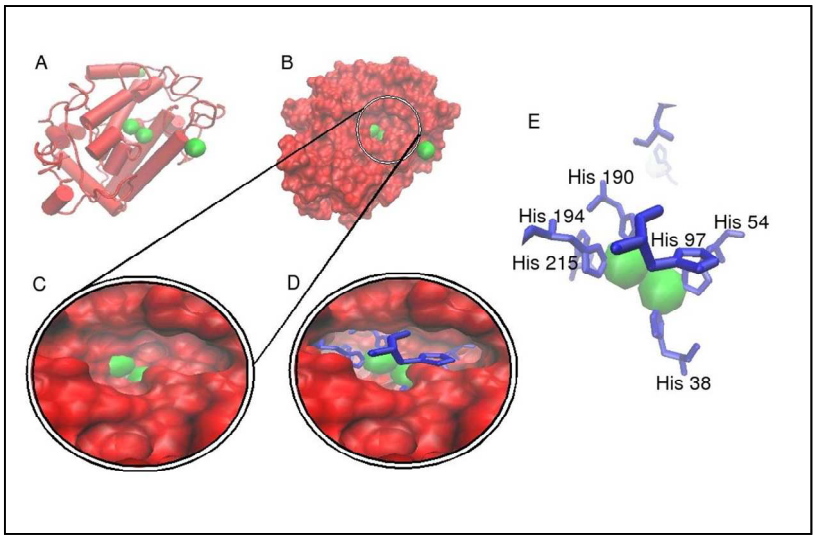



**Figure 1.9.** The active site of the tyrosinase molecule (the copper atoms are shown in green, the molecular surface is indicated in red and histidine amino acids are shown as a blue line) (http://en.wikipedia.org/wiki/Tyrosinase).

Figure 1.9. shows (models C and D) that the active site for this protein lies on the surface of the molecule. The two copper atoms within the active site of tyrosinase enzymes interact with dioxygen to form a highly reactive chemical intermediate that then oxidizes the substrate.

Chemical and spectroscopic studies of tyrosinase have indicated that the active site of the enzyme is similar to haemocyanins and catechol oxidases (Sanchez-Ferrer *et al*., 1995; Halaouli *et al*., 2006). They are also similar with respect to spectroscopic and paramagnetic properties (Himmelwright *et al*., 1980), primary sequence (van Gelder *et al*., 1997) and in the reactions catalysed by the enzymes (Decker and Rimke, 1998; Espin *et al*., 1998; Jaenicke and Decker, 2004).

*Tyrosinase-catalysed reactions*

Tyrosinases use molecular oxygen to catalyse two different enzymatic reactions (Figure 1.10; Solomon *et al*., 1996; van Gelder *et al*., 1997; Land *et al*., 2003; Claus and Decker, 2006): a) the *ortho*-hydroxylation of monophenols to *o*-diphenols (monophenolase, cresolase activity) and b) the oxidation of *o*-diphenols to *o*-quinones (diphenolase, catecholase activity).

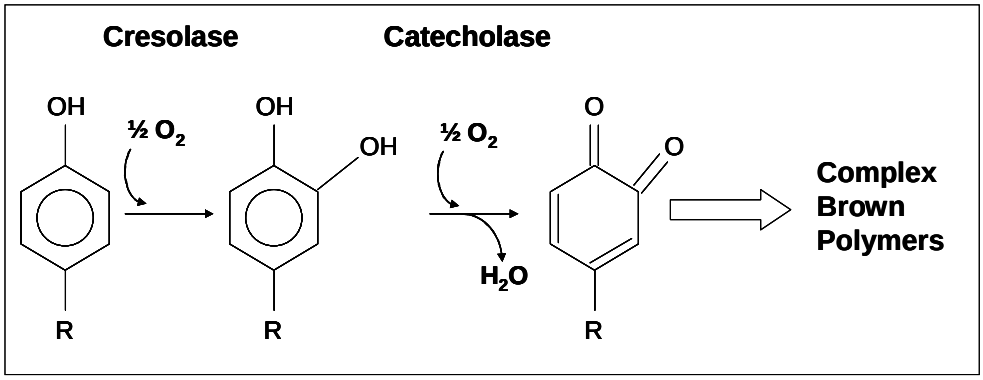



**Figure 1.10.** Cresolase and catecholase activity of tyrosinase. Molecular oxygen is used in both reactions (taken from van Gelder *et al*., 1997).

These reactions may be separate or sequential. The quinone formed generally undergoes further, non-enzymic reactions resulting in polymerization, particularly in aqueous medium, producing intermediates which associate spontaneously in dark brown pigments and related polyphenolic polymers (Soler-Rivas *et al*., 1997; Burton, 2003). The cresolase activity of tyrosinases is often much lower than their catecholase activity (Selinheimo *et al*., 2007). In particular, plant tyrosinases show low or no monophenolase activity (Martinez and Whitaker, 1995; Selinheimo *et al*., 2007).

### 1.3.4. The possible roles of tyrosinase

As discussed above, the best-documented function of the enzyme is melanization. The enzyme is responsible for the first steps of melanin synthesis from L-tyrosine to the formation of L-dopaquinone and L-dopachrome. These first steps from monophenol/diphenol to *o*-quinone are the most critical from a biological point of view (Figure 1.11; Sanchez-Ferrer *et al*., 1995; Halaouli *et al*., 2006).

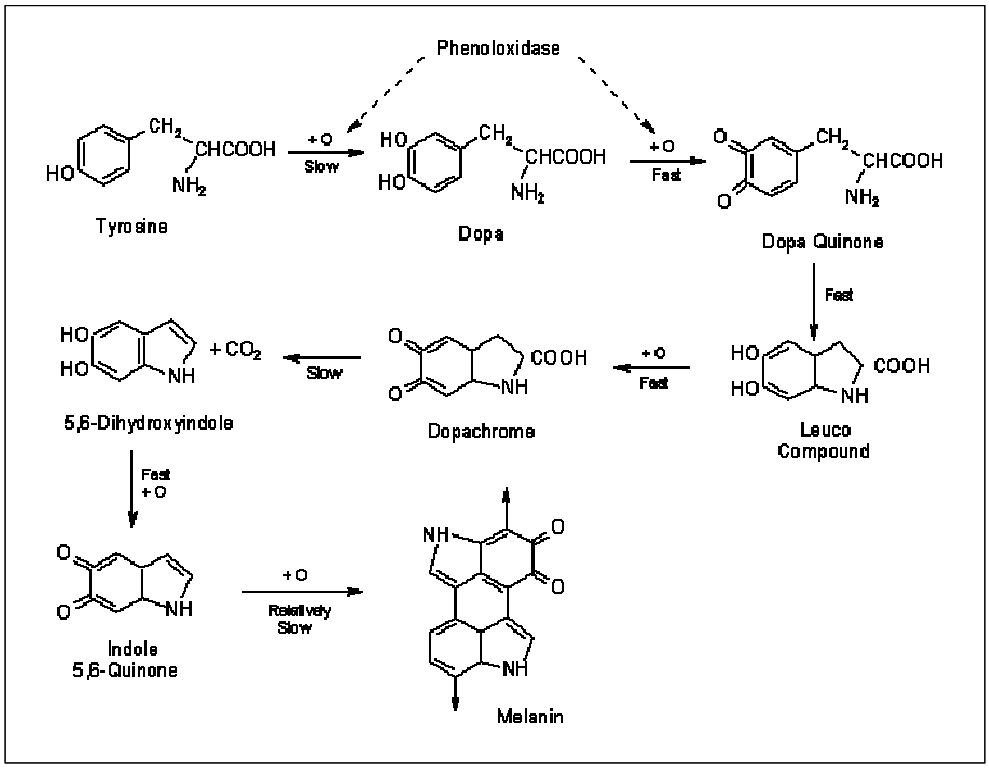



**Figure 1.11.** Formation of melanin from tyrosine (taken from Lerner, 1953).

The dark pigments, melanin, protect the cells of bacteria, fungi, plants and animals against UV radiation (Butler and Day, 1998), free radicals and other oxidants, heat and enzymatic hydrolysis (Claus and Decker, 2006). Melanin protects the spores of many organisms against the harmful radiation and promotes the processes of sporulation and its stability. Jacobson (2000) reviewed the role of fungal melanin in pathogenesis. In addition to the protective role of melanin, it was found to play an important function in the osmotic penetration of the plant cell wall by the appressorium of the fungus (Jacobson, 2000; Mayer, 2006). Melanin has been demonstrated in lichens where the pigment participates in the protection of lichens against high light intensities and extreme climatic conditions (Stepanenko *et al*., 2002). It is recognized that melanin can be induced by UV-B radiation in many lichen species such as *Cladonia rangiferina* (Nybakken and Julkunen-Tiitto, 2006), *Lobaria pulmonaria* (Solhaug *et al*., 2003) or *Umbilicaria rossica* (Stepanenko *et al*., 2002). It is well-known that tyrosinase derived from the members of most families of the Caryophyllales such as *Portulaca grandiflora* and *Beta vulgaris* are able to participate in betalain formation (Mueller *et al*., 1997; Steiner *et al*., 1999; Strack *et al*., 2003). Unexpectedly, betalains were also found in some higher fungi (Strack *et al*., 2003). Mueller *et al*. (1996) suggested that tyrosinase isolated from *Amanita muscaria* is involved in the biosynthesis of betalains. Later, Gandia-Herrero *et al*. (2005) presented similar results but these suggestions require more convincing evidence (Mayer, 2006). Tyrosinases

have also some important role not only in biosynthetic processes but also in the resistance of plants to stress, pathogens and herbivores. These observations are based on the fact that tyrosinases are involved in the generation of reactive oxygen species (Thipyapong *et al*., 2004). Claus and Filip (1990) suggested that extracellular tyrosinases may have a role in the polymerization and detoxification of plant phenolic compounds and the formation of humic acid, in the soil environment. Further, tyrosinases also play an essential role in defence reactions and sclerotization in invertebrates (Sugumaran, 2002) and in regulation of oxidation-reduction potential and in the wound healing systems in plants (Mayer, 1987; Walker and Ferrar, 1998).

### 1.3.5. Applications of tyrosinases

Tyrosinases have many applications in biotechnology, food processing, medicine and textile, pulp and paper industry, due to their ability to polymerize compounds (Selinheimo, 2008). Tyrosinases are used in food and non-food processes due to their crosslinking abilities (Selinheimo *et al*., 2007; Selinheimo, 2008). The enzymes can detoxify the phenol-containing waste water and contaminated soils (Claus and Filip, 1990) and can act as biosensors for monitoring of phenols in the environmental technology (Claus and Decker, 2006). Tyrosinase is also used in pharmaceutical industries due to its ability to produce *o*-diphenols such as L-DOPA which is the precursor of dopamine involved in the treatment of Parkinson's disease (Raju *et al*., 1993).

## 1.4. Introduction to the present study

Recently, Beckett *et al*. (2003) showed that lichens in the suborder Peltigerineae have high rates of extracellular redox activity. The authors suggested these species can defend themselves against pathogens by producing ROS that may be directly toxic to invading pathogens. In higher plants, the rapid production of extracellular ROS, often called the 'oxidative burst', is believed to play an important role in plant defense against pathogenic infections (Murphy *et al*., 1998). In higher plants, potential extracellular ROS-producing enzymes include plasma membrane NAD(P)H dehydrogenases or oxidases, and/or cell wall peroxidases (Luthje *et al*., 2000; Blee *et al.*, 2001). The aims of this study were to determine which enzymes are responsible for redox activity in Peltigeralean lichens, and to try and elucidate their roles in lichen biology. Potential extracellular radical generating enzymes in fungi include peroxidases, laccases and tyrosinases. Preliminary results indicated that lichens had very low surface peroxidase activities, and therefore the basic properties of laccase and tyrosinase were investigated, using the locally abundant *Pseudocyphellaria aurata* as a test species. The molecular masses of the enzymes were estimated using polyacrylamide gel electrophoresis. The substrate specificity of the enzyme and the effect of inhibitors, pH and temperature on enzyme activity were determined. The potential applications of lichen laccases and tyrosinases for the detoxification of xenobiotics were also evaluated.

# CHAPTER 2

## MATERIALS AND METHODS

*Pseudocyphellaria aurata* (Ach.) Vaino was used as test species.

Most specimens were collected from *Leucosidea sericea* (*Rosaceae*)

(seen here).

## 2.1. Lichen material

Table 2.1. lists the species of lichens examined in this study and their collection localities. Specimens collected from the field fully hydrated were equilibrated in a controlled environment chamber for 48 h at 15°C and a photosynthetic photon fluence rate (PPFR) of 75 µmol photons $m^{-2}$ $s^{-1}$ of continuous fluorescent light. All light intensities were measured across photosynthetically active wavebands using the light meter in a Parkinson leaf chamber designed for an Analytical Development Corporation Mark III portable infra-red gas analyzer (Hoddeston, UK). Lichens collected dry were gradually rehydrated using air at a relative humidity of 100% for 48 h (over distilled water) at 15°C and a PPFR of 75 µmol $m^{-2}$ $s^{-1}$, followed by contact with wet filter paper for a further 24 h. They were then used immediately. Exceptions to the above were the lichens collected in Russia and Estonia. If collected moist, they were allowed to slowly dry between sheets of newspaper. All specimens were then stored refrigerated for up to 6 weeks after collection, and then rehydrated as above.

*Preparation of lichen discs or fragments*

For experiments on whole lichens, to reduce variability, in species with large lobes 6 mm disks were cut from the thalli. For species in which disks could not be cut, thalli fragments were cut into 1 cm strips. In all cases, large collections of lichens were made, and the thalli used were randomly sampled from those thalli that appeared most healthy. Disks or thallus strips were stored as above for least 12 h before an experiment to minimize any effect of mechanical disruption on enzyme activity, and then used immediately.

*Preparation of lichen leachates*

Lichen leachates were prepared by shaking lichens thalli (*ca*. 10 g fresh mass) in 50 ml distilled water with the pH adjusted to 7.0 for 1 h. The resulting leachates were filtered through Whatman number 1 filter paper and used immediately or concentrated by dialysis. Dialysis tubing (molecular mass cut off 10 kilodalton (kDa), Sigma, St. Louis, MO) was placed either directly on sucrose or suspended in a 20% solution of polyethylene glycol (PEG) 20000 (Fluka, Seelze, Germany) at 4°C; typically, dialysis for 10 h reduced the volume to ca. 2 ml. Concentrated leachates were stored at -60°C until needed. On average, 52% of activity was lost during dialysis. Absorption spectra of concentrated leachates were determined from 250 to 700 nm. For electrophoresis, laccase activity was further increased by centrifuging dialyzed

leachates at 4000 × *g* for 20 min in “Ultrafree” microconcentrators with a molecular mass cut off 10 kDa (Millipore, Bedford, MA).

**Table 2.1.** Lichens that were tested in our experiments and their collection localities.

| Species | Collection locality |
|---|---|
| **Lichens from suborder Peltigerineae** | |
| *Collema flaccidum* (Ach.) Ach. | Drakensberg, Cape Town, RSA |
| *Leptogium saturninum* (Dicks.) Nyl. | Drakensberg, RSA |
| *Leptogium sessile* Vainio | Drakensberg, Umgeni Valley Nature Reserve, RSA |
| *Lobaria pulmonaria* (L.) Hoffm. | Sortavala, Russia |
| *Lobaria scrobiculata* (Scop.) DC. | Drakensberg, RSA |
| *Nephroma arcticum* (L.) Torss. | White Sea, Russia |
| *Nephroma parile* (Ach.) Ach. | Lahemma, Estonia |
| *Nephroma rufum* (C. Bab.) | Drakensberg, RSA |
| *Peltigera apthosa* (L.) Willd. | Sortavala, Russia |
| *Peltigera canina* (L.) Willd. | Sortavala, White Sea, Russia |
| *Peltigera didactyla* (With.) J R Laundon | Sortavala, Russia |
| *Peltigera horizontalis* (Huds.) Baumg. | Sortavala, Russia |
| *Peltigera hymenina* (Ach.) Delise | Drakensberg, RSA |
| *Peltigera leucophlebia* (Nyl.) Gyelnik | Sortavala, White Sea, Russia |
| *Peltigera malacea* (Ach.) Funck | Sortavala, Russia |
| *Peltigera neopolydactyla* (Gyelnik) Gyelnik | Sortavala, White Sea, Russia |
| *Peltigera polydactyla* (Necker) Hoffm. | Sortavala, White Sea, Russia |
| *Peltigera praetextata* (Flörke ex Sommerf) Zopf. | Drakensberg, RSA; Sortavala, Russia |
| *Peltigera rufescens* (Weiss) Humb. | Drakensberg, RSA; White Sea, Russia |
| *Peltigera scabrosa* Th. Fr. | Sortavala, White Sea, Russia |
| *Pseudocyphellaria aurata* (Ach.) Vaino | Nottingham Road, Dargle Valley, Cape Town, RSA |
| *Pseudocyphellaria gilva* (Ach.) Malme | Cape Town, RSA |
| *Solorina crocea* (L.) Ach. | Murmansk Oblast, Russia |
| *Sticta fuliginosa* (Dicks.) Ach. | Nottingham Road, Drakensberg, RSA |
| *Sticta cf. limbata* (Sm.) Ach. | Pietermaritzburg, Cape Town, RSA |
| *Sticta cf. sublimbata* (Steiner) Swinscow and Krog | Drakensberg, Umgeni Valley Nature Reserve, RSA |
| *Sticta sp.* | Cape Town, RSA |
| **Lichens from non-suborder Peltigerineae** | |
| *Alectoria sarmentosa* (Ach.) Ach. | White Sea, Russia |
| *Anaptychia ciliaris* (L.) Körb. | Sararemma, Estonia |
| *Bryoria simplicior* (Vanio) Brodo and D. Hawksw. | Sortavala, Russia |
| *Cetraria islandica* (L.) Ach. | Sortavala, Russia |
| *Cladonia cariosa* (Ach.) Spreng. | Sortavala, Russia |
| *Cladonia cf. rangiferina* Hoffm. | White Sea, Russia |
| *Cladonia stellaris* (Opiz) Pouzar and Vezda | Sortavala, Russia |
| *Cladonia cf. uncialis* (L.) F. H. Wigg. | White Sea, Russia |
| *Evernia prunastri* (L.) Ach. | Sortavala, Russia |
| *Flavocetraria nivalis* (L.) Karnefelt and Thell | Sortavala, Russia |
| *Heterodermia speciosa* (Wulf.) Trevis. | Drakesnberg, RSA |
| *Hypogymnia physodes* (L.) Nyl. | Sortavala, Russia |
| *Parmelia cetrarioides* (Delise ex Duby) Nyl. | Umgeni Valley, RSA |
| *Platismatia glauca* (L.) W. L. Culb. and C. F. Culb. | Sortavala, Russia |
| *Pseudevernia furfuracea* (L.) Zopf | Sortavala, Russia |
| *Ramalina celastri* (Sprengel) Krog and Swinscow | Pietermaritzburg, RSA |
| *Ramalina farinacea* (L.) Ach. | Sortavala, Russia |
| *Ramalina pollinaria* (Westr.) Ach. | Sortavala, Russia |
| *Roccella montagnei* Bél. | Umgeni Valley, RSA |
| *Stereocaulon tomentosum* Fr. | Sortavala, Russia |
| *Umbilicaria deusta* (L.) Baumg. | Sortavala, Russia |
| *Umbilicaria pustulata* (L.) Hoffm. | Sortavala, Russia |
| *Usnea undulata* Stirton | Pietermaritzburg, RSA |

## 2.2. Enzyme and protein assays

*ABTS assay method for laccase*

Laccase activity was investigated by the oxidation of ABTS (Fluka, Buchs, Switzerland) to the more stable cation radical ($ABTS^+$) (Figure 2.1; Bourbonnais and Paice, 1990; Min *et al*., 2001). The cation radical is responsible for the blue-green colour.

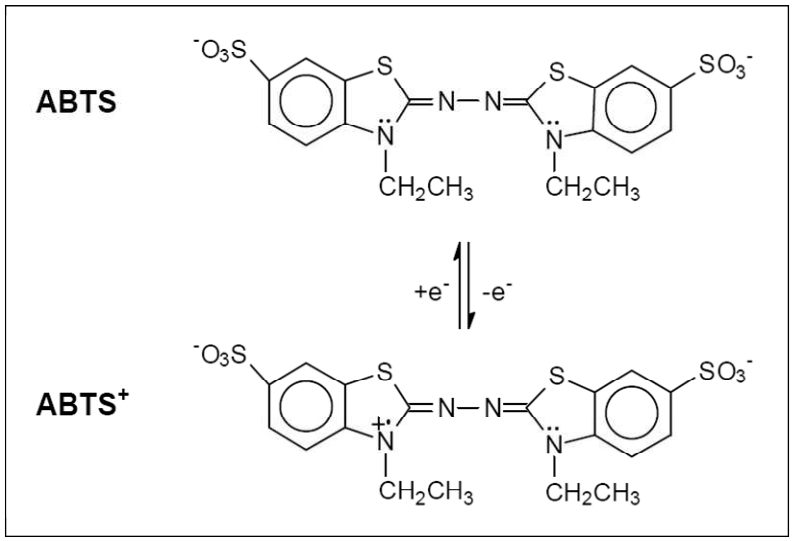



**Figure 2.1.** The laccase-catalysed oxidation of ABTS to a cation radical ($ABTS^+$) (taken from Majcherczyk *et al*., 1998).

For whole thallus assays using ABTS, 30 mg fresh mass of lichen tissue was shaken by a circulating water bath at 60 revolutions $min^{-1}$ (rpm) in 5 ml of 1 mM ABTS dissolved in 25 mM phosphate buffer, pH 5.0, for 15 min at 30°C. To assay leachates or cellular fractions, 10-100 µl of extract, 100 µl of 10 mM ABTS (final concentration 1 mM) and 25 mM phosphate buffer pH 5.0 to give a final volume of 1 ml were incubated for 15 min at 30°C. A stock solution of ABTS was prepared by dissolving the substrate in distilled water. The absorbance of the cation radical was monitored by a Cary 50 UV-Vis spectrophotometer (Varian, USA) at 420 nm. The extinction coefficient of the product measured at $A_{420}$ is 36 $mM^{-1}$ $cm^{-1}$. Enzyme activity was expressed in µmol production $g^{-1}$ dry mass $h^{-1}$ in all assays.

*Syringaldazine assay method for laccase*

This assay is based on the oxidation of 4,4'-[azinobis(methanylylidene)]bis(2,6-dimethoxyphenol) (syringaldazine, Sigma) to its corresponding quinone, 4,4'-[azinobis(methanylylidene)]bis(2,6-dimethoxycyclohexa-2,5-diene-1-one) (Figure 2.2; Medeiros *et al*., 1999). The quinone radical is responsible for the pink-reddish colour.

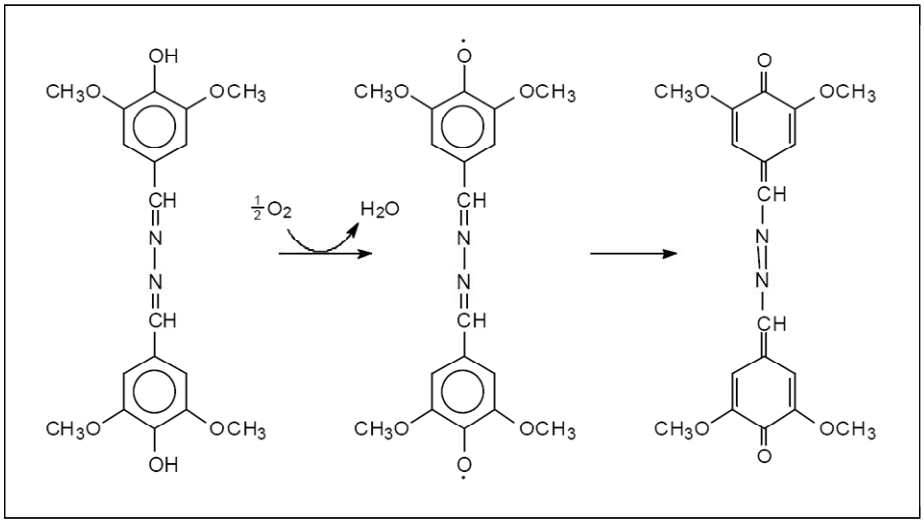



**Figure 2.2.** The laccase-catalysed oxidation of syringaldazine to its corresponding quinone (taken from Sanchez-Amat and Solano, 1997).

Lichen thallus (about 30 mg dry mass of lichen thallus or fragments) was shaken and incubated in 5 ml of 10 µM syringaldazine dissolved in 25 mM phosphate buffer, pH 6.5, for 15 min at 25°C. To assay leachates, 10-100 µl of extract, 100 µl of 100 µM syringaldazine (final concentration 10 µM) and 25 mM phosphate buffer pH 6.5 to give a final volume of 1 ml were incubated for 15 min at 25°C. A stock solution of syringaldazine was prepared by dissolving the substrate in dimethyl sulfoxide (DMSO) (50% of the total volume) then slowly adding the remaining volume of distilled water. The absorbance of the quinone radical was monitored at 525 nm. The extinction coefficient of the product at $A_{525}$ is 65 $mM^{-1}cm^{-1}$.

*o-tolidine assay method for laccase*

This assay method was adapted from a method described by Miller *et al*. (1997). Lichen leachates (200 µl of extract) were incubated in 100 µl of 10 mM 3,3'-dimethylbenzidine (*o*-tolidine, Sigma) (final concentration is 1 mM) dissolved in 25 mM acetate buffer, pH 5.0 to give a final volume of 1 ml were incubated for 15 min at 20°C. The blue coloured product was

measured at $A_{630}$, but the extinction coefficient of the product is unknown. A stock solution of *o*-tolidine was prepared by dissolving the substrate in the mixture of ethanol (50%) and distilled water (50%).

*Epinephrine assay method for tyrosinase*

Tyrosinase activity was estimated by the oxidation of epinephrine (Sigma) to adrenochrome (Figure 2.3; Sugumaran *et al*., 1987).

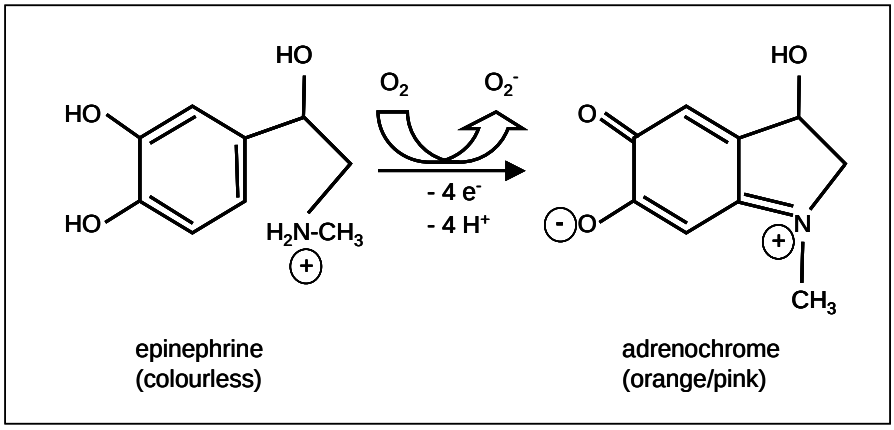



**Figure 2.3.** Schematic illustration about the oxidation of epinephrine to adrenochrome (http://www.csun.edu/~hcchm001/sodassay.pdf).

For whole thallus assays, 150 mg of fresh mass of lichen tissue was shaken by a circulating water bath at 60 rpm in 3.5 ml of 1 mM epinephrine solution, pH 7 (pH adjusted with HCl and NaOH), for 15 min in the dark at 25°C. Leachates or cellular fractions were not tested with this method. The reddish coloured product was monitored at 490 nm. The extinction coefficient of adrenochrome measured at $A_{490}$ is 4.47 $mM^{-1}$ $cm^{-1}$. A stock solution of epinephrine (7 mM) was prepared by dissolving the substrate in 0.5 M HCl, then adding distilled water and adjusting the pH to 7.0.

*L-DOPA assay method for tyrosinase*

Tyrosinase activity was determined by the method of Horowitz *et al*. (1970). This method is based on the oxidation of L-dihydroxyphenylalanine (L-DOPA, Sigma) to 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) (Figure 2.4).

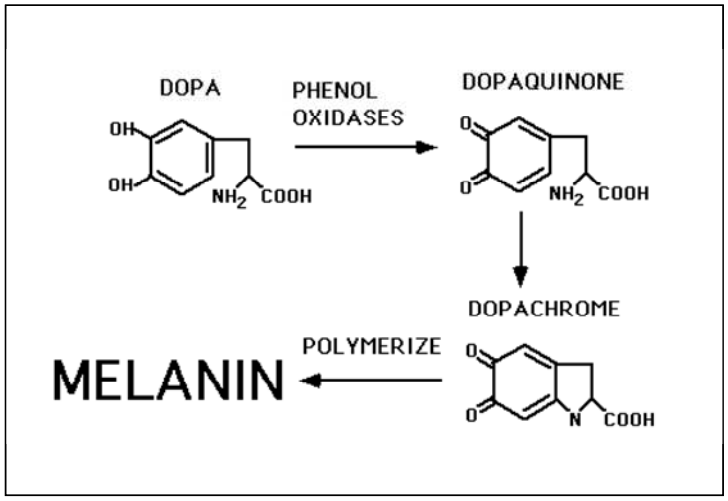



**Figure 2.4.** Oxidation of L-DOPA to dopachrome via dopaquinone. Dopachrome can polymerize spontaneously to melanin pigments (taken from Buchanan and Murphy, 1998).

Tyrosinase activity in lichen thallus was determined assaying the follows; 150 mg of fresh mass of lichen tissue was shaken at 60 rpm in 5 ml of 50 mM phosphate buffer, pH 6.0, and 2 mM L-DOPA, for 10 min at 25°C. To assay leachates or cytosolic and cell wall fractions, 50-200 µl of extract, 200 µl of 10 mM DOPA (final concentration is 2 mM) and 50 mM phosphate buffer pH 6 to give a final volume of 1 ml were incubated for 10-15 min at 25°C. The extinction coefficient of the orange coloured product measured at $A_{475}$ is 3.6 $mM^{-1}$ $cm^{-1}$. L-DOPA (10 mM) was dissolved in distilled water. To test the presence of latent tyrosinase 2 mM final concentration of SDS was added to the reaction mixture.

*L-tyrosine assay method for tyrosinase*

L-tyrosine (Sigma) was also used to detect tyrosinase activity using the method described by Mueller *et al*. (1996). It is an additional test for the ability of lichens to metabolize tyrosinase substrates that is based on the monophenol oxidase activity rather than the diphenol oxidase activity of tyrosinase; 150 mg of fresh mass of lichen tissue was shaken at 60 rpm in 3.5 ml of 50 mM phosphate buffer, pH 6.0, and 2 mM L-tyrosine, for 30 min at 25°C. For leachates, 200 µl of extract, 200 µl of 10 mM L-tyrosine (final concentration is 2 mM) and 600 µl of 50 mM phosphate buffer, pH 6.0 were mixed to give a final volume of 1 ml and then the mixture incubated for 30 min at 25°C. The extinction coefficient of the yellow coloured product measured at $A_{490}$ is 3.3 $mM^{-1}$ $cm^{-1}$. A stock solution of L-tyrosine was prepared by dissolving the substrate in 2.5 M HCl, adding distilled water and adjusting the pH to 6.0.

*Hydrogen peroxide assay method for peroxidase*

Peroxidase activity was estimated for cellular fractions only as the increase in the rate of reaction when $H_2O_2$ was added to give a final concentration of 10 mM in a 1 mM ABTS assay solution at pH 6.0.

*Calculation of laccase, tyrosinase and peroxidase activity*

Enzyme activity was expressed in µmol production $g^{-1}$ dry mass $h^{-1}$ and calculated as follows:

$$\frac{Abs \times v}{\varepsilon/1000 \times t \times m}$$

where

| | |
|---|---|
| Abs | - absorbance at certain wavelength |
| v | - total reaction volume in litre |
| ε | - extinction coefficient of the substrate |
| t | - time in hour |
| m | - dry mass in gram |

## 2.3. Electrophoretic analysis

*SDS- and Native-PAGE*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), or gel electrophoresis, was used to determine the approximate molecular masses of the active forms of laccases and tyrosinases. Mostly native-PAGE i.e. non-denaturing PAGE was used. The protocol is similar to that of denaturing SDS-PAGE with the exception that running buffer and gels contained SDS (0.1%), but samples were not heated, and β-mercaptoethanol was omitted from the loading buffer. SDS-PAGE was performed using a Hoefer Mini-Vertical Electrophoresis System (Hoefer Scientific Instruments). The separating gels were 5, 6, or 7.5% for laccases, or 10% for tyrosinases with a 3 or 4% of stacking gel (Laemmli, 1970). Molecular mass markers (“High Range,” Bio-Rad, Hercules, CA) were loaded onto the gel with 2% SDS and 5% β-mercaptoethanol and were heated for 5 min at 95°C. Electrophoresis was typically run for 15 min at 120 V then at 150 V for a further 45-50 min.

After electrophoresis, protein standards were stained by Coomassie blue dye solution for 20-30 min then destained overnight with a mixture of 40% methanol, 10% acetic acid, and 50% distilled water. The staining solution contained Coomassie brilliant blue R250 (Sigma), methanol and glacial acetic acid supplement with distilled water. Laccase bands were visualized by incubating gels in 10 mM 2,6-dimethoxyphenol (DMP, Fluka) in 0.2 M 2-morpholino-ethane-sulfonic acid (MES, Sigma) buffer, pH 6.0. After ca. 20 min a yellow-brown colour appeared at the position of the laccase. To test for the presence of haem- or copper-containing proteins, gels were incubated with 6.3 mM 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) for 2 h and then $H_2O_2$ added to give a final concentration of 30 mM (Thomas *et al*., 1976). To check the reproducibility of results, extracellular laccases were concentrated and gels run using between two and four different collections of lichens. In the case of four species, we were able to collect material from widely separated sites to test for geographical variations in laccase isozymes. To determine the approximate molecular mass of tyrosinase, bands were visualized by incubating gels in 10 mM DOPA in 0.1 M phosphate buffer pH 6.0, and 10 mM epinephrine in 0.1 M phosphate buffer pH 7.0. After approximately 20 min, black (dopachrome) and orange (adrenochrome) bands appeared at the position of the tyrosinase. Gels were also stained with the 'Silver Plus' Kit (Bio-Rad, Hercules, CA, USA) (Merril *et al*., 1984) with and without heating with mercaptoethanol.

*Isoelectric focussing (IEF)*

To determine the molecular mass of the laccase monomer, two-dimensional electrophoresis was carried out as follows. Initially, the pI of the laccase was determined using a Bio-Rad 'Fast Gel System' with wide-range ampholytes (pH 3.0 to 9.0). Gels were stained using TMB and $H_2O_2$ as above, yielding bands that were clear, but feint and rapidly fading. IEF standards (Bio-Rad) were run on the same gel and stained as above using Coomassie brilliant blue. Bands were excised and macerated in 1.5 ml eppendorf tubes containing 0.4 ml of 50 mM Tris-HCl buffer pH 7.5 with 150 mM NaCl and 0.1 mM EDTA. The tubes were then shaken overnight at room temperature, centrifuged for 20 min at 4000 × *g*, and the volume of the supernatants reduced to approximately 45 µl by centrifugation at 1000 × *g* for 40 min in 'Ultrafree-MC' concentrators. Electrophoresis was then carried out using a 10% gel as above but under denaturing conditions. After electrophoresis, the resulting single laccase monomer protein band was visualised using the 'Silver-Plus' staining kit as above.

## 2.4. Cellular location of enzymes in *Pseudocyphellaria aurata* and *Flavocetraria nivalis*

Cellular location of redox enzymes was determined using a modification of the method of Rast *et al*. (2003). Fresh material (3.5 g) was freeze dried, ground in 35 ml of ice cold 0.25 M Tris-HCl buffer pH 8.0, and then centrifuged at 4°C for 15 min at 4000 × *g*, and the supernatant representing the cytosolic fraction ("C"). The pellet was suspended in 20 ml of 50 mM phosphate buffer pH 7.0, and then centrifuged as above. This was repeated three times, the sum of the supernatants representing enzymes loosely bound to the cell wall e.g. by hydrogen bonds ("B1"). The pellet was re-suspended in 15 ml of phosphate buffer and solid digitonin (Sigma) added to give a final concentration of 0.3%. The solution was stirred for 3 h at 4°C, centrifuged as above, and then the step repeated. The combination of the two supernatants represented enzymes bound by van der Waals forces and hydrophobic interactions ("B2"). The pellet was re-suspended in 15 ml buffer of phosphate buffer, NaCl added to give a final concentration of 2 M. The solution was then stirred for 3 h at 4°C, centrifuged as above, and then the step repeated. The combination of the two supernatants represents enzymes bound by strong electrostatic forces ("B3"). Finally, the remaining pellet was re-suspended in 10 ml of phosphate buffer, and represents enzymes bound by covalent linkages. This last fraction was used directly in assays ("B4"). The cell wall fragments were pelleted after the assay to allow measurement of absorption. All the above fractions were assayed for laccase, tyrosinase and peroxidase activity. To test for the presence latent enzyme forms, assays for tyrosinases were carried out with and without 2 mM sodium dodecyl sulphate (SDS) as recommend by Moore and Flurkey (1990).

## 2.5. Effect of wounding and desiccation on laccase and tyrosinase activity in *Pseudocyphellaria aurata*

Each treatment comprised five replicates, and each replicate comprised five 6 mm disks. Three treatments were used. Treatment one comprised undesiccated and unwounded material. Treatment two comprised material in which enzyme activities (with ABTS and epinephrine as substrates) were measured for 0.5 h, the disks cut into quarters using a scalpel, and then activity measured over selected 15 min intervals for 2.5 h. Treatment three comprised material in which enzyme activity was measured for 0.5 h, the material allowed to dry over 2.5 h to a relative water content of 0.05, rehydrated by the addition of liquid water then enzyme activity measured over selected 15 min intervals for 2.5 h. Leakage of cytosolic enzymes was quantified by

measuring the proportion of the strictly cytosolic enzyme glucose-6-phosphate dehydrogenase (G-6-PD) released into the medium following stress. The assay mixture contained glucose-6-phosphate (1 mM), NADP (0.2 mM), $MgCl_2$ (1 mM), Tris-HCl buffer pH 8.0 (0.1 mM) and sample, and production of NADPH was measured at 340 nm.

## 2.6. Effect of inducers of laccase activity in *Pseudocyphellaria aurata*

*P. aurata* was collected and prepared as above. Some material was left untreated, while the rest was divided into replicates of 60 discs and shaken for 1 h in 10 ml of each of the following solutions: distilled water, 0.5-25 mM 2,5-xylidine (Sigma), 0.25-0.7 mM copper ions ($Cu^{+2}$) (supplied at copper sulphate), 0.5-10 µM ferulic acid, 0.1-20 µM syringaldazine and 0.05-0.76 M ethanol. Laccase activity was measured initially using ABTS as a substrate, and at the same times on the first, third and fifth days after pretreatment in five replicates of four discs. Some experiments with compounds that apparently induced laccases were repeated, except that rather than use intact discs total thallus activity was measured. Here, freeze-dried tissues were ground in buffer and then activity tested in crude homogenate.

## 2.7. pH optimum of unpurified laccase

The effect of pH on laccase activity was studied spectrophotometrically. ABTS, syringaldazine and *o*-tolidine as substrates were used to determine the optimum pH of laccases. *Pseudocyphellaria aurata* from suborder Peltigerineae was chosed as test species, because it is locally abundant species with good enzyme activity, and six other lichen species belong to other taxonomic group within Peltigerales. Lichen leachates and the different assays were applied as described above under the assay methods. Measurements were made at room temperature (ca. 20°C). To test the influence of pH on enzyme activity, phosphate and acetate buffer were used from pH 3.5 to 5.5 (acetate buffer) and pH 5.0 to 7.8 (phosphate buffer). All assays were repeated three times.

## 2.8. Temperature optimum

The effect of temperature on laccase activity was followed by the laccase-catalysed oxidation of ABTS, syringaldazine and *o*-tolidine. The experiments were carried out at temperatures ranging from 7°C to 70°C at 10°C intervals using a spectrophotometer. The

preparation of leachates from test species and the assay methods were the same as above. All experiments were carried out at optimal pH and done in triplicate.

## 2.9. Thermostability

Thermostability of different laccases extracted from *Collema flaccidum*, *Lobaria scrobiculata*, *Peltigera rufescens* and *Pseudocyphellaria aurata* was determined by spectrophotometrically. Unconcentrated leachates were pretreated at 40, 50 and 60°C for different periods, and then laccase activity measured as above at 30°C using ABTS as substrate.

## 2.10. Substrate specificity

Laccase and tyrosinase enzymes have overlapping substrate specificity, and therefore substrates used were chosen with care. Three different substrates (ABTS, syringaldazine and *o*-tolidine) were used to investigate the specificity of laccases toward different phenolic compounds. ABTS is a most commonly used synthetic substrate (Min *et al*., 2001) while syringaldazine is a natural compound specific to laccase (Medeiros *et al*., 1999). *O*-tolidine like ABTS belongs to a group of synthetic substrates (Miller *et al*., 1997). Identification of tyrosinase was investigated using epinephrine (Sugumaran *et al*., 1987), L-DOPA (Horowitz *et al*., 1970) and L-tyrosine (Mueller *et al*., 1996), the latter being the most specific compound to tyrosinase. Enzymes activity was measured using 6 mm discs or thallus parts from all the investigated lichens. For each measurement, five replicates of equivalent to ca. 20 mg dry mass were incubated in 5 ml substrate solution. The oxidation of the substrates was followed by spectrophotometer at optimal pH and temperature.

Kinetic studies were performed for each 'laccase substrate'. At least six different concentrations of substrate were used for each substrate at optimal pH and temperature, and the kinetic parameters ($K_M$, $V_{max}$) determined.

## 2.11. Inhibition and activation studies

Many compounds have been reported to inhibit or activate laccase and tyrosinase activity (Baldrian, 2006; Mayer, 2006). Five potential inhibitors and four compounds termed as activators were used, including sodium azide ($NaN_3$), potassium cyanide (KCN), ethylenediaminetetraacetic acid (EDTA), sodium fluoride (NaF) and 4-hexylresorcinol, while

2,5-xylidine and three type of metal salts such as calcium chloride ($CaCl_2$), manganese chloride ($MnCl_2$) and copper chloride ($CuCl_2$) (Crowe and Olsson, 2001; Baldrian, 2004). $NaN_3$, KCN, NaF and EDTA are metal chelators that can irreversible bind to the copper centre of the enzyme (Edens *et al*., 1999) while 4-hexylresorcinol is specific inhibitor of tyrosinases but not laccases (Dawley and Flurkey, 1993a, b). 2,5-xylidine is an inducer of laccases in the long term (Garcia *et al*., 2006). Metal ions are essential to the fungal partner and can induce laccase enzyme on long term but can be toxic as well (Baldrian, 2003; Lorenzo *et al*., 2005). The effect of activators on laccase activity was investigated on short- and long-term.

Lichen leachates were incubated in the different type of inhibitors or activators for 20 min, substrate was added (ABTS, syringaldazine, *o*-tolidine and L-DOPA) and then the formation of the product was measured over the next 20 min at optimal pH and temperature. At least nine different concentrations of $NaN_3$ and KCN were used to test the effect of inhibitors on laccase activity using the three 'laccase substrates'. In the experiments of EDTA, NaF, 2,5-xylidine and metal ions ABTS was used to follow the effect of inhibitors and activators on laccase activity. In case of EDTA inhibition, the leachates derived from four different lichens while in all the other measurements *Pseudocyphellaria aurata* was used. The effect of different inhibitors such as KCN, $NaN_3$, NaF and 4-hexylresorcinol on tyrosinase activity was tested by leachates extracted from *Pseudocyphellaria aurata* using L-DOPA as substrate. The percentage of inhibition was then calculated. Five replicates were done in each experiment.

*Tests for the mechanism of wound-induced increase in enzyme activity and whether enzymes are glycosylated*

*Pseudocyphellaria aurata* whole and wounded discs were used to investigate the effect of 0.2 mM cycloheximide (inhibitor of translation), 0.05 and 0.1 mM monensin (inhibit glycoprotein secretion) and 15 µg/ml concentration of actinomycin D (inhibitor of transcription) on laccase activity. The inhibitor compounds were used to test whether the increase in enzyme activity that follows wounding is the result of activation or de novo synthesis of the protein. 20 discs (wounded and unwounded) were shaken in 10 ml of inhibition solutions for 1 h and kept them on wet paper. Discs were cut to quarter were used as wounded species. ABTS as substrate was used to measure laccase activity. Cycloheximide and monensin as well as actinomycin D and monensin were used simultaneously. Enzyme activity was measured after three hours and three days of incubation. Four different concentrations of concanavalin A were used to test whether the laccases were glycosylated (concanavalin A deactivates glycoproteins). Leachates

of *Pseudocyphellaria aurata* were prepared, and ABTS used to measure enzyme activity immediately after treatment. All experiments were repeated two times.

## 2.12. Decolourization of dyes

The ability of eight species of lichens to decolourize different types of dyes was investigated. Five species belonged to suborder Peltigerineae, and for these species the decolourizing potential of unconcentrated leachates were also studied. Lichen thalli and unconcentrated leachates were prepared as above. For whole thalli four and for leachates five types of dyes in 2 ml of solution (0.01% final concentration of dye) were used. The dyes tested were the indigoid dye Acid Blue 74, the anthraquinone-type dye Remazol Brilliant Blue R (RBBR), the azo dye Chicago Sky Blue 6B, Acid Red 103 (belonging to the quinone-imine dye class) and the triarylmethane-type dye Fast Green FCF.

In experiments with discs, 10 discs from each species were used, while in experiments with leachates 1 ml was used. Specimen bottles were shaken at room temperature and the degree of the decolourization processes were followed spectrophotometrically after 3, 6, 24 and 48 h. Changes in absorbance was monitored at 590 nm (RBBR), 610 nm (Acid Blue 74), 505 nm (Acid Red 103), 620 nm (Chicago Sky Blue 6B) and at 625 nm (Fast Green FCF).

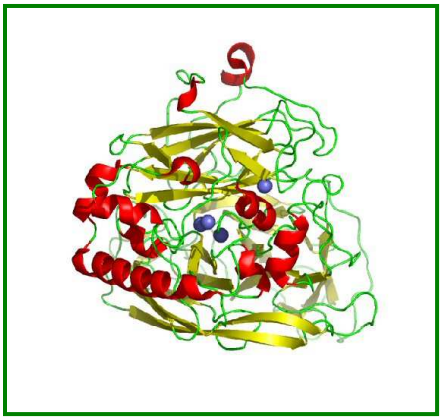
In addition, the ability of 1-hydroxybenzotriazole (1-HBT) to act as a “mediator” for laccase/tyrosinase was tested to enhance the decolourization processes by increasing the enzyme activity. A final concentration of 0.6 mM 1-HBT was used. Decolourization of dye was expressed in the amount of dye remaining as a function of time.

## 2.13. Statistical analysis

All measurements were done using 2-6 replicates. F and t-statistics were calculated and the least significant difference between means (three replicates) at $P < 0.05$ probability was used for inspection of differences between values.

# CHAPTER 3

## BASIC PROPERTIES OF LICHEN LACCASES



Laccase from *Melanocarpus albomyces* (1gw0)
(http://chemistry.umeche.maine.edu/CHY431/Wood2.html).

*Lobaria scrobiculata* (Scop.) DC.
growing in Monk's Cowl State Forest in the Republic of South Africa.

## 3.1. Introduction

The general characteristics of laccase enzymes were reviewed in Chapter 1. As discussed, perhaps surprising, no reports exist for laccases in an important group of fungi, the lichenized ascomycetes. Some earlier works (Minibayeva and Beckett, 2001; Beckett *et al*., 2003) demonstrated the presence of strong extracellular redox activity in some species of lichens. While several surveys had reported the existence of laccases in free-living ascomycetes (e.g. Lyons *et al*., 2003; Pointing *et al*., 2003, 2005; Tetsch *et al*., 2005), at the time this study was started it appeared to be the first for lichens. Extracellular laccase activity was measured in 50 species of lichens from different taxonomic groupings and contrasting habitats using ABTS and syringaldazine as substrates. As our results confirmed the presence of laccases in species from suborder Peltigerineae, we selected four species from different families in the suborder, namely *Collema flaccidum* (Collemataceae), *Lobaria scrobiculata* and *Pseudocyphellaria aurata* (Lobariaceae) and *Peltigera rufescens* (Peltigeraceae). Most of the studies such as the effects of temperature, pH and inducers of laccase synthesis on long-term activity and substrate specificity of laccases were performed by *Pseudocyphellaria aurata*, a locally abundant species in South Africa. However, the effect of pH on laccase activity was studied and compared between *Pseudocyphellaria aurata* and six non-Peltigerineae lichen species. Using the selected species the thermostability and the presence of inhibitors on enzyme activity, were examined. We also studied the effect of Concanavalin A (Con A) on laccase activity using *P. aurata* to test whether lichen laccases are glycosylated. Finally, we determined the molecular weight, isoelectric point and the structure of the active form of laccase from *Peltigera malacea*.

## 3.2. Results

*Occurrence of laccases in lichens*

Laccase activity was detected in almost all lichens in Peltigerineae tested (out of 27 species tested), but was barely detectable in species belonging to other suborders (Table 3.1). The only exception was that limited laccase activity was detected in *Ramalina celastri*, (13 ± 3 µmol $g^{-1}$ dry mass $h^{-1}$).

**Table 3.1.** Extracellular laccase activity, measured using the substrates ABTS and syringaldazine in a range of lichens.

| Species | Laccase activity, ABTS (µmol $g^{-1}$ dry mass $h^{-1}$) | Laccase activity, syringaldazine (µmol $g^{-1}$ dry mass $h^{-1}$) |
|---|---|---|
| **Lichens from suborder Peltigerineae** | | |
| *Collema flaccidum* | 28 ± 9 | 1.3 ± 0.3 |
| *Leptogium saturninum* | 1 ± 1 | 0.4 ± 0.2 |
| *Leptogium sessile* | 18 ± 4 | 0.3 ± 0.1 |
| *Lobaria pulmonaria* | 16 ± 2 | 1.1 ± 0.4 |
| *Lobaria scrobiculata* | 68 ± 7 | 3.0 ± 1.9 |
| *Nephroma arcticum* | 37 ± 14 | 0.1 ± 0.0 |
| *Nephroma parile* | 25 ± 3 | 0.7 ± 0.4 |
| *Nephroma rufum* | 32 ± 2 | 1.4 ± 0.3 |
| *Peltigera apthosa* | 83 ± 22 | 3.0 ± 0.3 |
| *Peltigera canina* | 130 ± 10 | 2.3 ± 0.2 |
| *Peltigera didactyla* | 162 ± 11 | 2.2 ± 0.3 |
| *Peltigera horizontalis* | 9 ± 2 | 1.0 ± 0.4 |
| *Peltigera hymenina* | 156 ± 22 | 6.8 ± 1.9 |
| *Peltigera leucophlebia* | 25 ± 13 | 1.6 ± 0.3 |
| *Peltigera malacea* | 62 ± 13 | 1.0 ± 0.4 |
| *Peltigera neopolydactyla* | 113 ± 33 | 2.4 ± 0.5 |
| *Peltigera polydactylon* | 76 ± 16 | 1.2 ± 0.4 |
| *Peltigera praetextata* | 120 ± 12 | 2.4 ± 0.7 |
| *Peltigera rufescens* | 100 ± 23 | 1.8 ± 0.4 |
| *Peltigera scabrosa* | 121 ± 19 | 2.6 ± 0.5 |
| *Pseudocyphellaria aurata* | 19 ± 4 | 1.7 ± 0.6 |
| *Pseudocyphellaria gilva* | 8 ± 2 | 0.1 ± 0.0 |
| *Solorina crocea* | 137 ± 2 | 17.3 ± 4.5 |
| *Sticta fuliginosa* | 6 ± 1 | 3.8 ± 0.6 |
| *Sticta cf. limbata* | 54 ± 17 | 2.5 ± 0.1 |
| *Sticta cf. sublimbata* | 32 ± 10 | 1.5 ± 0.9 |
| *Sticta sp.* | 42 ± 8 | 2.4 ± 0.3 |
| **Lichens from non-suborder Peltigerineae** | | |
| *Alectoria sarmentosa* | 2 ± 0 | n.d. |
| *Anaptychia ciliaris* | 3 ± 2 | 0.0 ± 0.0 |
| *Bryoria simplicior* | 0 ± 0 | 0.3 ± 0.0 |
| *Cetraria islandica* | 1 ± 0 | 0.1 ± 0.0 |
| *Cladonia cariosa* | 3 ± 0 | 0.1 ± 0.0 |
| *Cladonia cf. rangiferina* | 2 ± 1 | n.d. |
| *Cladonia stellaris* | 1 ± 0 | 0.0 ± 0.0 |
| *Cladonia cf. uncialis* | 2 ± 0 | n.d. |
| *Evernia prunastri* | 6 ± 2 | 0.0 ± 0.0 |
| *Flavocetraria nivalis* | 2 ± 1 | 0.0 ± 0.0 |
| *Heterodermia speciosa* | 2 ± 1 | 0.2 ± 0.0 |
| *Hypogymnia physodes* | 5 ± 2 | 0.1 ± 0.0 |
| *Parmelia cetrarioides* | 1 ± 0 | 0.5 ± 0.2 |
| *Platismatia glauca* | 5 ± 1 | 0.0 ± 0.0 |
| *Pseudevernia furfuracea* | 2 ± 1 | 0.1 ± 0.0 |
| *Ramalina celastri* | 13 ± 3 | 1.5 ± 0.4 |
| *Ramalina farinacea* | 1 ± 0 | 0.0 ± 0.0 |
| *Ramalina pollinaria* | 1 ± 0 | 0.1 ± 0.0 |
| *Roccella montagnei* | 0 ± 0 | 0.0 ± 0.0 |
| *Stereocaulon tomentosum* | 0 ± 0 | 0.1 ± 0.1 |
| *Umbilicaria deusta* | 0 ± 0 | 0.1 ± 0.0 |
| *Umbilicaria pustulata* | 0 ± 0 | 0.2 ± 0.0 |

| Species | Laccase activity, ABTS (µmol $g^{-1}$ dry mass $h^{-1}$) | Laccase activity, syringaldazine (µmol $g^{-1}$ dry mass $h^{-1}$) |
|---|---|---|
| *Usnea undulata* | 2 ± 2 | 0.1 ± 0.1 |
| Mean for suborder Peltigerineae (n = 27) | 62 ± 11 | 2.4 ± 0.6 |
| Mean for non-suborder Peltigerineae (n = 23) | 2 ± 1 | 0.2 ± 0.0 |

Figures are given as the mean ± S.D., n = 5. n.d. = not determined.

The mean laccase activity was 62 ± 11 µmol $g^{-1}$ dry mass $h^{-1}$ in species from Peltigerineae while in other lichens it appeared to be only 2 ± 1 µmol $g^{-1}$ dry mass $h^{-1}$ using ABTS as a substrate. The genus *Peltigera* had the highest activity, mostly higher than 100 µmol $g^{-1}$ dry mass $h^{-1}$; that value was also exceeded by *Solorina crocea*. Activity was less in all lichens tested using syringaldazine as a substrate; mean rates of metabolism for Peltigeralean and non-Peltigeralean species were 2.4 ± 0.6 µmol $g^{-1}$ dry mass $h^{-1}$ and 0.2 µmol $g^{-1}$ dry mass $h^{-1}$, respectively.

*Effect of temperature on lichen laccases*

The basic properties of *Pseudocyphellaria aurata* laccases such as the effect of temperature, pH, different type of inhibitors and inducers were investigated using classic laccase substrates. In some experiments (thermostability, pH and inhibition) the results were compared with the results of other lichen laccases (see above).

Conditions for optimum temperature of laccase activity was substrates dependent and varied between 7°C and 40°C in *P. aurata* (Figure 3.1). In case of the substrate ABTS and syringaldazine laccase activity had the optimum temperature of 40 and 30°C, respectively. Interestingly, the optimum temperature for laccase activity using *o*-tolidine was only 7°C and with increasing temperature the activity dropped rapidly.

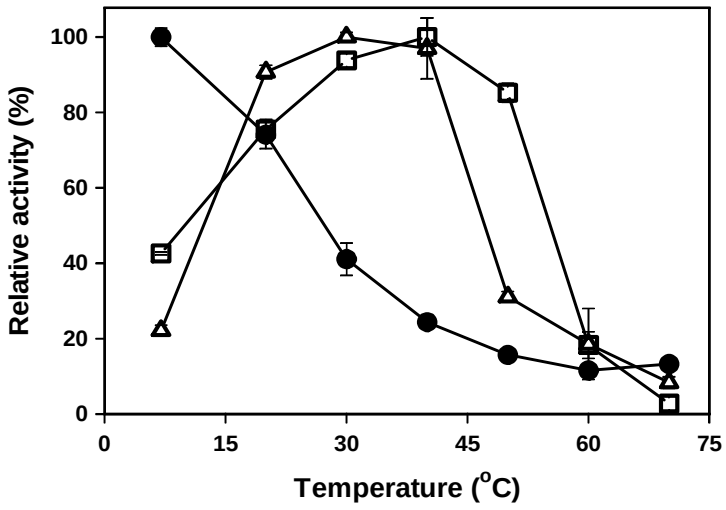



**Figure 3.1.** Effect of temperature on the activity of *Pseudocyphellaria aurata* laccase. Activity was measured in *Pseudocyphellaria* leachates using ABTS (□), syringaldazine (Δ) and *o*-tolidine (●) as substrates. Error bars indicate the standard deviation, n = 3.

Investigating the thermostability of lichen laccases we found that the enzymes isolated from *Collema flaccidum*, *Lobaria scrobiculata*, *Peltigera rufescens* and *Pseudocyphellaria aurata* displayed only moderate thermostability at 40°C, with species losing between 30-60% of their activity after 5 h (Figure 3.2/A). At 50°C all species tested lost more than half of their activity after 0.5 h (Figure 3.2/B). Of the four species tested, the laccase from *Peltigera rufescens* had the highest stability, and still retained about 30% of this original activity after 2 h at 50°C (Figure 3.2/B, Δ). At 60°C the laccases from all species lost activity almost immediately (data not shown).

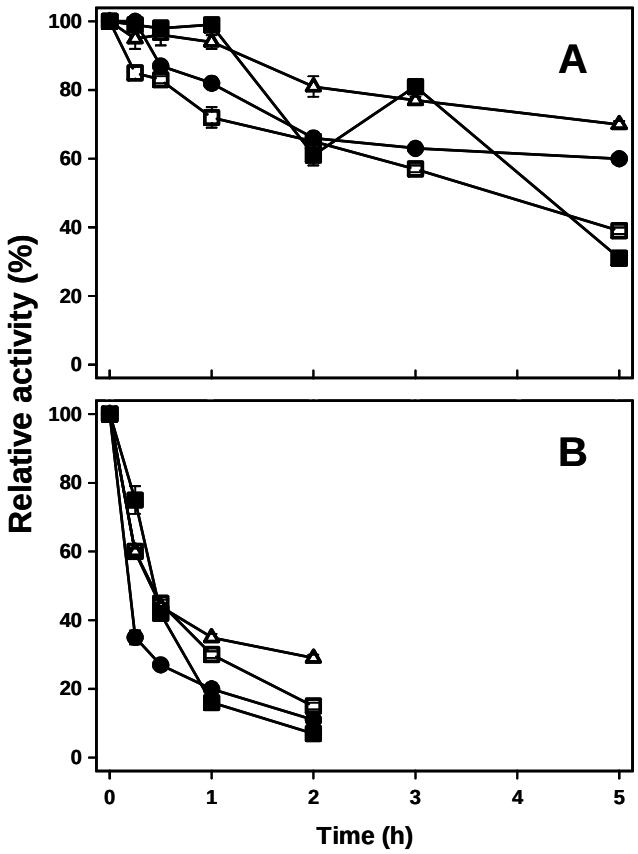



**Figure 3.2.** Thermostability of laccases at **A**) 40°C and **B**) 50°C in *Collema flaccidum* (●), *Pseudocyphellaria aurata* (□), *Lobaria scrobiculata* (■) and *Peltigera rufescens* (Δ). The actual rates of laccase activity at time zero were (in µmol product $g^{-1}$ dry mass $h^{-1}$): *Collema flaccidum*: 29.0 ± 0.7, *Pseudocyphellaria aurata*: 9.8 ± 0.2, *Lobaria scrobiculata*: 38.9 ± 1.9, *Peltigera rufescens*: 76.2 ± 7.6. Error bars indicate the standard deviation, n = 3.

*Effect of pH on laccase activity in lichen leachates*

The effect of pH on laccase activity was determined in *Pseudocyphellaria aurata* and six non-Peltigerineae lichen species (Table 3.2), *Alectoria sarmentosa*, *Cetraria islandica*, *Cladonia cf. rangiferina*, *Cladonia stellaris*, *Cladonia cf. uncialis* and *Flavocetraria nivalis* using the three laccase substrates.

**Table 3.2.** Optimal pH values of laccase from six non-Peltigerineae lichen species with ABTS as substrate.

| Species | Optimum pH (ABTS) |
|---|---|
| *Alectoria sarmentosa* | 5.0 |
| *Cetraria islandica* | 4.0 |
| *Cladonia cf. rangiferina* | 5.0 |
| *Cladonia stellaris* | 5.0 |
| *Cladonia cf. uncialis* | 5.0 |
| *Flavocetraria nivalis* | 5.0 |

All measurements showed that conditions for optimal activity were substrate dependent, and the optimum pH varied between 4.0 and 6.5 (Table 3.2, Figure 3.3). In *P. aurata* laccase had an optimum activity at pH 5.0 in case of the substrate *o*-tolidine while the optimum pH was 5.5 and 6.5 using ABTS and syringaldazine, respectively (Figure 3.3).

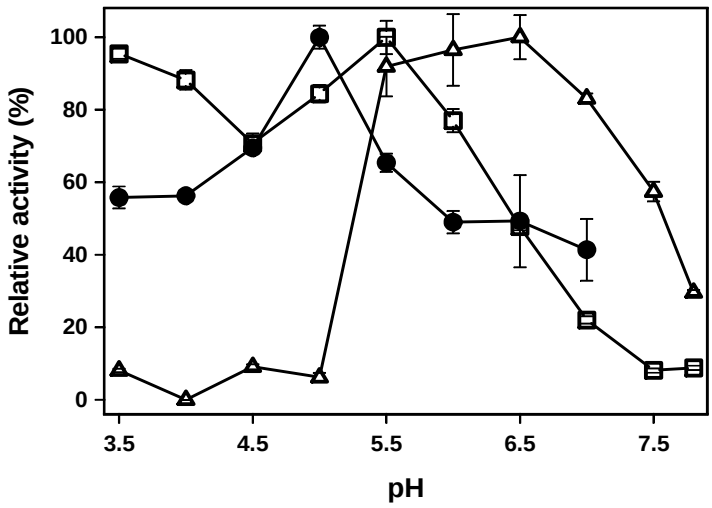



**Figure 3.3.** The effect of pH on the activity of laccase from *Pseudocyphellaria aurata* leachates using ABTS (□), syringaldazine (Δ) and *o*-tolidine (●) as substrates. Error bars indicate the standard deviation, n = 3.

The investigated non-Peltigerineae lichen species had an optimum pH of 5.0 using the substrate ABTS except *Cetraria islandica* where pH 4.0 was optimal (Table 3.2).

*Enzyme kinetic parameters of* Pseudocyphellaria *laccase*

Kinetic parameters ($K_M$, $V_{max}$) of laccases derived from *Pseudocyphellaria aurata* were determined for three substrates (ABTS, syringaldazine and *o*-tolidine; Figure 3.4). The

experimental kinetic data were fitted to single substrate (Michaelis-Menten) kinetics by nonlinear regression analysis (curve-fit).

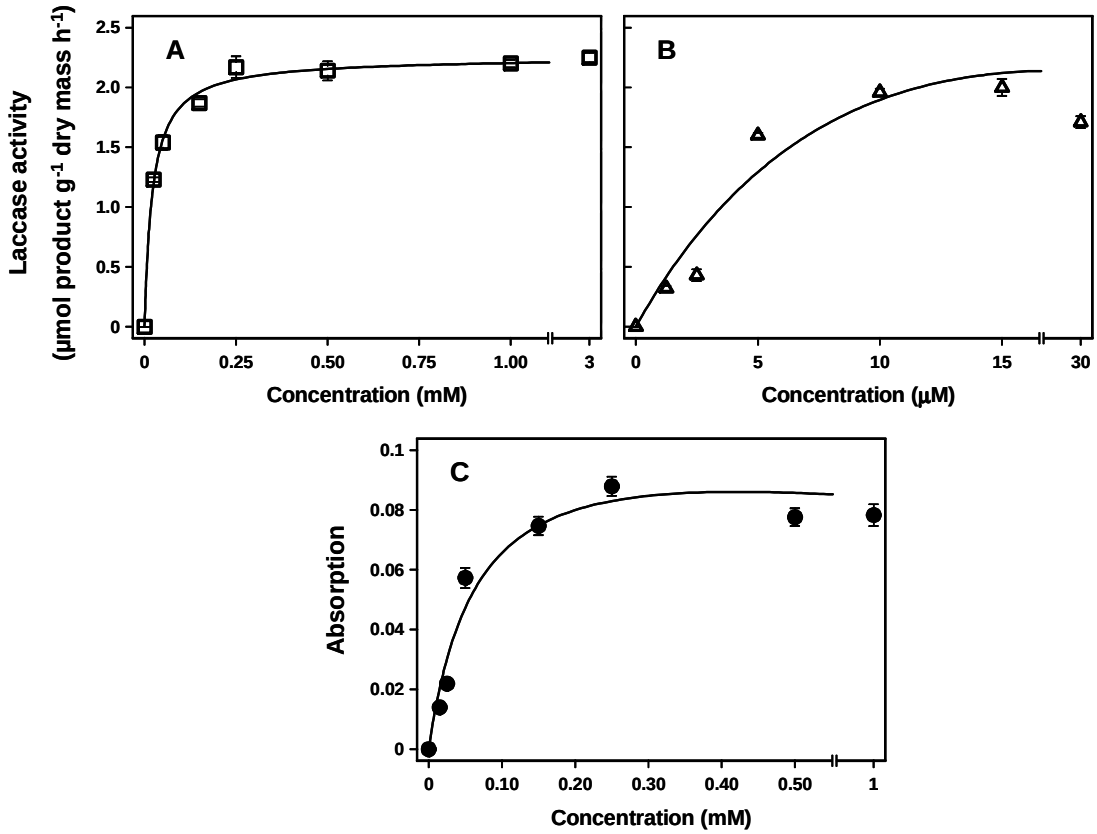



**Figure 3.4.** Michaelis-Menten kinetics of the oxidation of ABTS (A), syringaldazine (B) and *o*-tolidine (C) by laccases from *Pseudocyphellaria aurata*. Error bars indicate the standard deviation, n = 3.

Our results showed that laccase activity measured as a function of substrate concentration always displayed classical Michealis-Menten kinetics (Figure 3.4), with the $K_M$ varying between 18 and 68 µM (Table 3.3). The value of $V_{max}$ was 9.17 and 2.24 µmol product $g^{-1}$ dry mass $h^{-1}$ in case of the substrate syringaldazine and ABTS, respectively.

**Table 3.3.** Kinetic parameters for the oxidation of three different substrates by laccases isolated from *Pseudocyphellaria aurata*.

| | ABTS | Syringaldazine | *o*-tolidine |
|---|---|---|---|
| $K_M$ (µM) | 21 | 18 | 68 |
| $V_{max}$ (µmol product $g^{-1}$ dry mass $h^{-1}$) | 2.24 | 9.17 | -* |
| $V_m/K_M$ | 0.10 | 0.51 | - |

* cannot be calculated, as extinction coefficient of the product is unknown.

*Stress factors affecting laccase activity in* Pseudocyphellaria aurata

The effect of stress such as wounding and desiccation on laccase activity in *P. aurata* was investigated (Figure 3.5). Shaking discs of *P. aurata* in distilled water tended to gradually increase laccase activity, but activity was rapidly increased by both wounding and desiccating then rehydrating the thalli.

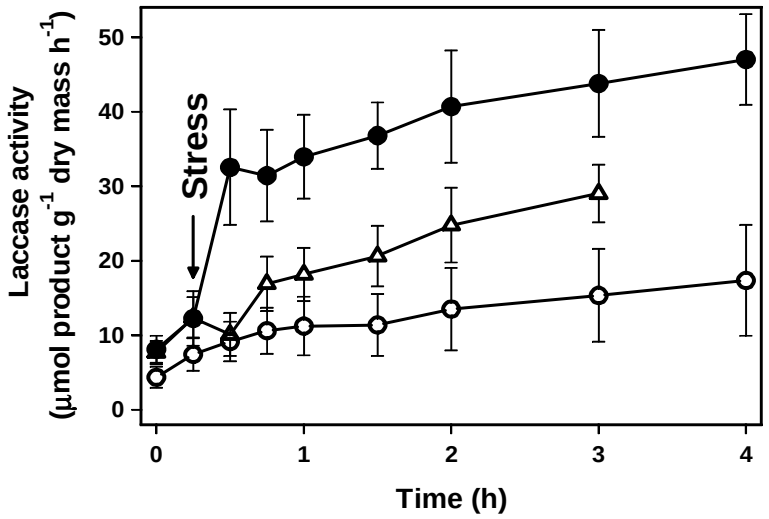



**Figure 3.5.** Effect of wounding and desiccation on laccase activity in *Pseudocyphellaria aurata* discs using ABTS as substrate. The arrow indicates the time that material was stressed, either by cutting discs into quarters (solid circles), or by desiccating it to a relative water content of 0.05 over 2.5 h then suddenly rehydrating it (open triangles). Open circles represent control (unstressed) material. Error bars indicate the standard deviation, n = 3.

Wounding caused considerable increase in laccase activity in *P. aurata* discs using ABTS therefore we investigated whether wounding causes release of cell wall bound enzymes such as laccases or the enzymes are secreted (Table 3.4). The activity of laccases and the typical intracellular enzyme, glucose-6-phosphate-dehydrogenase (G-6-PD) were examined in this experiment.

**Table 3.4.** Investigation of the cytoplasmic enzyme G-6-PD and the cell wall enzyme laccase activity in *Pseudocyphellaria aurata*. Results were expressed in µmol product $g^{-1}$ dry mass $h^{-1}$.

| | **Activity of G-6-PD** | **Laccase activity** |
|---|---|---|
| Total activity in material[a] | 150 ± 9 | 459 ± 201 |
| Material was shaken in water for 1 h, freeze-dryed overnight and ground in liquid N | 147 ± 6 | 180 ± 43 |
| Material was shaken in water for 1 h, ABTS added to discs in fresh solution | - | 20 ± 7 |
| Solution from above[b] | 4 ± 3 | 0.03 ± 0.01 |
| Material was wounded, shaken in water for 1 h, freeze-dryed overnight and ground in liquid N | 153 ± 10 | 164 ± 31 |
| Material was wounded, shaken in water for 1 h, ABTS added to discs in fresh solution | - | 47 ± 9 |
| Solution from above[b] | 6 ± 3 | 0.14 ± 0.04 |

Note: [a]It means enzyme activity of material that was freeze-dried overnight then ground in liquid nitrogen finally measured enzyme activity. [b]Material (wounded or non-wounded) was shaken in water then moved to fresh solution and the remaining solution was tested to enzyme activity. Values are given as the mean ± S.D., n = 3.

Our results show that wounding caused only a small proportion of leakage of the cytosolic enzyme G-6-PD (Table 3.4); rate of the G-6-PD activity of wounded material was 153 ± 10 µmol product $g^{-1}$ dry mass $h^{-1}$ - similar to total and firstly shaken then freeze-dried and measured disc's enzyme activity (150 ± 9 and 147 ± 6 µmol product $g^{-1}$ dry mass $h^{-1}$) - while the solution showed 6 ± 3 µmol product $g^{-1}$ dry mass $h^{-1}$ enzyme activity. Laccase activity showed similar pattern to G-6-PD. Wounding caused only small release of laccase into the solution; 0.14 ± 0.04 µmol product $g^{-1}$ dry mass $h^{-1}$ activity was found in the solution while about 25% of the total rate of laccase activity was appeared in the fresh solution (47 ± 9 µmol product $g^{-1}$ dry mass $h^{-1}$) using discs.

*Inhibition of laccase activity by several compounds*

Effect of a wide range of inhibitors on lichen laccases was investigated. Figure 3.6. shows the inhibition of laccase from *Pseudocyphellaria aurata* by sodium azide ($NaN_3$) and potassium cyanide (KCN). Results indicated that concentrations of $NaN_3$ needed to inhibit activity by 50% for ABTS, syringaldazine and *o*-tolidine were 5, 20 and 5 µM, while the concentrations of cyanide required to inhibit laccase activity by 50% were 1000, 750 and 750 µM. Laccase activity was strongly inhibited by $NaN_3$ (Figure 3.6/A, C, E) and to a lesser extent by KCN (Figure 3.6/B, D, F) and it was most sensitive to inhibitors when *o*-tolidine was used as a substrate (Figure 3.6/E, F).

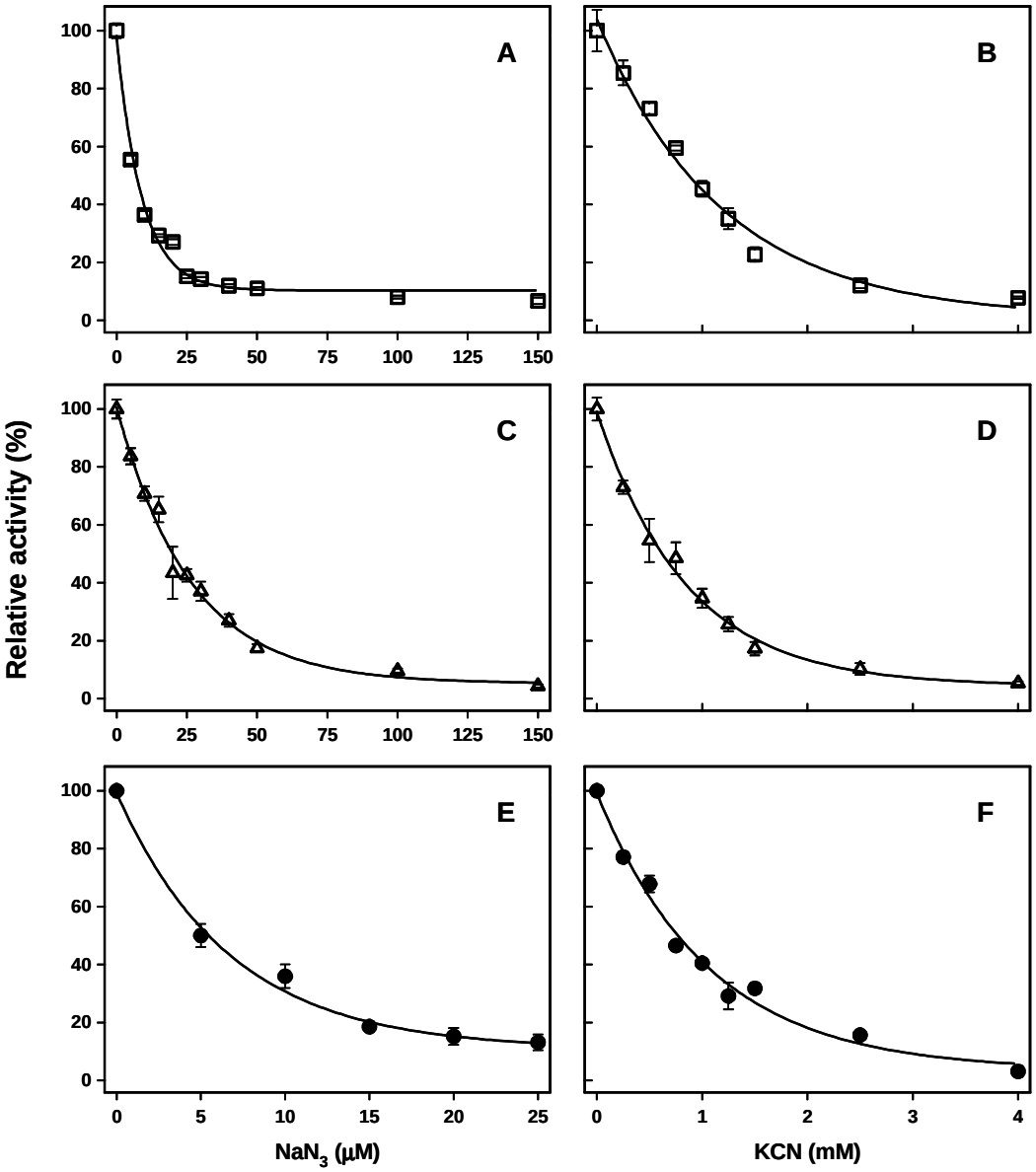



**Figure 3.6.** Inhibition of laccase activity from *Pseudocyphellaria aurata* by sodium azide ($NaN_3$) and potassium cyanide (KCN). ABTS (A, B), syringaldazine (C, D) and *o*-tolidine (E, F) were used as substrate. Error bars indicate the standard deviation, n = 5.

Sodium fluoride (NaF) is a general inhibitor of multicopper oxidases. NaF inhibited laccase activity from *P. aurata* higher extent than cyanide (Figure 3.6 and 3.7).

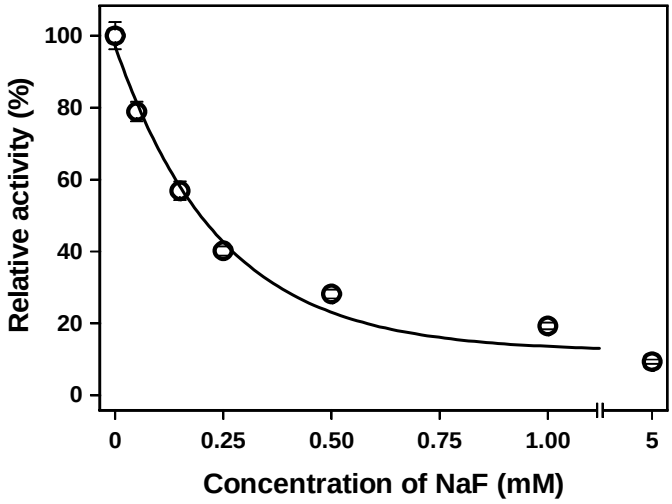



**Figure 3.7.** Relative activity of laccase from *Pseudocyphellaria aurata* is shown using sodium fluoride (NaF) as inhibitors. ABTS was used as substrate. Initial activity of laccase was 0.3550 ± 0.0134 µmol product $g^{-1}$ dry mass $h^{-1}$. All data represent five replicates of measurements and error bars indicate the standard deviation.

The effect on laccase activity of the organic compound EDTA and several metal ions ($Ca^{2+}$, $Cu^{2+}$ and $Mn^{2+}$) were investigated using the laccases from *Pseudocyphellaria aurata*, *Collema flaccidum*, *Lobaria scrobiculata* and *Peltigera rufescens* (Table 3.5). For this purpose, laccases were incubated in solutions of the above mentioned compounds at concentrations ranging from 0.01 to 10.0 mM. EDTA had very strong effect on laccase activity isolated from *Collema flaccidum* where even 0.01 mM EDTA significantly inhibited laccase activity while 0.1 mM concentration of inhibitor had an inhibition by 50% ($P < 0.01$). Laccases from *Lobaria scrobiculata* and *Peltigera rufescens* were also inhibited to a certain extent at the concentration of 1 mM ($P < 0.01$), while higher concentrations of EDTA induced laccase activity. EDTA had significant effect on *Pseudocyphellaria* laccase even at the concentration of 0.1 mM ($P < 0.05$) while using 1.0 and 10.0 mM the significance was increased ($P < 0.01$). In case of the ion $Ca^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ laccase activity was considerably inhibited by all investigated ions in *P. aurata* even at very low concentration.

**Table 3.5.** Inhibition of laccase activity from *Pseudocyphellaria aurata*, *Collema flaccidum*, *Lobaria scrobiculata* and *Peltigera rufescens* by different concentration of inhibitors using ABTS as substrate. Results indicated the relative activity of laccases after the inhibition processes.

| Inhibitor | Inhibitor concentr. (mM) | *Pseudocyphellaria aurata* | *Collema flaccidum* | *Lobaria scrobiculata* | *Peltigera rufescens* |
|---|---|---|---|---|---|
| EDTA | 0.0 | 100 | 100 | 100 | 100 |
| | 0.01 | 102 | 85** | 94 | 93** |
| | 0.1 | 97* | 53** | 73** | 100 |
| | 1.0 | 92** | 36** | 88** | 88** |
| | 10.0 | 85** | 27** | 115 | 89* |
| $CaCl_2$ | 0.0 | 100 | | | |
| | 0.1 | 102 | | | |
| | 1.0 | 93* | | | |
| | 5.0 | 79** | | | |
| | 10.0 | 84** | | | |
| $CuCl_2$ | 0.0 | 100 | | | |
| | 0.1 | 86** | | | |
| | 0.25 | 82** | | | |
| | 0.5 | 78** | | | |
| | 0.7 | 78** | | | |
| $MnCl_2$ | 0.0 | 100 | | | |
| | 0.1 | 96* | | | |
| | 1.0 | 87** | | | |
| | 5.0 | 69** | | | |
| | 10.0 | 64** | | | |

* indicates significant difference at $P < 0.05$ while ** signifies significant difference at $P < 0.01$. Comparisons were made relative to the control.

*Effect of a wide range of inducers on* Pseudocyphellaria *laccase*

Discs from *Pseudocyphellaria aurata* were used to test the effect of typical laccase inducers on laccase activity. Results show that during storage in moist conditions, laccase activity tended to decline slightly in untreated material, but pretreating material with different concentrations of $Cu^{2+}$, 2,5-xylidine (Table 3.6), ferulic acid, syringaldazine and ethanol had no significant effect on laccase activity (data not shown).

**Table 3.6.** Effect of inducers such as the $Cu^{2+}$ and 2,5-xylidine on extracellular and total thallus laccase activity (after 1, 3 and 5 days of treatment).Values are expressed in µmol product $g^{-1}$ dry mass $h^{-1}$.

| | 0. day | 1. day | 3. day | 5. day |
|---|---|---|---|---|
| Control | 16 ± 3 | 11 ± 2** | 11 ± 1** | 10 ± 3** |
| Control – total | 336 ± 81 | 273 ± 44 | 193 ± 51* | 208 ± 50* |
| **$Cu^{2+}$** - 0.5 mM | | 21 ± 2** | 25 ± 6** | 30 ± 6** |
| 0.5 mM – total | | 367 ± 10** | 93 ± 32* | 99 ± 34* |
| 0.7 mM | | 14 ± 7 | 29 ± 11* | 27 ± 9** |
| 0.7 mM – total | | 193 ± 11* | 66 ± 18** | 62 ± 10** |
| **2,5-xylidine** - 5 mM | | 5 ± 3** | 10 ± 5 | 8 ± 3 |

| | 0. day | 1. day | 3. day | 5. day |
|---|---|---|---|---|
| 5 mM – total | | 203 ± 30* | 202 ± 43 | 201 ± 58 |
| 10 mM | | 8 ± 3 | 8 ± 5 | 19 ± 9 |
| 10 mM – total | | 209 ± 25* | 48 ± 10** | 12 ± 3** |

Figures are given as the mean ± S.D., n = 3. * indicates significant difference at $P < 0.05$ while ** signifies significant difference at $P < 0.01$. Comparisons were made relative to the control.

Although $Cu^{2+}$ and xylidine increased activity measured on intact discs, subsequent measurement of total thallus laccase activity indicated that the increase measured was a result of inducer-induced membrane damage, and the subsequent leakage of intracellular laccases (Table 3.6).

*Investigation of glycosylation on* Pseudocyphellaria *laccase*

To test whether lichen laccases are glycosylated, the effect of Con A on laccase activity was tested (Table 3.7).

**Table 3.7.** The effect of Concanavalin A on laccase from *Pseudocyphellaria aurata*.

| Concentration of Con A ($\mu g\ ml^{-1}$) | Laccase activity ($\mu mol\ product\ g^{-1}\ dry\ mass\ h^{-1}$) |
|---|---|
| 0 | 8.9 ± 0.1 |
| 5 | 8.6 ± 0.3 |
| 10 | 8.0 ± 0.3* |
| 50 | 7.9 ± 0.3* |
| 100 | 7.9 ± 0.1** |

Figures are given as the mean ± S.D., n = 3. * indicates significant difference at $P < 0.01$ while ** signifies significant difference at $P < 0.001$. Comparisons were made between the control and the Con A treatments.

Table 3.7. shows the inhibitory effect of different concentration of Con A on the glycosylated laccase activity. 10 $\mu g\ ml^{-1}$ or higher concentration of Con A significantly inhibited laccase activity ($P < 0.01$ and $P < 0.001$) indicating that lichen laccases from *P. aurata* are might glycosylated.

*Preliminary biochemical characterization of laccases isolated from* Peltigera malacea

Extracellular leachates of *Peltigera malacea* became clear blue solutions after concentration for electrophoresis, with an absorption peak around 614 nm (data not shown). After electrophoresis of the leachates, native gels stained with DMP revealed a single brown

band, while TMB staining for iron and copper functional groups revealed a single blue–green band (Figure 3.8).

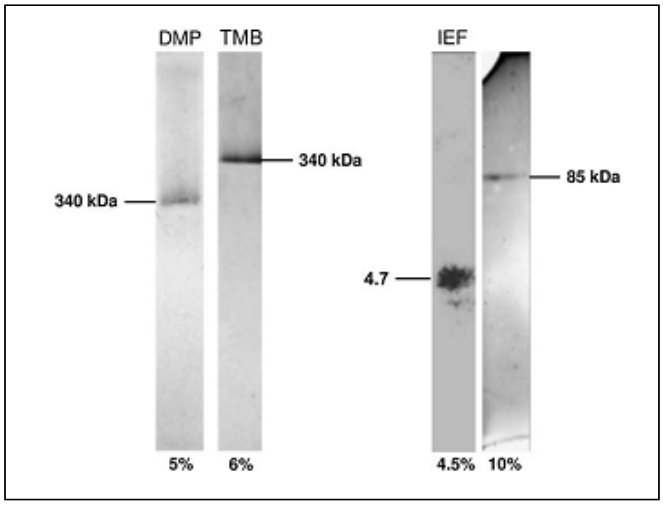



**Figure 3.8.** Characterization of laccases from *Peltigera malacea*. From left to right: native 5% gel, laccase activity staining with DMP; native 6% gel, haem and copper groups stained with TMB; isoelectric focusing followed by TMB staining; denaturing 10% gel (laccase monomer) using protein eluted from the isoelectric focusing gel.

The molecular mass of the active form of the laccase was estimated as ca. 340 kDa. Further analysis was performed by two–dimensional electrophoresis, using a combination of IEF followed by a denaturing 10% polyacrylamide gel. After IEF and TMB staining, a single clear but feint blue–green band was visible corresponding to a pI of ca 4.7 (Figure 3.8). This band was excised, the proteins extracted and run on a denaturing 10% gel. Silver staining revealed a single band with a molecular mass of 85 kDa, indicating that the active form is a tetramer.

## 3.3. Discussion

*Occurrence of laccases in lichens*

The present study is one of the first report of laccase activity in lichenized ascomycetes. After the start of our investigation, Zavarzina and Zavarzin (2006) demonstrated the occurrence of surface laccases and tyrosinases in lichens but they used different species and obtained slightly different results. Demonstration of laccase activity was based on several observations, all consistent with the characteristics of laccases from free-living fungi (Thurston, 1994;

Leonowicz *et al*., 2001; Mayer and Staples, 2002; Baldrian, 2006). First, lichens and their leachates could readily metabolize classic laccase substrates such as ABTS, syringaldazine and *o*-tolidine in the absence of $H_2O_2$ (Table 3.1 and 3.3). The ability to metabolize syringaldazine in particular is considered diagnostic for laccases, as it is not metabolized by other phenol oxidases (Thurston, 1994). Our results illustrate that out of 27 species belonging to the suborder Peltigerineae, 23 showed clear oxidation of laccase substrates (Table 3.1). The highest laccase activity was found in the lichens *Peltigera didactyla*, *P. hymenina* and *Solorina crocea* using ABTS. The soil-stabilizing lichen *Solorina crocea* exhibited the highest activity using the specific laccase substrate, syringaldazine. Similar results were found by Zavarzina and Zavarzin (2006). They investigated 72 species of lichens and found that 20 species out of 21 from the order Peltigerineae displayed considerable laccase activity while only a few lichens from the order Lecanorales showed phenoloxidase activity using ABTS as substrate in their field experiments. Interestingly, they showed positive reaction with ABTS from 32 of 43 species belonging to the order Lecanorales in laboratory experiments collected during a different field season from the one in which the field measurements were made. Zavarzina and Zavarzin (2006) studied the water-soluble phenoloxidases and found that *Solorina crocea* was the most active producer of these enzymes. Lichens from the Lecanorales showed very low water-soluble phenoloxidase activity, and they hypothesized that these laccases were strongly associated with thalli in these species.

In lichens, it seems probable that laccases are produced by the fungal rather than the algal or cyanobacterial symbionts. Laccase activity occurs in members of Peltigerineae that have both cyanobacterial and chlorophycean photobionts. Chlorophycean algae have never been shown to display laccase activity (Quinn *et al.*, 2000). Although sequence analyses suggest that laccase-like genes may be present in cyanobacteria (Nakamura *et al*., 2003), no evidence exists that genes are actually expressed, or what the functions of their corresponding proteins may be. Furthermore, free-living *Nostoc* collected close to some of our Peltigerineae displayed no laccase activity. Nevertheless, these results are confirmed by the observation of Zavarzina and Zavarzine (2006) by the investigation of colour of the lower (fungal) thallus surface.

*Effect of temperature on lichen laccases*

The effect of temperature on laccase activity in *Pseudocyphellaria aurata* was determined using different laccase substrates (Figure 3.1). ABTS, syringaldazine and *o*-tolidine were used as substrates and interestingly, optimum temperature was 7°C of laccase activity using *o*-

tolidine in contrast with values reported in the literature (Kim *et al*., 2008). Optimum temperature is affected by the assay used and is related to the type of organism from which the enzyme was isolated. According to the literature the typical temperature optimum for laccase activity using ABTS ranges between 30°C and 70°C (Table 1.1; Baldrian, 2006); therefore, *P. aurata* laccase displays a typical optimum temperature.

The thermostability of laccases isolated from *Collema flaccidum*, *Lobaria scrobiculata*, *Peltigera rufescens* and *Pseudocyphellaria aurata* was investigated. All lichen laccases tested showed moderate thermostability at 40°C, much less at 50°C (Figure 3.2) and laccases were immediately inactivated at 60°C (data not shown). Similarly to our results, *Solorina crocea* and *Peltigera apthosa* needed only a few minutes and 25 min respectively for 50% inactivation at 50°C and few minutes at 60°C for both species (Lisov *et al*., 2007). In free-living fungi, the half life of the enzyme at 50°C ranges from minutes in *Botrytis cinnerea*, to 2-3 h in *Lentinula edodes* and *Agaricus bisporus*, and to 50-70 h in *Trametes* sp. (for review, see Baldrian, 2006). The rather low stability of lichen laccases is possibly surprising, because even in temperate climates the temperature of dry thalli can reach 60°C (Kappen, 1974). However, while the heat tolerance of dry lichen thalli is high, that of hydrated lichens is actually lower than that of higher plants. Most lichens, including tropical species, die when the thallus temperature exceeds 35-43°C (Beckett *et al*., 2008). Normally, in the field, lichens will dry before they reach these temperatures, and therefore high thermostability will not be needed. Poor thermostability may limit any biotechnological applications of lichen laccases.

*Effect of pH on lichen laccases*

Determining the effect of pH on laccase activity we found that the pH optima of laccases isolated from lichens differ from those from plants and bacteria (typically from 6.0 to 9.5, Table 1.1; Table 3.2; Figure 3.3; Shiba *et al*., 2000). However, they are slightly more alkaline than fungal laccases, which tend to have rather more acidic pH optima, typically from 2.0 to 4.5 using ABTS and *o*-tolidine as substrates (Miller *et al*., 1997; Baldrian, 2006). Results with syringaldazine show typical fungal pH optima (Baldrian, 2006). This nearly neutral pH range of lichen laccases using syringaldazine may provide better biotechnological applications in the process of biobleaching that requires substantial enzyme activities at neutral to alkaline conditions of pH (Heinzkill *et al*., 1998; Bar, 2001).

Syringaldazine is a phenolic- while ABTS and *o*-tolidine are regarded as non-phenolic substrates (Figure 3.9). Xu (1997) discussed the connection between pH profile and type of

substrate. Phenolic substrates usually show a bell-shaped pH profile due to the different redox potential between the reducing substrates and the type 1 copper centre of laccase (rising part of the graph; Xu, 1997; Bar, 2001). The decreasing part of the graph at higher pH indicate the increasing amount of hydroxide anion that can bind to the type 2/type 3 copper centre of laccase and inhibit the binding of $O_2$. Therefore the pH profile of lichen laccase activity using syringaldazine displays the typical effect of pH for laccases acting on phenolic substrates (Figure 3.3). ABTS rather show a monotonic decline than a bell-shaped profile, a result that other workers have often observed (e.g. Xu, 1997; Bar, 2001). Interestingly, laccase activity also shows a bell-shaped pH profile using *o*-tolidine (Figure 3.3) even though the substrate is a non-phenolic compound. The pH profile of *o*-tolidine is might caused by similar interaction between substrate and enzyme than in case of the phenolic compound syringaldazine.

*Enzyme kinetic parameters of* Pseudocyphellaria *laccase*

Evaluating the kinetic parameters of *P. aurata* laccase, $K_M$ values indicated that the binding affinities toward the different substrates were in the order syringaldazine > ABTS > *o*-tolidine. Similar $K_M$ values and binding affinities of substrates have been found in laccases from most of the fungi species (Baldrian, 2006). Syringaldazine contains methoxy- (-$OCH_3$) and hydroxyl groups (-OH; Figure 3.9). The high $V_M/K_M$ ratio displayed when syringaldazine is used as a substrate suggests that methoxy groups allow the adjacent hydroxyl group to be oxidised efficiently (Bar, 2001). In addition, syringaldazine has three adjacent substituents that can increase binding affinity, because the active site of the enzyme has two possible ways to bind to the substrate (Bar, 2001).

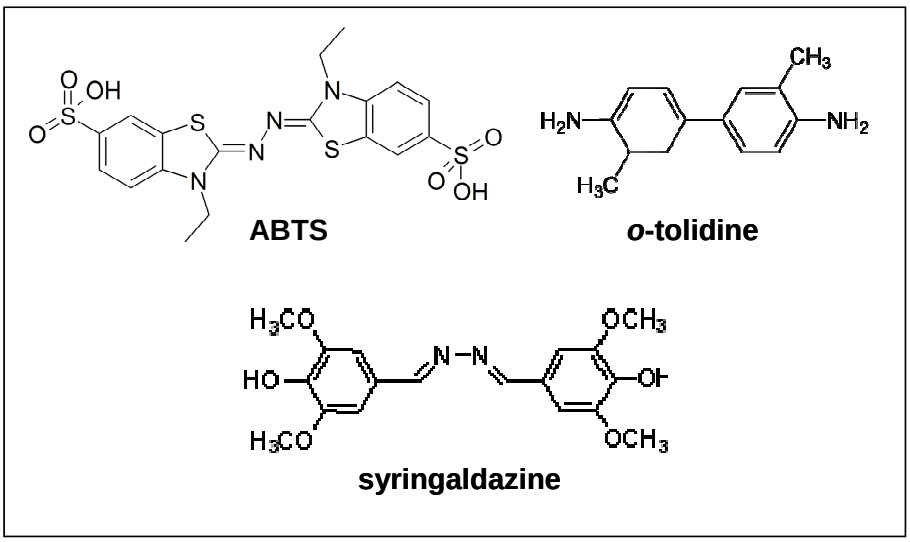


**Figure 3.9.** Chemical structure of phenolic- and non-phenolic substrates used in the experiments of substrate specificity of lichen laccases.

The non-phenolic compound ABTS contains sulfonic acid (-$SO_3H$) groups (Figure 3.9). The $SO_3H$ group has a lone electron pair, and therefore it can be oxidised by laccase but it does not have adjacent substituents to increase binding affinity. *O*-tolidine had shown the lowest biding affinity to the enzyme, possibly because it possesses methyl- (-$CH_3$) and amino substituent groups (-$NH_2$).

*Stress factors affecting laccase activity in* Pseudocyphellaria aurata

Lichens are special organisms because they have ability to tolerate extreme stresses such as low thallus water content, temperature extremes, mechanical injury and ultraviolet radiation (Zavarzina and Zavarzin, 2006; Beckett *et al*., 2008). A possible influence of two stress factors on laccase activity from *Pseudocyphellaria aurata* was studied. Desiccation and wounding stimulated laccase activity (Figure 3.5), but leakage of cytoplasmic enzymes cannot explain the stress-induced increases in activity. Based on the observation that wounding causes the release of only 4% of the strictly cytosolic G-6-PD activity (Table 3.4), it was estimated that leakage of intracellular enzymes contributes only a small proportion of the stress-induced increases in enzyme activity. Further investigations showed that high proportion of laccase activity from wounded lichen discs appeared in fresh solution indicating that laccases from *Pseudocyphellaria aurata* are bound extracellularly to the cell wall. Zavarzina and Zavarzin (2006) found similar results; phenoloxidase activity was stimulated in *Peltigera apthosa* and *Lobaria pulmonaria* after one and two desiccation/rehydration cycles. These and our data may suggest that laccases associated with lichen thallus are either resistant to desiccation or the transition of the thallus from anabiosis to a metabolically active state stimulates their production (Zavarzina and Zavarzin, 2006). The mechanisms for such fast increases occur after desiccation and wounding in lichen laccase activity were not studied in this project, but could include phosphorylation (Larrondo *et al*., 2003), changes in apoplastic pH, assembly of the active form of the enzyme from existing monomers, or alterations of protein conformation.

*Inhibition of laccase activity by several compounds*

A wide range of compounds have been reported to inhibit laccase activity (Baldrian, 2006) therefore the effect of a number of inhibitors on laccase activity from different lichen

species (*Pseudocyphellaria aurata*, *Collema flaccidum*, *Lobaria scrobiculata* and *Peltigera rufescens*) was investigated. The general inhibitors of metal-containing oxidases are cyanide, sodium azide and fluoride (Edens *et al*., 1999). The effects of sodium azide, potassium cyanide and sodium fluoride were tested on *P. aurata* laccase (Figure 3.6; 3.7). All of three inhibitors strongly reduced laccase activity that was more sensitive to azide than cyanide. Similar results were found in the ascomycete *Mauginiella* sp. (Palonen *et al*., 2003) while in *Melanocarpus albomyces* cyanide and azide had similar inhibitory effect on enzyme activity while sodium fluoride had weaker effect, only 40% inhibition using 1 mM concentration of flouride (Kiiskinen *et al*., 2002). In case of the inhibitor sodium flouride we found greater inhibitory effect on laccase activity than with cyanide that can be attributed to the limited accessibility of the type 2/3 trinuclear copper site of laccase by $F^-$ (Xu, 1996).

EDTA significantly inhibited laccase activity, but the degree of inhibition between the investigated lichen species differed remarkably (Table 3.5). Similar results were found in the study of *Pycnoporus sanguineus* and *Coprinus micaceus* by Bar (2001) and in the experiments of *Trametes versicolor* by Lorenzo *et al*. (2005). EDTA usually has inhibitory effect on laccase activity because it can take out copper ions from enzymes forming a complex, thus, modifying the laccase active site (Zavarzina *et al*., 2004; Lorenzo *et al*., 2005). $Ca^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ are heavy metal ions that can act as inducers of laccase in fungus cultivation media whereas they are toxic on extracellular laccases and when present in excess (Baldrian and Gabriel, 2002; Baldrian, 2003, 2004). Results showed a considerably inhibition of laccase activity using the heavy metal ions, especially the $Mn^{2+}$. Similar results were found in the fungus *Daedalea quercina* (Baldrian, 2004) where $Mn^{2+}$ was the most efficient inhibitor of laccase activity with a 91% inhibition. Most of the literature deals with $Cu^{2+}$ as an important inhibitor of laccase activity that inhibits enzyme activity even at low concentrations (Baldrian and Gabriel, 2002). We found similar results, but Lorenzo *et al*. (2005) and Baldrian (2004) observed that $Cu^{2+}$ stimulated laccase activity in *Trametes versicolor* and *Daedalea quercina* at concentrations lower than 1 mM while enzyme activity was significantly inhibited at higher concentrations. Most studies show $Ca^{2+}$ as an inducer of laccase. Crowe and Olsson (2001) reported that calcium ionophore and lithium- and calcium chloride all induced laccase activity through mobilizing calcium across membranes by various means. For reasons that remain unclear, $Ca^{2+}$ can sometimes act as an inhibitor (Munoz *et al*., 1997a). The authors showed that inhibition of laccase activity by calcium ion occurs in *Pleurotus eryngii* where 2.5 mM $CaCl_2$ had approximately 60% inhibition on laccase activity. In the present study we also found inhibition of enzyme activity using calcium ion. However, recent studies found that rather chlorides then

metal ions (such as $Ca^{2+}$, $Cu^{2+}$ and $Mn^{2+}$) are responsible for laccase inhibition (Farnet *et al*., 2008).

*Effect of a wide range of inducers on* Pseudocyphellaria *laccase*

Various ions and molecules can induce laccase synthesis in long term in free-living fungi, e.g. $Cu^{2+}$, ethanol, ferulic, vanillic and veratric acids, syringaldazine and xylidine (Palmieri *et al*., 2000; Crowe and Olsson, 2001; Leonowicz *et al*., 2001). However, in many free-living fungi the production of laccases is often constitutive, and not suppressed even by high nutrient availability (ten Have and Teunissen, 2001). In *P. aurata* laccase activity was not influenced by normal laccase inducers, suggesting that in this species laccases are constitutively expressed.

*Investigation of glycosylation on* Pseudocyphellaria *laccase*

Most of the laccases described in plants and fungi are glycoproteins with different degree of carbohydrate content (Baldrian, 2006). Concanavalin A (Con A, a lectin protein) can bind specifically to certain groups of glycoproteins and deactivate them. Laccases from fungi described so far are glycoproteins with approximately 10-25% carbohydrate content (Baldrian, 2006). There are some exceptions; for example *Coriolopsis fulvocinnerea* contains 32% (Shleev *et al*., 2004) while *Pleurotus pulmonarius* laccase was found to contain 44% sugars (de Souza and Peralta, 2003). Very low degree of glycosylation was observed in *Pleurotus eryngii*, where glycosylation was only 1-7% (Munoz *et al*., 1997b). Carbohydrates are mostly N-linked to the polypeptide chain (Saparrat *et al*., 2002; Baldrian, 2006) and their suggested roles are to protect the enzyme from proteolysis (Yoshitake *et al*., 1993) and to render enzymes soluble. Our results showed that 10 $\mu g\ ml^{-1}$ concentration of Con A was high enough to significantly inhibit lichen laccase activity (Table 3.7). Deactivation of enzyme activity suggested that the laccase from *Pseudocyphellaria aurata* is a glycoprotein. In the future more work is needed to determine the degree and type of laccase glycosylation in lichen laccases.

*Biochemical characterization of laccase isolated from* Peltigera malacea

Using electrophoresis, we found that the active form of the laccase in *Peltigera malacea* is a tetramer with a molecular mass of 340 kDa (Figure 3.8). Although most laccases from free-living fungi appear to be monomers (Thurston, 1994; Leonowicz *et al*., 2001; Baldrian, 2006), a tetrameric laccase has been reported from the ascomycete *Podospora anserina* (Durrens, 1981).

While the molecular mass of the active form of the enzyme is quite high, the molecular mass of the monomer (85 kDa) is within the range of other molecular masses reported for other laccases (Baldrian, 2006). Microcharacterization of the enzymes revealed other properties consistent with their identification as laccases. These included an acidic pI of ca. 4.7, transient staining by TMB (Figure 3.8), and an absorption spectrum with a peak at 614 nm (see Chapter 5 for more details) due to a type 1 or paramagnetic 'blue' copper atom (Claus, 2004). Although TMB staining is often used as an indicator of haem groups, this substance also stains Cu-containing functional groups (Miller and Nicholas, 1984). Many fungi that display laccase activity possess more than one isoforms (Mayer and Staples, 2002), and up to 20 occur in *Trametes gallica* (Dong *et al*., 2005), but *P. malacea* has only one isoform (Figure 3.8).

## 3.4. Conclusions

The present study suggests that in lichenized Ascomycota significant laccase activity seems to occur mainly in Peltigerineae. There are several possible explanations for high laccase activity in this particular group of lichens. Members of Peltigerineae are characterized by high growth rates, and high metabolic activity in general, and a tendency to grow in wetter, more productive habitats (Palmqvist *et al*., 2002). Possibly, high cell wall redox activity is necessary to provide enough nutrients to support fast growth. Recent phylogenetic studies support the view that Peltigerineae form a monophyletic group within Lecanorales (Wiklund and Wedin, 2003; Miadlikowska and Lutzoni, 2004; Wedin and Wiklund, 2005) and share traits that are uncommon in other lichen groups (Grube and Winka, 2002). More generally, within the Ascomycota, the Lecanorales are considered advanced (Lutzoni *et al*., 2004). Although laccase activity is generally considered to be best developed in the Basidiomycota, possession of laccases may enable lichens in Peltigerineae to more efficiently exploit their environment, giving a selective advantage to these highly developed lichens.

# CHAPTER 4

## BASIC PROPERTIES OF LICHEN TYROSINASES


The structures of *Streptomyces castaneoglobisporus* tyrosinase; red indicates the mature tyrosinase protein while the carrier protein is blue (Matoba *et al*., 2006).

*Solorina crocea* (L.) Ach.
was collected in Russia (photo by D. Cserhalmi).

## 4.1. Introduction

Tyrosinases are a family of multicopper oxidase proteins found in some higher plants, fungi, prokaryotes and animals (Seo *et al*., 2003; Claus and Decker, 2006). Perhaps surprisingly, this is one of the first studies for the existence of tyrosinases in an important group of fungi, the lichenized ascomycetes. However, in lichens, strong evidence exists that melanins act as screens for UV light (Gauslaa and Solhaug, 2001; Stepanenko *et al*., 2002; Solhaug *et al*., 2003). Zavarzina and Zavarzin (2006) carried out some experiments showing that in the field lichens can metabolize tyrosinase substrates, and this was confirmed by our own preliminary results. The aims of the work presented here were to verify the presence of tyrosinases in lichens, distinguish them from laccases on the bases of substrate specificity, sensitivity to inhibitors, activation by SDS and stress and the microcharacteristics of the enzymes such as the cellular location and molecular masses. We also determined the relationship between laccase activity and tyrosinases activity in a range of species. Determination of the taxonomic distribution of tyrosinase activity and characterization of the enzymes will be essential for elucidating the biological roles of these redox enzymes in lichen physiology.

## 4.2. Results

*Occurrence of tyrosinases in lichens*

Results from a survey of extracellular tyrosinase activity in 47 lichen species using epinephrine as substrate showed that while activity could be readily demonstrated in members of the Peltigerineae, other species had very low activities (Table 4.1).

**Table 4.1.** Tyrosinase activity, detected as oxidation of epinephrine to adrenochrome in a range of lichens.

| **Species** | **Tyrosinase activity (µmol $g^{-1}$ dry mass $h^{-1}$)** |
|---|---|
| **Lichens from suborder Peltigerineae** | |
| *Collema flaccidum* | 56 ± 13 |
| *Leptogium saturninum* | 24 ± 7 |
| *Leptogium sessile* | 19 ± 3 |
| *Lobaria pulmonaria* | 22 ± 3 |
| *Lobaria scrobiculata* | 25 ± 2 |
| *Nephroma arcticum* | 131 ± 14 |
| *Nephroma parile* | 62 ± 9 |
| *Nephroma rufum* | 28 ± 7 |
| *Peltigera apthosa* | 109 ± 11 |
| *Peltigera canina* | 145 ± 11 |

| Species | Tyrosinase activity (µmol $g^{-1}$ dry mass $h^{-1}$) |
|---|---|
| *Peltigera didactyla* | 224 ± 11 |
| *Peltigera horizontalis* | 55 ± 17 |
| *Peltigera hymenina* | 234 ± 28 |
| *Peltigera leucophlebia* | 134 ± 28 |
| *Peltigera malacea* | 85 ± 15 |
| *Peltigera neopolydactyla* | 175 ± 10 |
| *Peltigera polydactylon* | 151 ± 16 |
| *Peltigera praetextata* | 138 ± 18 |
| *Peltigera rufescens* | 117 ± 15 |
| *Peltigera scabrosa* | 227 ± 31 |
| *Pseudocyphellaria aurata* | 60 ± 13 |
| *Pseudocyphellaria gilva* | 19 ± 2 |
| *Solorina crocea* | 131 ± 31 |
| *Sticta fuliginosa* | 78 ± 7 |
| *Sticta cf. limbata* | 94 ± 13 |
| *Sticta cf. sublimbata* | 46 ± 13 |
| *Sticta sp.* | 123 ± 11 |
| **Lichens from non-suborder Peltigerineae** | |
| *Anaptychia ciliaris* | 11 ± 4 |
| *Bryoria simplicior* | 4 ± 0 |
| *Cetraria islandica* | 1 ± 0 |
| *Cladonia cariosa* | 6 ± 1 |
| *Cladonia stellaris* | 3 ± 1 |
| *Evernia prunastri* | 5 ± 1 |
| *Flavocetraria nivalis* | 1 ± 0 |
| *Heterodermia speciosa* | 4 ± 2 |
| *Hypogymnia physodes* | 4 ± 1 |
| *Parmelia cetrarioides* | 6 ± 1 |
| *Platismatia glauca* | 5 ± 2 |
| *Pseudevernia furfuracea* | 4 ± 1 |
| *Ramalina celastri* | 13 ± 4 |
| *Ramalina farinacea* | 4 ± 1 |
| *Ramalina pollinaria* | 3 ± 1 |
| *Roccella montagnei* | 4 ± 2 |
| *Stereocaulon tomentosum* | 4 ± 1 |
| *Umbilicaria deusta* | 3 ± 1 |
| *Umbilicaria pustulata* | 7 ± 1 |
| *Usnea undulata* | 3 ± 1 |
| Mean for suborder Peltigerineae (n = 27) | 100 ± 13 |
| Mean for non-suborder Peltigerineae (n = 20) | 5 ± 1 |

Figures are given as the mean ± S.D., n = 3.

The mean of tyrosinase activity was 100 ± 13 µmol $g^{-1}$ dry mass $h^{-1}$ in species from Peltigerineae while in lichens out of the suborder Peltigerineae it appeared to be only 5 ± 1 µmol $g^{-1}$ dry mass $h^{-1}$ using epinephrine as a substrate. The highest tyrosinase activity occurred within the genus *Peltigera*, mostly higher than 100 µmol $g^{-1}$ dry mass $h^{-1}$. High enzyme activity also occurred in *Nephroma arcticum*, *Solorina crocea* and one *Sticta* species. The lichen *Anaptychia ciliaris* and *Ramalina celastri* showed the highest tyrosinase activity among the lichens out of Peltigerineae, higher than 10 µmol $g^{-1}$ dry mass $h^{-1}$.

Tyrosinase activity was significantly correlated with laccase activity within the Peltigerineae (Figure 4.1).

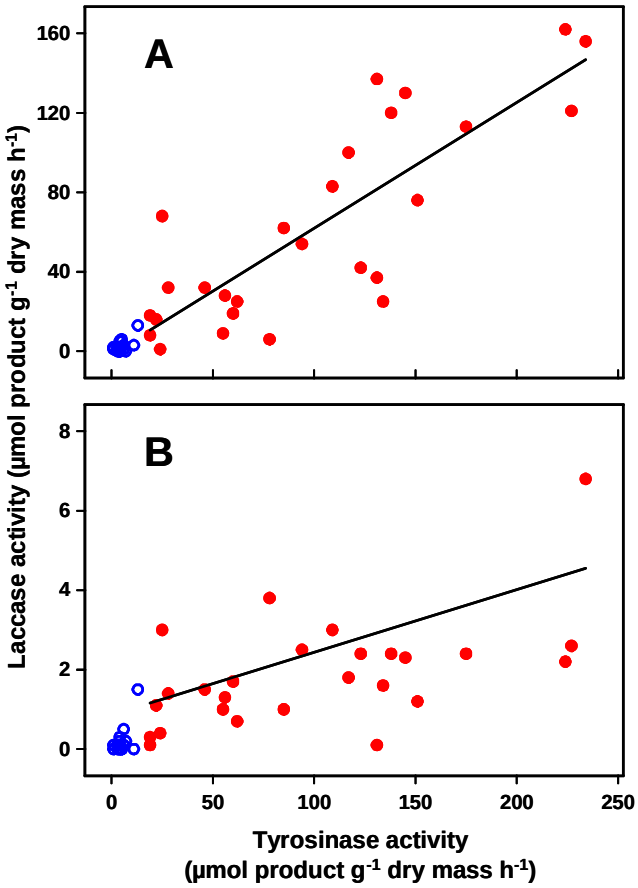



**Figure 4.1.** Rates of metabolism of the laccase substrates ABTS (A) and syringaldazine (B) in a range of lichens are positively correlated to rates of metabolism of the tyrosinase substrate epinephrine ($P < 0.001$). Each symbol denotes a single species; filled circles (red) indicate lichens in suborder Peltigerineae, and open circles (blue) lichens from other suborders.

*Properties of tyrosinases in lichens*

Lichens and leachates derived from Peltigerineae also readily metabolized L-tyrosine, although at only approx. 10% of the typical rates observed with epinephrine and DOPA (Table 4.2). For example, in *Pseudocyphellaria aurata*, rates were 7 ± 3 µmol $g^{-1}$ dry mass $h^{-1}$ for thallus disks, and 1 ± 0 µmol $g^{-1}$ dry mass $h^{-1}$ for leachates derived by shaking lichens in water while the activity of tyrosinases appeared to be 63 ± 7 and 60 ± 13 µmol $g^{-1}$ dry mass $h^{-1}$ in *Pseudocyphellaria* disks using the substrate L-DOPA and epinephrine, respectively (Table 4.1 and 4.2).

**Table 4.2.** Extracellular tyrosinase activity of *Pseudocyphellaria aurata* using L-DOPA and L-tyrosine as substrates.

| | Tyrosinase activity ($\mu$mol $g^{-1}$ dry mass $h^{-1}$) | |
|---|---|---|
| | L-DOPA | L-tyrosine |
| discs | 63 ± 7 | 7 ± 3 |
| leachates | 4 ± 0 | 1 ± 0 |

Figures are given as the mean ± S.D., n = 5.

Tyrosinases and laccases could be distinguished on the basis of their sensitivity to inhibitors (Table 4.3). The inhibitors cyanide, azide and sodium fluoride all strongly reduced tyrosinase activity. Tyrosinase activity was more resistant to cyanide and azide, and while these inhibitors reduced tyrosinase activity equally, laccase activity was more sensitive to azide than cyanide. The tyrosinase-specific inhibitor 4-hexylresorcinol (0.2 mM) reduced tyrosinase activity by approx. 20%.

**Table 4.3.** The effect of inhibitors on tyrosinase and laccase activity in the lichen *Pseudocyphellaria aurata*. L-DOPA and ABTS were used as substrates to determine tyrosinase and laccase activity, respectively.

| Inhibitor | Enzyme activity ($\mu$mol product $g^{-1}$ dry mass $h^{-1}$) | Inhibition (%) |
|---|---|---|
| **Tyrosinase (L-DOPA)** | | |
| Control | 10.8 ± 0.4 | 0 |
| 2 mM $NaN_3$ | 2.4 ± 0.4 | 77 |
| 1 mM KCN | 2.4 ± 0.4 | 78 |
| 40 mM NaF | 1.8 + 0.2 | 84 |
| 0.2 mM Hexylresorcinol | 8.5 ± 0.2 | 21 |
| **Laccase (ABTS)** | | |
| Control | 17.5 ± 0.6 | 0 |
| 2 mM $NaN_3$ | 0.4 ± 0.1 | 96 |
| 1 mM KCN | 2.5 ± 0.1 | 86 |
| 40 mM NaF | 0.3 ± 0.0 | 98 |
| 0.2 mM Hexylresorcinol | n.d. | |

Figures are given ± S.D., n = 4.
n.d. = not determined

Wounding stress strongly stimulated while desiccation stress had no effect on tyrosinase activity (Figure 4.2).

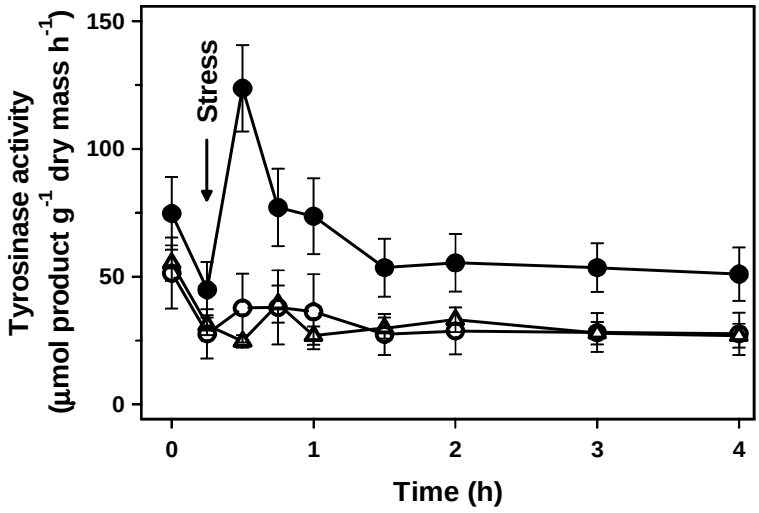



**Figure 4.2.** The effect of wounding and desiccation on tyrosinase activity (measured with epinephrine as a substrate) in *Pseudocyphellaria aurata*. The arrow indicates the time that material was stressed, either by cutting disks into quarters (filled circles) or by desiccating it to a relative water content of 0.05 over 2.5 h then suddenly rehydrating it (open triangles). Open circles represent control (unstressed) material. Error bars indicate standard deviation, n = 3.

2 mM SDS stimulated tyrosinase activity of intact discs by 80% while about 50% of tyrosinases derived from thallus discs being 'latent' (Table 4.4). However, SDS had no effect on laccase activity measured using ABTS.

**Table 4.4.** The effect of SDS (2 mM) on laccase and tyrosinase activity of *Pseudocyphellaria aurata* discs and leachates using ABTS and L-DOPA as substrates.

| | Laccase activity (µmol $g^{-1}$ dry mass $h^{-1}$) | | Tyrosinase activity (µmol $g^{-1}$ dry mass $h^{-1}$) | |
|---|---|---|---|---|
| | control | with SDS | control | with SDS |
| discs | 17 ± 1 | 15 ± 2 | 63 ± 7 | 134 ± 39* |
| leachates | | | 4 ± 0 | 31 ± 3** |

Figures are given as the mean ± S.D., n = 3. * indicates significant difference at $P < 0.05$ while ** signifies significant difference at $P < 0.01$.
Comparisons were made between control and treatment with SDS.

*Comparison of the cellular location and molecular masses of tyrosinases and laccases*

Low activities of laccase and peroxidase, but not tyrosinase, were detected in the non-Peltigeralean lichen *Flavocetraria nivalis* (Figure 4.3/A; Table 4.5). Laccase activity was

mostly intracellular, while peroxidase activity was equally distributed between the intracellular and the hydrophobic cellular locations.

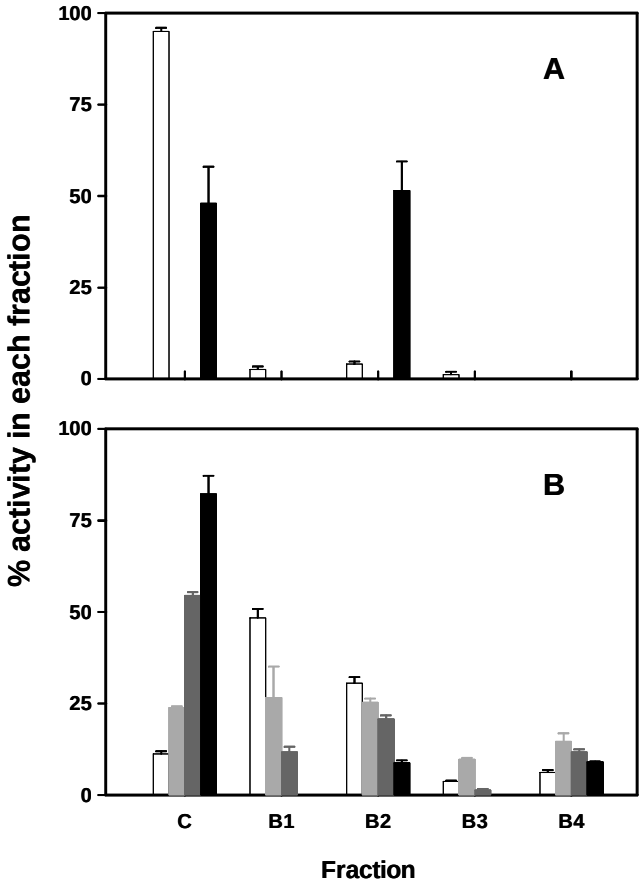



**Figure 4.3.** Cellular location in *Flavocetraria nivalis* (A) and *Pseudocyphellaria aurata* (B) of laccases (open bars), tyrosinases without SDS (light grey), tyrosinases with 2 mM SDS (dark grey) and peroxidases (solid bars). Values are expressed as percentages of the total activity. C, cytosolic fraction; B1, loosely bound to the cell wall, e.g. by hydrogen bonds; B2, bound by van der Waals forces and hydrophobic interactions; B3, bound by strong electrostatic forces; B4, bound by covalent linkages. Table 4.5 gives the actual enzyme activities. Error bars indicate the standard deviation, n = 5.

*Pseudocyphellaria aurata* displayed activities of all three enzymes (Figure 4.3/B; Table 4.5). Laccase was located mostly in the loosely and hydrophobically bound cell wall fractions. Compared with the laccases, a greater proportion of tyrosinases occurred intracellularly. Adding SDS stimulated tyrosinase activity in all fractions except those bound by electrostatic interactions (Figure 4.3/B fraction B3, Table 4.5). Separate experiments showed that SDS had no effect on laccase activity measured with ABTS (Table 4.4).

**Table 4.5.** Activities of the enzymes laccase, tyrosinase and peroxidase in the lichens *Pseudocyphellaria aurata* and *Flavocetraria nivalis* in various cellular fractions (see text for details). Values are expressed in µmol product $g^{-1}$ dry mass $h^{-1}$.

| | *Pseudocyphellaria aurata* | *Flavocetraria nivalis* |
|---|---|---|
| Laccase activity | | |
| C | 9 ± 1 | 0.60 ± 0.01 |
| B1 | 36 ± 2 | 0.01 ± 0.01 |
| B2 | 23 ± 1 | 0.02 ± 0.01 |
| B3 | 3 ± 0 | 0.10 ± 0.01 |
| B4 | 5 ± 0 | 0 |
| Tyrosinase activity – SDS | | |
| C | 46 ± 1 | 0 |
| B1 | 51 ± 16 | 0 |
| B2 | 48 ± 2 | 0 |
| B3 | 18 ± 1 | 0 |
| B4 | 28 ± 4 | 0 |
| Tyrosinase activity + SDS | | |
| C | 370 ± 6 | 0 |
| B1 | 79 ± 10 | 0 |
| B2 | 141 ± 7 | 0 |
| B3 | 9 ± 3 | 0 |
| B4 | 79 ± 5 | 0 |
| Peroxidase activity | | |
| C | 4.3 ± 0.3 | 0 |
| B1 | 0 | 0.02 ± 0.01 |
| B2 | 0.5 ± 0.0 | 0 |
| B3 | 0 | 0.02 ± 0.01 |
| B4 | 0.5 ± 0.0 | 0 |

Activities of tyrosinases are given with and without the addition of 2 mM SDS to the assay medium. Figures are given as the mean ± S.D., n = 5.

Following electrophoresis, tyrosinase isolated from *Pseudocyphellaria aurata* visualized with L-DOPA and epinephrine revealed one main band with a molecular mass of approx. 56 kDa (Figure 4.4). *Peltigera malacea* tyrosinase had a molecular weight of approximately 58 kDa and also possessed a smaller amount of enzyme with a molecular mass of approx. 160 kDa stained by L-DOPA.

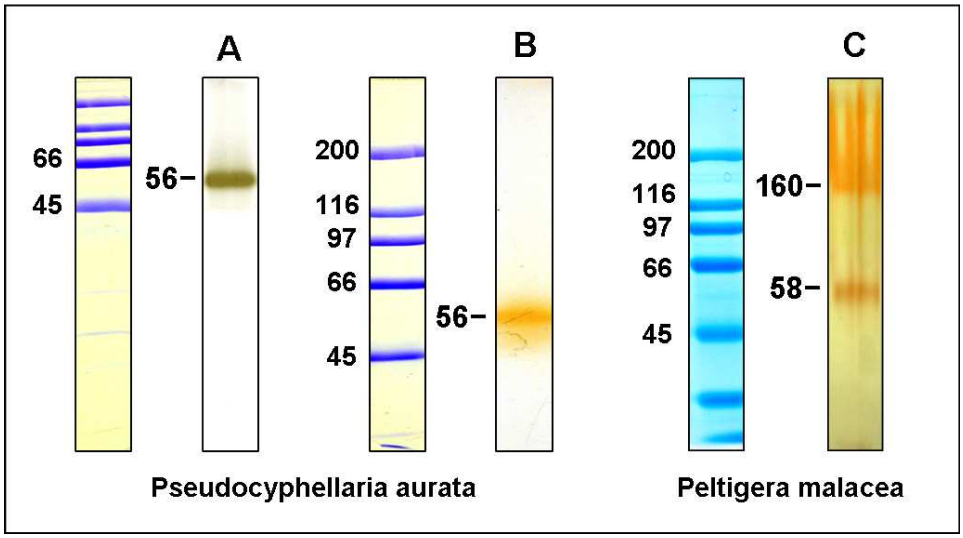



**Figure 4.4.** Characterization of tyrosinases from *Pseudocyphellaria aurata* and *Peltigera malacea*. A native 12.5% (A), 7.5% (B) and 10% (C) polyacrylamide gel was used; tyrosinase activity was stained by L-DOPA (A, C) and epinephrine (B). Molecular mass markers were stained using Coomassie brilliant blue G250. Approximate weights of the molecular markers and tyrosinase bands are indicated in kDa.

The approximate molecular masses of the isoforms derived from *Pseudocyphellaria aurata* were the same in the cytoplasmic and cell wall fractions (Figure 4.5). Laccase visualized by DMP revealed one band with a molecular weight of approximately 165 kDa. Tyrosinase had an approximate molecular weight of 55 kDa. Activity staining with guaiacol was used to detect peroxidase activity. Interestingly, some minutes after incubating the gel in guaiacol an orange band appeared with a molecular weight of approx. 80 kDa that disappeared later; after 20 h of incubation in distilled water another band became visible with a heavier molecular weight of approximately 165 kDa (data not shown). TMB was used to test for the presence of haem- or copper-containing proteins such as peroxidase and laccase. Two greenish-blue bands were detected at the same position as with guaiacol staining with an approximate molecular weight of 80 and 165 kDa (Figure 4.5).

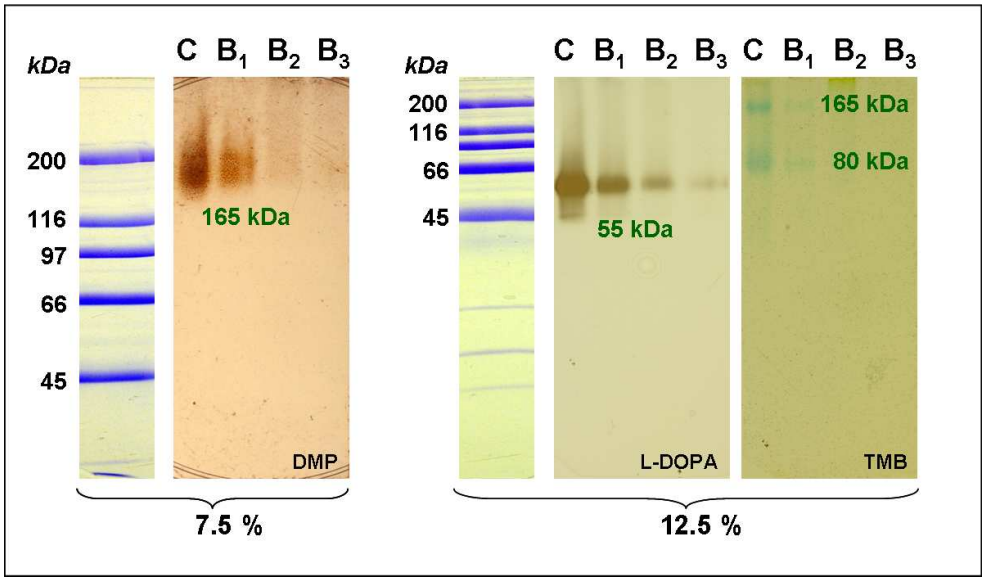



**Figure 4.5.** Characterization of laccase, tyrosinase and peroxidase from the fractions of *Pseudocyphellaria aurata*. A native 7.5% (in case of laccase) and 12.5% (for tyrosinase and peroxidase) polyacrylamide gel was used; laccase activity were visualized by DMP, tyrosinase activity was stained by L-DOPA and TMB was used to detect peroxidase activity. Molecular mass markers were stained using Coomassie brilliant blue G250. Different fractions are described in the legend of Figure 4.3. Approximate weights of the molecular markers and the enzyme bands are indicated in kDa.

Silver staining also revealed a protein in B1 and B2 with a molecular mass identical to that of the activity stain (data not shown). The position of these bands did not change if samples were heated in mercaptoethanol before loading, suggesting that estimates of laccase, tyrosinase and peroxidase molecular mass are reasonably accurate.

## 4.3. Discussion

*Occurrence of tyrosinases in lichens*

Occurrence of tyrosinases is described in many fungi including some ascomycetes (Kupper *et al*., 1989; Nakamura *et al*., 2000; Selinheimo *et al*., 2006) but interestingly there are no reports of the presence of tyrosinases in lichens. The main finding of this study is that in addition to laccase activity, high extracellular tyrosinase activity occurs in lichens in the Peltigerineae (Table 4.1 and Figure 4.1). It was possible to distinguish tyrosinase activity from

laccase activity based on several observations, all consistent with the characteristics of tyrosinases from free-living fungi (Halaouli *et al*., 2006). These include the ability of lichens and their leachates to readily metabolize classic tyrosinase substrates such as L-DOPA, epinephrine and L-tyrosine (Table 4.1 and 4.2). The latter is the most specific compound to tyrosinase (Mueller *et al*., 1996). Almost all lichens belonging to suborder Peltigerineae displayed significant oxidation of tyrosinase substrates (Table 4.1). Similar to the results of assays of laccase activity, the highest tyrosinase activity occurred within the genus *Peltigera*. Interestingly, two lichens from other suborder, the lichen *Anaptychia ciliaris* and *Ramalina celastri*, displayed moderate enzyme activities. Zavarzina and Zavarzin (2006) reported that *Solorina crocea* seemed to be the most active producer of the water soluble enzyme, in case of both laccase and tyrosinase. However, we found only moderate production of tyrosinase (131 ± 31 µmol $g^{-1}$ dry mass $h^{-1}$) while laccase activity in *Solorina* showed one of the highest rates between the studied species (Table 3.1).

Our results showed that tyrosinase activity was significantly correlated with laccase activity within the Peltigerineae. Guillen *et al*. (2000) suggested that laccases catalyse a reaction which produces superoxide radicals ($O_2\cdot^-$) that can break down to $H_2O_2$ (for more details see Chapter 1). Interestingly, Beckett and Minibayeva (2007) reported that tyrosinase activity was considerably correlated to the rate of $H_2O_2$ breakdown. Therefore it is possible that the laccase catalysed extracellular $H_2O_2$ production can be break down by lichen cell wall tyrosinases from suborder Peltigerineae at least in a certain degree, in a catalase-like reaction (Beckett and Minibayeva, 2007).

As no reports of tyrosinases exist in free-living cyanobacteria or algae, it seems most likely that the lichen mycobiont being responsible for tyrosinase synthesis. Results presented here are consistent with the findings of Zavarzina and Zavarzin (2006) who deduced the presence of tyrosinase based up colour changes following the application of tyrosinase substrates to the lower (exclusively fungal) thallus surface.

*Properties of tyrosinases in lichens*

Our results show that lichens from the suborder Peltigerineae could oxidise the three typical tyrosinase substrates; L-DOPA, L-tyrosine and epinephrine (Figure 4.6, Mayer, 1987, 2006; Claus and Decker, 2006). We found that the oxidation rate of L-tyrosine was much lower comparing to the oxidation rate of L-DOPA and epinephrine (Table 4.1 and 4.2) as it was observed in the reports of Zavarzina and Zavarzin (2006) and Lisov *et al*. (2007).

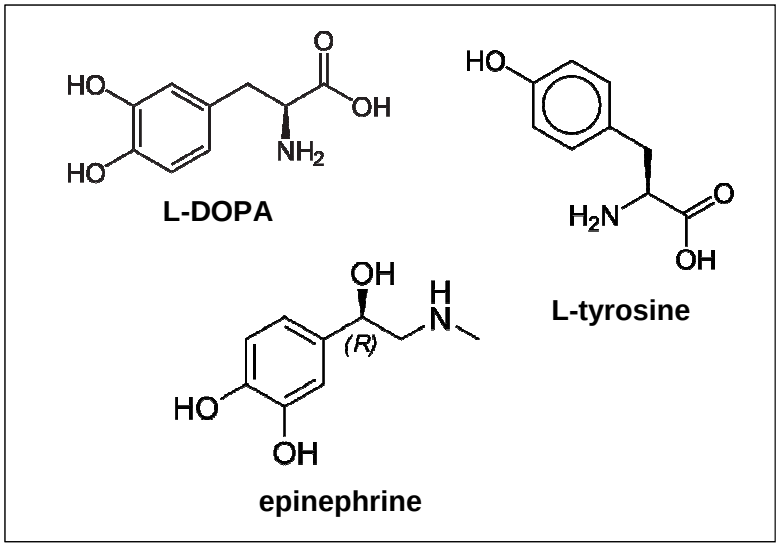



**Figure 4.6.** Structures of phenolic compounds used in the study of substrate specificity of tyrosinase.

It is well known that tyrosinase can only oxidize *ortho*-diphenols and *para*-monophenols (Messerschmidt, 1997). The structure of phenolic compounds may indicate the degree of oxidation by tyrosinase enzyme as was shown in case of laccases in Chapter 3. Our observations suggest that the *ortho*-position of the –OH groups in case of L-DOPA and epinephrine may cause greatest oxidation extent by tyrosinase in contrast to L-tyrosine that is a *p*-monophenol (Figure 4.6).

Many inhibitors of tyrosinases have been reported, for example sodium azide, copper chelators, gallic acid, kojic acid, some flavanols and 4-hexylresorcinol (4HR) (Ratcliffe *et al*., 1994; Burton, 2003; Mayer, 2006; Lisov *et al*., 2007). Our results show strong inhibition of lichen tyrosinases by cyanide, azide and fluoride. 4-hexylresorcinol also inhibited tyrosinase that compound is a specific inhibitor of tyrosinases, which is useful for differentiating between laccase and tyrosinase (Dawley and Flurkey, 1993a; Burton, 2003). Dawley and Flurkey (1993b) reported that 100 µM concentration of 4HR caused 90% loss of mushroom tyrosinase activity. However, 4HR was not as effective on lichen tyrosinase as on mushroom tyrosinase because it reduced enzyme activity by approximately 20% (Table 4.3).

Interestingly, both wounding and desiccation increased laccase activity while only wounding caused strong activation in tyrosinase activity (Figure 4.2). As mentioned in Chapter 3, the leakage of cytoplasmic enzymes cannot be the explanation of the stress-induced increases

in activity. Mayer (2006) suggests that wounding can cause localized acidification which results in conversion of a latent to an active enzyme. However, the mechanisms for such fast increases in activity could include activation by modifying protein conformation and many other mechanisms (Larrondo *et al*., 2003).

Many fungal and plant tyrosinases (about 60-90% of the total enzyme) are present in latent forms and can be activated by SDS, proteolysis, acid shock or by stresses (van Gelder *et al*., 1997; Mayer, 2006). Latent form of tyrosinases seems to be very stable but it becomes more sensitive to temperature and pH after activation (Söderhäll, 1995). SDS activation has been widely reported for different fungal and plant tyrosinases (van Gelder *et al*., 1997; Halaouli *et al*., 2006; Marusek *et al*., 2006). Our results show that lichen tyrosinases can be also strongly activated by SDS (Table 4.4) but the mechanism is unknown. The activation mechanisms of fungal and plant tyrosinases is also unclear today, but has been attributed to the slow conformational enzymatic changes, solubilization of the enzyme or removal of an inhibitor (Mayer and Harel, 1979; van Gelder *et al*., 1997; Espin and Wichers, 1999; Halaouli *et al*., 2006). However, Ichishima *et al*. (1984) suggested that two endogenous serine proteases are involved in the activation of *Aspergillus oryzae* tyrosinase. Activation of latent forms may also allow fungi to increase tyrosinase activity rapidly in response to stresses such as wounding (Figure 4.2).

*Separation of tyrosinases from laccases on the basis of some biochemical properties*

Cellular fractionation and molecular weight estimates were carried out to distinguish tyrosinases and laccases. The cellular locations of laccases and tyrosinases were clearly different (Figure 4.3; Table 4.5); while most laccase activity was in the cell wall, a much greater proportion of tyrosinase was intracellular. Cellular fractionation of other species (data not shown) indicated that *Peltigera* spp. displayed similar patterns to *Pseudocyphellaria*, while *Cladonia* spp. was similar to those of *Flavocetraria*. It was also found that small but significant amounts of enzymes were released simply by shaking lichens in water, for *Pseudocyphellaria aurata* corresponding to approx. 0.8 and 0.4% of the total cellular activities of laccases and tyrosinases, respectively. These activities were probably released in the first supernatant of the cellular fractionation, and therefore included in the cytoplasmic fraction ‘C’. While these activities are too small to influence the enzyme distribution presented in Figure 4.3, in the field continual release of small amounts of redox enzymes could be important for lichens.

PAGE separation of cytosolic and cell wall fractions from *Peltigera malacaea* and *Pseudocyphellaria aurata* showed that a band with a molecular mass of approx. 60 kDa appeared within minutes of incubation in the tyrosinase substrates L-DOPA and epinephrine (Figure 4.4). This mass is similar to those reported for other ascomycete tyrosinases (van Gelder *et al*., 1997; Halaouli *et al*., 2006; Marusek *et al*., 2006). The approximate molecular mass of the laccases from *Peltigera malacea* was much higher at well over 300 kDa (Figure 3.8), while the laccase from *Pseudocyphellaria aurata* had a molecular mass of approx. 160 kDa (Figure 5.2). Interestingly, after incubating gels for several hours with DOPA, faint bands appeared at the same location as those of the laccase bands visualized by DMP, suggesting that lichen laccases have a limited ability to metabolize DOPA. Conversely, even after incubation of the gels for several days, DMP never visualized the same bands as DOPA. Similar phenomenon was detected using guaiacol staining that is specific for peroxidase and also an important substrate of laccases.

Separation of the laccases and tyrosinases was achieved by gel filtration chromatography, and substrate specificity was tested that are clearly distinguished laccases from tyrosinases (experiments were carried out by R.P. Beckett in the laboratory of the Biocenter Klein Flottbek, University of Hamburg). Size exclusion chromatography (SEC) of concentrated cell wall enzymes from *Peltigera malacea* revealed one broad peak of laccase activity with a molecular mass of ca. 380 kDa, and one sharper peak of tyrosinase activity (Figure 4.7) with a mass of ca. 60 kDa.

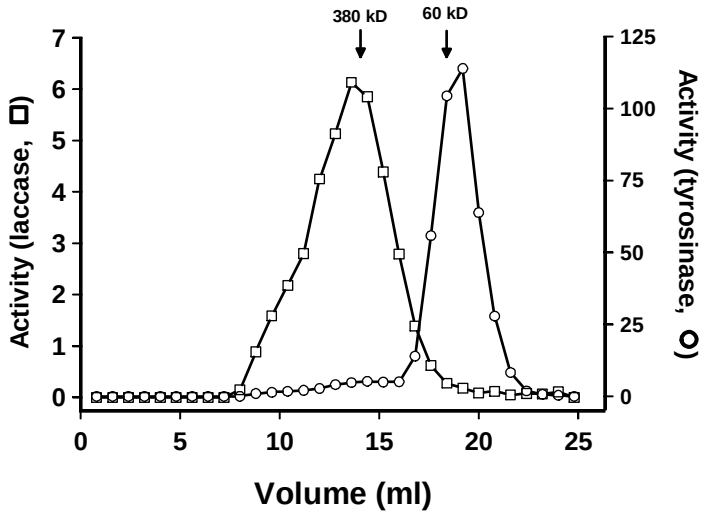



**Figure 4.7.** Elution profiles of the activities of laccase (assayed with ABTS as a substrate) and tyrosinase (assayed with epinephrine as a substrate) from *Peltigera malacea* after size exclusion chromatography. Units are µmoles of substrate metabolized $ml^{-1}$ $min^{-1}$.

The molecular mass of the laccase from *Peltigera malacea* obtained by SEC was similar to that estimated by electrophoresis, suggesting that our electrophoretic estimates of molecular masses are reasonably accurate. Further, SEC clearly distinguished laccase from another multicopper oxidase, tyrosinase (Figure 4.7, Table 4.6).

While some overlap in substrate specificity occurred, fractions that comprised the laccase peak readily metabolized the normal laccase substrates DMP, ABTS and guaiacol but only poorly normal tyrosinase substrates such as L-tyrosine, L-DOPA and epinephrine (Table 4.6). Fractions derived from the tyrosinase peak readily metabolized normal tyrosinase but not laccase substrates (Table 4.6).

**Table 4.6.** The metabolism of laccase and tyrosinase substrates by *Peltigera malacea* leachates separated by size exclusion chromatography.

| Substrate | ε | λ | pH | Laccase peak activity (units $min^{-1}$ $ml^{-1}$eluate) | Tyrosinase peak activity (units $min^{-1}$ $ml^{-1}$eluate) |
|---|---|---|---|---|---|
| **Laccase substrates** | | | | | |
| DMP | 14.8 | $A_{477}$ | 6 | 33 ± 3 | 1 ± 1 |
| ABTS | 36 | $A_{420}$ | 5 | 56 ± 12 | 4 ± 0 |
| Guaiacol | 26.3 | $A_{470}$ | 6 | 0.80 ± 0.03 | 0.03 ± 0.05 |
| **Tyrosinase substrates** | | | | | |
| L-tyrosine | 3.3 | $A_{490}$ | 7 | 0 ± 0 | 23 ± 1 |
| L-DOPA | 3.6 | $A_{475}$ | 6 | 20 ± 0 | 620 ± 10 |
| Epinephrine | 4.5 | $A_{490}$ | 7 | 60 ± 10 | 1690 ± 30 |

Figures are given ± the standard deviation, n = 5.

These results confirm our earlier conclusion (based on substrate specificity and sensitivity of unpurified leachates) that both oxidases occur in the cell walls of lichens from suborder Peltigerineae. Substrate specificities measured using fractions derived from the peaks of laccase and tyrosinase activities were entirely consistent with published substrate preferences for these enzymes (Table 4.6) (Baldrian, 2006; Halaouli *et al*., 2006). Precise estimates for the molecular masses of the active forms of laccases are often difficult, because as is typical for many secreted enzymes, laccases are heavily glycosylated (Varki *et al*., 1999). Each “isoform” is glycosylated to various extents, and this will cause variation in molecular mass. This is almost certainly the

cause of the broad peaks of laccase activity found following size exclusion chromatography (Figure 4.7) and the broad, smearing bands visualized following electrophoresis (Figure 4.4, 4.5). Nevertheless, our results suggest that unlike the laccases of free-living fungi that usually occur as monomers (Baldrian, 2006), in lichens laccases occur as a variety of much higher molecular mass oligomers. However, a transient 80 kDa band appeared in the gel stained by guaiacol (data not shown) that might indicate a monomeric laccase in *Pseudocyphellaria aurata*. The latter is more likely and is supported by TMB (Figure 4.5) and silver staining (Figure 5.2).

## 4.4. Conclusions

This section of our study suggests that significant tyrosinase activity occur in lichenized Ascomycota, in suborder Peltigerineae. Tyrosinase activity significantly correlated with laccase activity. Co-occurrence of laccase and tyrosinase enzymes in lichens from suborder Peltigerineae may contribute to the characteristics of Peltigerineae's members such as the fast growth rates and high metabolic activity. Rast *et al*. (2003) reported that tyrosinases are ubiquitous and mostly cytosolic enzymes but in some cases it can occur extracellularly as it happened in our study. Most of the tyrosinases in organisms are in latent form that has probably a very important role in the protection mechanisms against stress (Mayer, 2006). Beckett and Minibayeva (2007) and Beckett *et al*. (2008) suggested that catalase-like tyrosinase activity are might involved in the protection of lichens against the harmful effects of ROS produced by laccases. This suggestion is supported by the results as tyrosinase activity was significantly correlated with laccase activity within suborder Peltigerineae. However, tyrosinases are very diverse in many of their properties, distribution, cellular location and function (Mayer, 2006). Tyrosinases cluster in groups for bacteria, fungi, animals and higher plants and within these groups the homologies are considerably higher than between them (Wichers *et al*., 2003; Mayer, 2006).

# CHAPTER 5

## DIVERSITY OF LACCASES IN LICHENS

Hoefer Mini-Vertical Electrophoresis System was used to perform polyacrylamide gel electrophoresis.

## 5.1. Introduction

The aim of the work described in this chapter was to characterize the diversity of laccases from a range of lichens from the suborder Peltigerineae with respect to such parameters as absorption spectra and approximate molecular mass. Discovery of new laccase isoforms could further extend the useful applications of these enzymes. Determination of the taxonomic distribution and characteristics of laccases will be essential to understand the biological roles of these redox enzymes in lichen physiology.

## 5.2. Results

*Absorption spectra of laccases from different lichen species*

As has been noted in many studies, laccase solution can be coloured (Leontievsky *et al*., 1997a, b). Following concentration, leachates became variously pigmented, but in most cases were either creamy-yellow or blue (Table 5.1). Species in which leachates became clearly blue following concentration had a definite absorption maximum at 614 nm (e.g. *Lobaria scrobiculata*, Figure 5.1/B) indicating a typical laccase type 1 Cu atom. Even in species where the leachate was not obviously blue (e.g., *Collema flaccidum*, Figure 5.1/C), careful inspection of absorption spectra revealed a small peak at 614 nm. However, this peak appeared completely lacking in some species with cream-colored concentrated leachates (e.g., *Pseudocyphellaria aurata* and *Solorina crocea*, Figure 5.1/A, D). A peak of absorption around 320 nm, indicative of a typical laccase type 3 Cu atom was often observed (Figure 5.1).

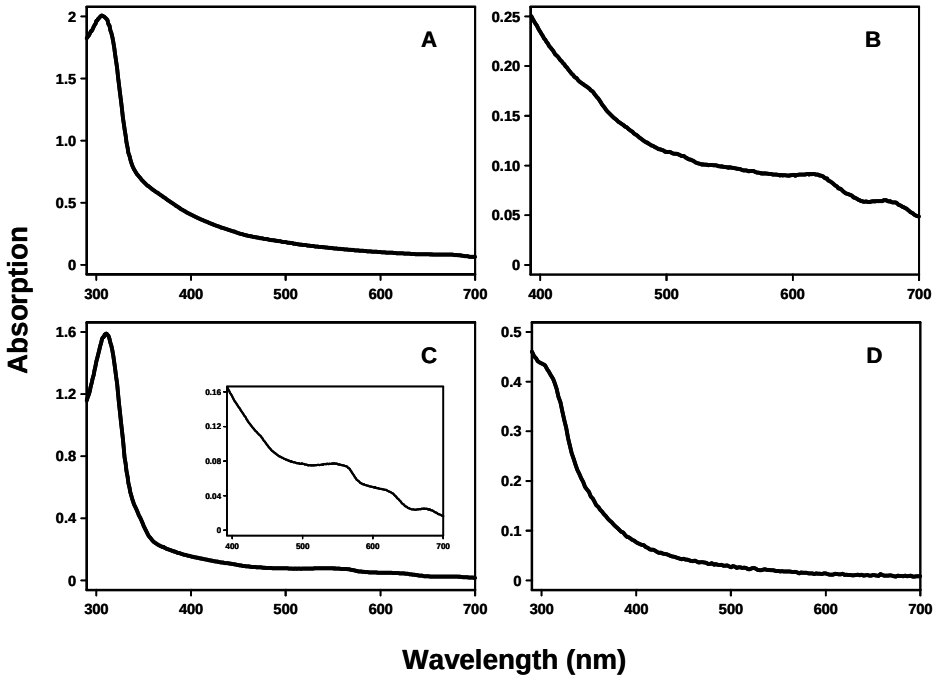



**Figure 5.1.** Absorption spectra of concentrated leachates from A) *Pseudocyphellaria aurata*, B) *Lobaria scrobiculata*, C) *Collema flaccidum* and D) *Solorina crocea*.

*Approximate molecular mass of laccases occur in lichens*

Lichen laccases show considerable diversity. While leachates derived from all lichens tested displayed some laccase activity, actual rates varied greatly between species (Table 5.1).

**Table 5.1.** Approximate molecular masses of lichen laccases, and their colour, activity and recovery following concentration in a range of lichens.

| Species | Approx. molecular mass (kDa) | Colour of concentrated leachates | Laccase activity (µmol product $g^{-1}$ dry mass $h^{-1}$, n = 5) | Recovery (%) |
|---|---|---|---|---|
| **Lichens from suborder Peltigerineae** | | | | |
| *Collema flaccidum* | 75, 160, 240 | pink | 23 ± 4 | 122 ± 20 |
| *Lobaria pulmonaria* | 140 | blue | 75 ± 5 | 51 ± 11 |
| *Lobaria scrobiculata* | 175 | blue | 33 ± 6 | 24 ± 4 |
| *Nephroma arcticum* | 300 | yellow | 6 ± 1 | 50 ± 6 |
| *Nephroma rufum* | 190 | yellow | 4 ± 1 | 25 ± 3 |
| *Peltigera canina* | 310 | pinkish purple | 34 ± 3 | 68 ± 7 |
| *Peltigera leucophlebia* | 200 | yellow | 49 ± 2 | 24 ± 2 |
| *Peltigera malacea* | 340 | blue | * | * |
| *Peltigera neopolydactyla* | 150 | yellow | 38 ± 3 | 32 ± 3 |

| Species | Approx. molecular mass (kDa) | Colour of concentrated leachates | Laccase activity (µmol product $g^{-1}$ dry mass $h^{-1}$, n = 5) | Recovery (%) |
|---|---|---|---|---|
| *Peltigera polydactyla* | 200 | pinkish yellow | 45 ± 4 | 69 ± 10 |
| *Peltigera praetextata* | >350 | blue | 84 ± 2 | 60 ± 5 |
| *Peltigera rufescens* | 340 | yellow | 65 ± 2 | 32 ± 2 |
| *Peltigera scabrosa* | >350 | blue | 21 ± 3 | 43 ± 3 |
| *Pseudocyphellaria aurata* | 160 | yellow | 9 ± 1 | 100 ± 7 |
| *Pseudocyphellaria gilva* | 160 | yellow | 3 ± 0 | 33 ± 2 |
| *Solorina crocea* | 135 | yellow | 571 ± 10 | 12 ± 1 |
| *Sticta cf. limbata* | 135 | cream | 6 ± 1 | 100 ± 5 |
| *Sticta* sp. | 155 | cream | 13 ± 2 | 77 ± 6 |
| *Sticta fuliginosa* | 160 | cream | 28 ± 6 | 54 ± 13 |
| *Sticta* cf. *sublimbata* | 165 | cream | 9 ± 0 | 22 ± 1 |

* not determined
Figures are given as the mean, n = 4.

Stability of laccases during concentration also varied greatly; some lost almost 90% of their activity, while in others recovery was 100% (Table 5.1). Electrophoretic estimates of approximate molecular masses showed that the active form of the laccases also varied considerably between species (Table 5.1). Masses greater than 350 kDa were difficult to estimate directly, and are therefore indicated in Table 5.1. as > 350 kDa. Apart from *Collema flaccidum*, *Nephroma arcticum* and many of the lichens in the Peltigeraceae, the approx. molecular masses of extracellular laccases varied between 135 and 190 kDa (Table 5.1; Figure 5.2). Generally, the laccases from lichens within the Peltigeraceae had the highest masses within the Peltigerineae, varying from 200 to over 350 kDa, although the approximate mass of the laccase from *Solorina crocea* was 135 kDa (Figure 5.2/F), resembling those of lichens from other families. All species appeared to contain only one laccase isoform, with the exception of *Collema flaccidum* where DMP visualized three bands (Table 5.1).

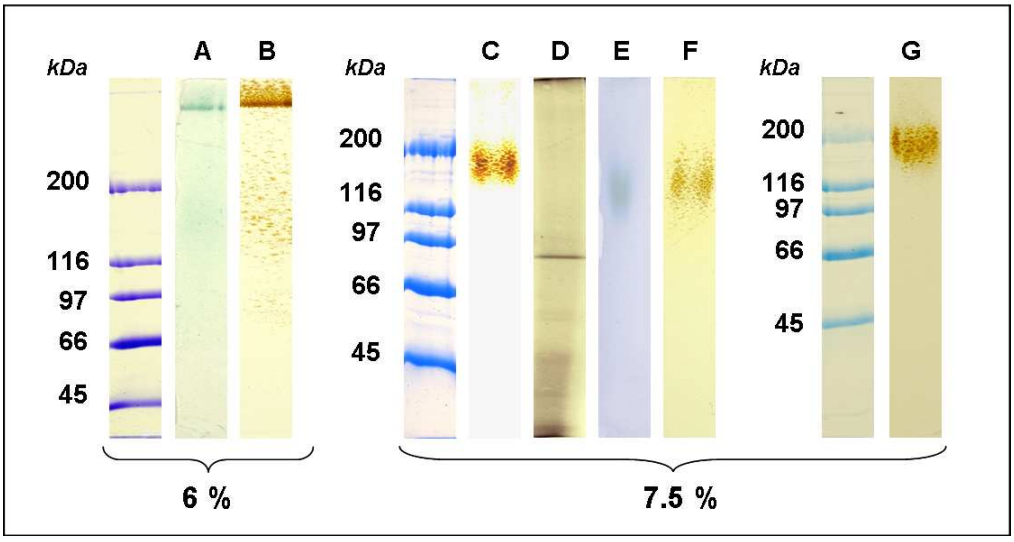



**Figure 5.2.** Characterization of laccases from A, B) *Peltigera rufescens*, C, D) *Pseudocyphellaria aurata*, E) *Sticta cf. limbata*, F) *Solorina crocea* and G) *Lobaria scrobiculata*. Native 6% and 7.5% polyacrylamide gel was used, and laccases visualized with TMB (A, E) and DMP (B, C, F, G) while laccase monomer was indicated by Silver staining (D). Molecular mass markers were stained using Coomassie brilliant blue G250. Masses of the molecular markers are indicated and Table 5.1 presents the approximate molecular masses of the laccases.

## 5.3. Discussion

Concentrated lichen leachates had a variety of colours (Table 5.1). For full catalytic activity, laccases need a minimum of four Cu atoms per active protein unit: a ‘‘Type 1’’ or paramagnetic ‘‘blue’’ Cu, responsible for absorbance at 614 nm; a ‘‘Type 2’’ or paramagnetic ‘‘non-blue’’ Cu; and a ‘‘Type 3’’ or diamagnetic spin-coupled Cu-Cu pair, responsible for absorbance at 320 nm (Claus, 2004). Many, but not all leachates had strong laccase activity but were not blue following concentration (Table 5.1; Figure 5.1). The results are comparable to those of Lisov *et al*. (2007), who found an absorption peak at 614 nm in extracts from *Solorina crocea* but not *Peltigera apthosa*. Laccases from some free-living fungi lack the characteristic blue colour originating from type 1 Cu, and have been termed ‘‘yellow’’ or ‘‘white’’ laccases (Leontievsky *et al*., 1997a, b). These laccases appear to form as a result of the binding of aromatic products of lignin degradation with the blue laccase. It has been suggested that the aromatic products may act as ‘‘mediators,’’ assisting in the oxidation of non-phenolic compounds. Some lichens apparently contain yellow laccases, and it will be interesting in the

future to test whether these species have an enhanced ability to metabolize non-phenolic compounds compared to species that containing typical blue laccases.

Results presented here clearly show that diversity exists within the extracellular laccases from lichens in the suborder Peltigerineae with respect to enzyme activity, stability during concentration and molecular mass (Table 5.1). Within the species sampled, no obvious trends existed between habitat preference and the taxonomic affinity of a species and the activity or stability of the laccases in its leachates. However, isoforms with the highest molecular masses seem to occur in the Peltigeraceae, a family that forms a separate clade in recently published phylogenies of suborder Peltigerineae (e.g., Miadlikowska and Lutzoni, 2004).

Free-living fungi typically contain laccases that vary in mass between 60 and 70 kDa (Baldrian, 2006), but lichen laccases are heavier (Table 5.1; Figure 5.2). However, even in free-living fungi, laccases with higher molecular mass have occasionally been reported, particularly from the Ascomycetes, e.g., 190 kDa for *Gaeumannomyces graminis* (Edens *et al*., 1999) and 383 kDa for *Podospora anserina* (Molitoris and Esser, 1970; Durrens, 1981). The approx. molecular mass of the active form in *Peltigera malacea* was 340 kDa (Table 5.1). Denaturing gels run with this laccase, separated from other proteins by isoelectric focusing, revealed a single band with a molecular mass of 85 kDa, indicating that the active form is a tetramer (see Chapter 3). If a monomer of ca. 85 kDa is common throughout the Peltigerineae, then the active forms of most extracellular laccases in this suborder are probably either dimers or tetramers. Comparing the molecular masses obtained here with those reported by Lisov *et al*. (2007), our estimate for *Solorina crocea* was similar, while our estimate for *Peltigera leucophlebia* resembles that of the related species *Peltigera apthosa* examined by Lisov *et al.* Interestingly, Lisov *et al*. (2007) reported the existence of lighter, apparently monomeric laccase isoforms in *S. crocea* and *P. apthosa*. In the present study, with the exception of *Collema*, monomeric isoforms were never observed. Possibly the lighter isoforms were artefacts because, apart from being not present in all samples of a particular species, Lisov *et al*. (2007) apparently stored their lichens for several months at room temperature, and also prepared their laccases by grinding up thalli.

The approximate molecular masses of the laccase isoforms were the same in the cytoplasmic and cell wall fractions (Figure 4.5 in Chapter 4). Running electrophoretic gels with higher (> 12.5%) and lower (< 5%) concentrations of polyacrylamide did not indicate the existence of lower and higher molecular mass isoforms. Interestingly, we found laccases with identical molecular masses in the same species collected from the same and different localities. At least

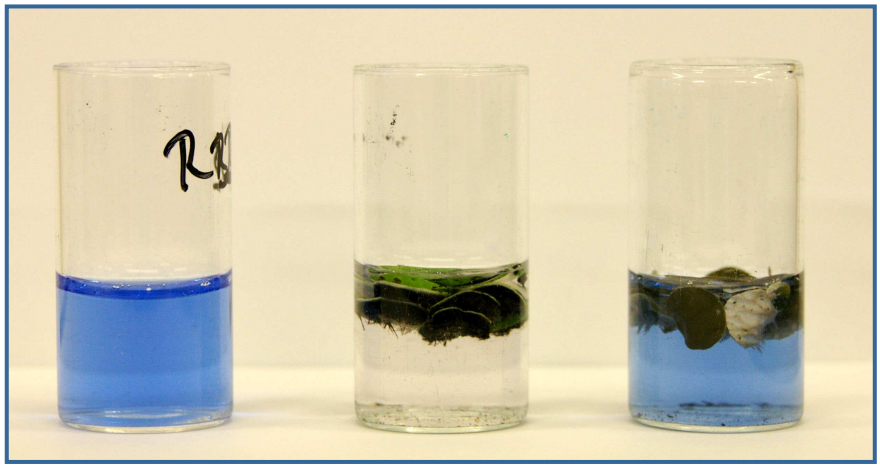
in the four species examined, extracellular laccase isoforms appear not to vary in molecular mass between locations differing in climate.

## 5.4. Conclusions

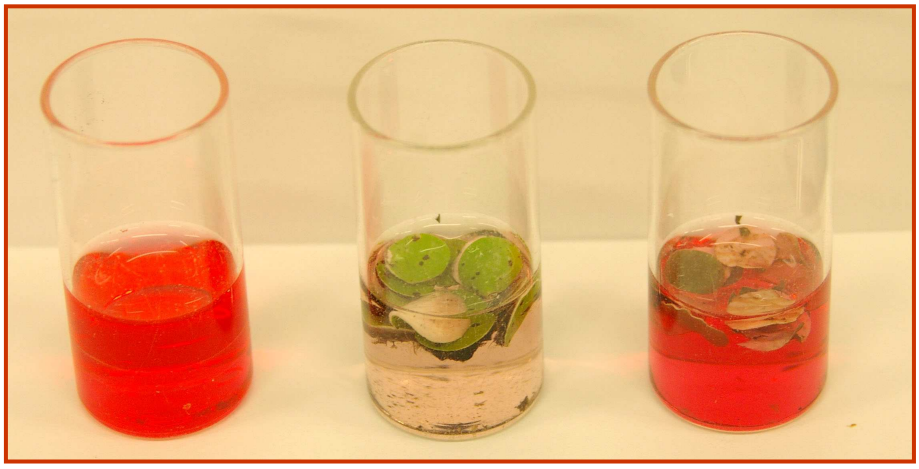
While almost all lichens in the Peltigerineae that have been tested display some extracellular laccase activity, species vary greatly in the amount of activity, stability and the types of laccases that they produce (Table 5.1). No obvious correlations can be discerned between on the one hand laccase activity, molecular mass and colour following concentration, and on the other species characteristics such as microhabitat (e.g., whether a lichen tends to grow on soil, rocks or bark) or taxonomic position. Therefore, the roles of laccases in lichen biology remain to be discovered. Further microcharacterization will enable us to learn more about their functions, and also their affiliation with laccases from free-living fungi. The considerable diversity that exists in extracellular lichen laccases suggests that they may have more than one role in lichen biology.

# CHAPTER 6

## DECOLOURIZATION OF SYNTHETIC DYES BY LICHENS



Decolourization of Remazol Brilliant Blue R by cell wall redox enzymes including laccases from *Peltigera leucophlebia* and *Peltigera rufescens* discs.



Decolourization of Acid Red 103 by cell wall redox enzymes including laccases from *Peltigera leucophlebia* and *Peltigera rufescens* discs.

## 6.1. Introduction

Laccases have broad range of application in biotechnology (Morozova *et al*., 2007a). These redox enzymes can produce free radicals during their catalytic processes (Guillen *et al*., 2000; Morozova *et al*., 2007a). During the last decade, most studies dealing with the use of laccases to decolourize dyes, especially synthetic dyes, have been experiments carried out in the presence of redox mediators (e.g. Young and Yu, 1997; Claus *et al*., 2002; Baldrian, 2004; Baldrian and Snajdr, 2006; Eichlerová *et al*., 2006). Redox mediators are required in many laccase-catalysed reactions where the substrate has very high redox potential, and therefore the mediator is needed to facilitate the reactions (Burton, 2003). Up to now, more than 100 possible mediator compounds have already been described, both natural and synthetic, but the most commonly used are still ABTS and 1-HBT. Johannes and Majcherczyk (2000) suggested that it is very important to choose the proper mediator substance because it plays a key role in the general applicability and effectiveness of the laccase mediator system (LMS, for more details see Chapter 1.2.5). Synthetic dyes are extensively used in industrial processes such as dying and printing. These commercial dyes have high stability to light, temperature, detergent and microbial attack (Rodriguez *et al*., 1999). About 10-15% of the total dye used is discharged into the environment where under anaerobic conditions the industrially important dyes (e.g. azo dyes) can decompose into mutagenic and/or carcinogenic amines (Young and Yu, 1997; Rodriguez *et al*., 1999; Claus *et al*., 2002).

Ligninolytic enzymes are able to degrade different xenobiotic compounds including polycyclic aromatic hydrocarbons, polychlorinated biphenyls and synthetic dyes (Baldrian, 2004). Most of the studies with biodegradation of synthetic dyes have involved the enzymes lignin peroxidase, manganese dependent peroxidase and laccase. Laccases have relatively low redox potential (430-800 mV) that allows the degradation of low redox potential compounds without the addition of hydrogen peroxide but not the oxidation of more recalcitrant aromatic compounds such as some synthetic dyes or polycyclic aromatic hydrocarbons except when redox mediators are used (Baldrian, 2006; Morozova *et al*., 2007b). The optimal redox mediator should be a good laccase substrate with cyclic redox conversion (see Figure 1.8 in Chapter 1), and the oxidized and reduced forms of the substrate must be stable and must not inhibit the enzymatic reaction (Johannes and Majcherczyk, 2000; Morozova *et al*., 2007b). Investigations have been carried out mostly with the basidiomycetes *Phanerochaete crysosporium*, *Trametes versicolor*, *Pleurotus ostreatus* and the ascomycete *Myceliophthora thermophila* (Young and Yu, 1997; Johannes and Majcherczyk, 2000; Claus *et al*., 2002; Kaushik and Malik, 2009). The aim of the

present study was to test the ability of lichens to decolourize synthetic dyes in the presence or absence of mediators. Lichens from the suborder Peltigerineae and from non-suborder Peltigerineae were chosen as well species that do not possess ligninolytic enzymes. The latter species were used to test whether non-Peltigeralean lichens may contain oxidases other than ligninolytic enzymes can cause decolourization of synthetic dyes.

## 6.2. Results

*Decolourization of synthetic dyes by lichen leachates*

The ability to decolourize different synthetic dyes (Table 6.1) by crude laccase containing leachates from a range of lichen species at pH 7.0 was assessed in this part of the project. Decolourization was monitored by lichen leachates of *Pseudocyphellaria aurata*, *Collema flaccidum*, *Lobaria scrobiculata*, *Peltigera rufescens* and *Sticta cf. sublimbata* from suborder Peltigerineae.

**Table 6.1.** The absorption maxima and type of synthetic dyes used in our decolourization experiments.

| Name | Dye type | $\lambda_{max}$ (nm) |
|---|---|---|
| Acid Blue 74 | indigoid | 610 |
| Acid Red 103 | quinone-imine | 505 |
| Chicago Sky Blue 6B | diazo | 620 |
| Fast Green FCF | triarylmethane | 625 |
| Remazol Brilliant Blue R | anthraquinone | 590 |

No differences in the decolourization rate of various dyes existed between the control and *Pseudocyphellaria aurata* leachates without mediator (Figure 6.1/■, □). However, 1-HBT inhibited rather than activated decolourization (Figure 6.1/▲, △). Similar results were found using RBBR, Acid Blue 74, Acid Red 103 and Fast Green FCF with *Collema flaccidum*, *Lobaria scrobiculata*, *Peltigera rufescens* and *Sticta cf. sublimbata* leachates (data not shown).

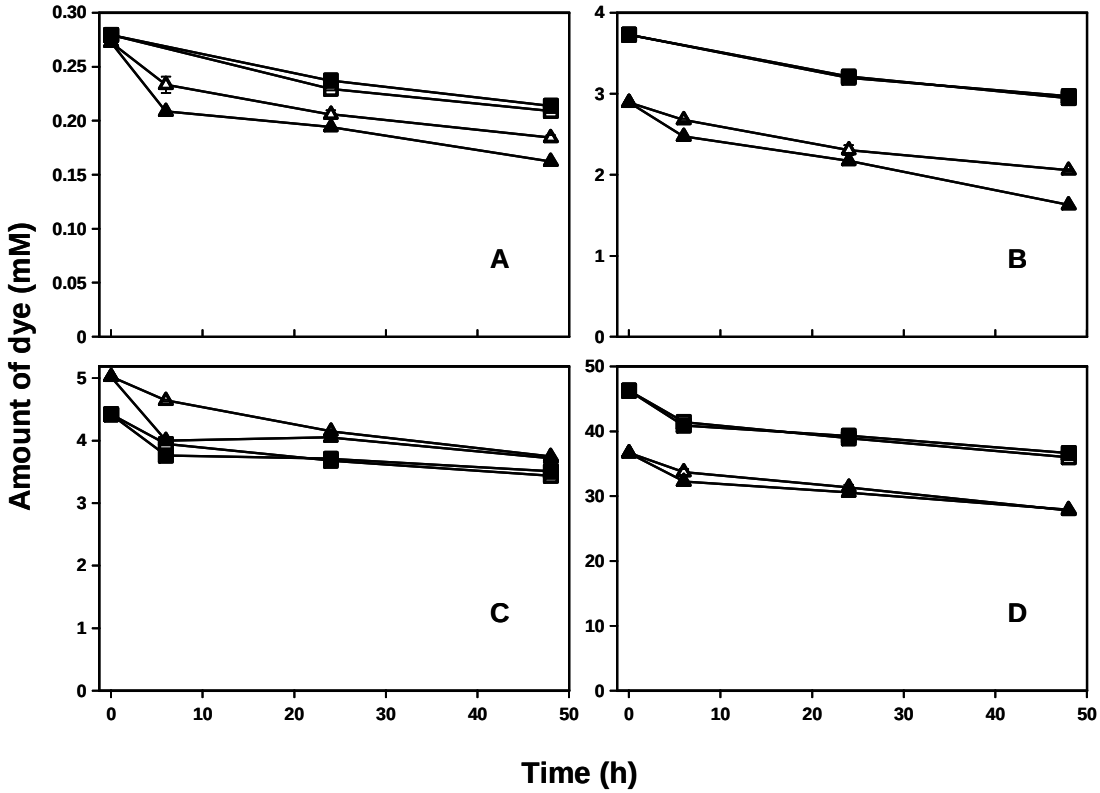



**Figure 6.1.** Degradation of the synthetic dye Remazol Brilliant Blue R (A), Acid Blue 74 (B), Acid Red 103 (C) and Fast Green FCF (D) by *Pseudocyphellaria aurata* leachates. Amount of dye in controls (dyes without lichen leachates) are indicated by solid square without the mediator 1-hydroxybenzotriazole (1-HBT) (■) and with 1-HBT it was followed by solid triangle (▲). Treatments containing lichen leachates they are showed by open square without the mediator 1-HBT (□) and with 1-HBT it was demonstrated by open triangle (△). Points are indicated by the average of three replicates while error bars show the standard deviation.

Interestingly, the only leachate that showed potential to decolourize a dye was derived from *Sticta cf. sublimbata*. This species decolourized the dye Chicago Sky Blue 6B within 48 h without and with mediator (by around 16 and 38%, respectively, Figure 6.2).

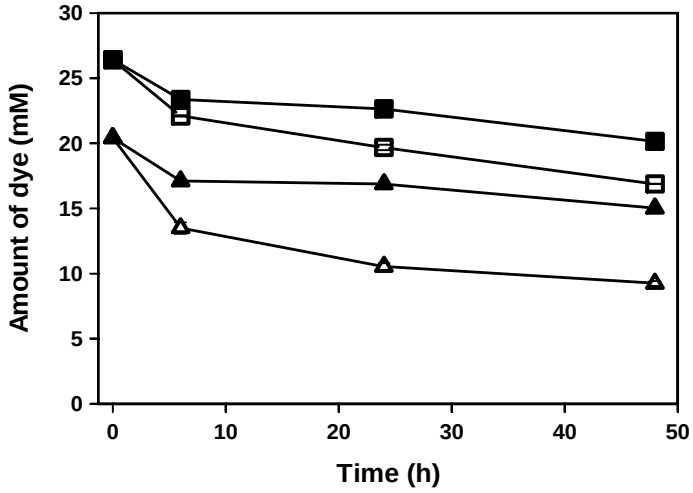



**Figure 6.2.** Decolourization of Chicago Sky Blue 6B by *Sticta cf. sublimbata* leachate. Controls (dyes without lichen leachate) without and with 1-HBT are indicated by solid square and solid triangle up (■, ▲), respectively while lichen leachates without and with 1-HBT are showed by open square and open triangle up (□, △), respectively. The data represent means of three replicates while error bars indicate the standard deviation.

*Decolourization of synthetic dyes by lichen discs*

Discs of the lichens from suborder Peltigerineae and species belonging to other suborders (*Cetraria islandica*, *Flavocetraria nivalis* and *Cladonia stellaris*) were used directly in decolourization experiments using different type of dyes. As shown in Figure 6.3, all dyes tested were decolourized by the discs of *Peltigera rufescens* and *Cetraria islandica*. Interestingly, enzymatic decolourization occurred at a higher rate after 6 h of incubation in RBBR and Acid Blue 74 in *C. islandica* (Figure 6.3/A, E) compared to *P. rufescens*. Chicago Sky Blue 6B and Acid Red 103 were decolourized to a greater extent by *P. rufescens* (Figure 6.3/C, G and D, H). In case of the latter two dyes 1-HBT was used as mediator to increase the decolourization rate. 1-HBT had a significant effect on the rate of decolourization of the azo dye Chicago Sky Blue 6B and the quinone-imine type of dye Acid Red 103 (Figure 6.3/C, G and D, H) using both *P. rufescens* and *C. islandica* discs. Chicago Sky Blue 6B was decolourized to the greatest extent but the degradation of all tested dyes were incomplete (Figure 6.3).

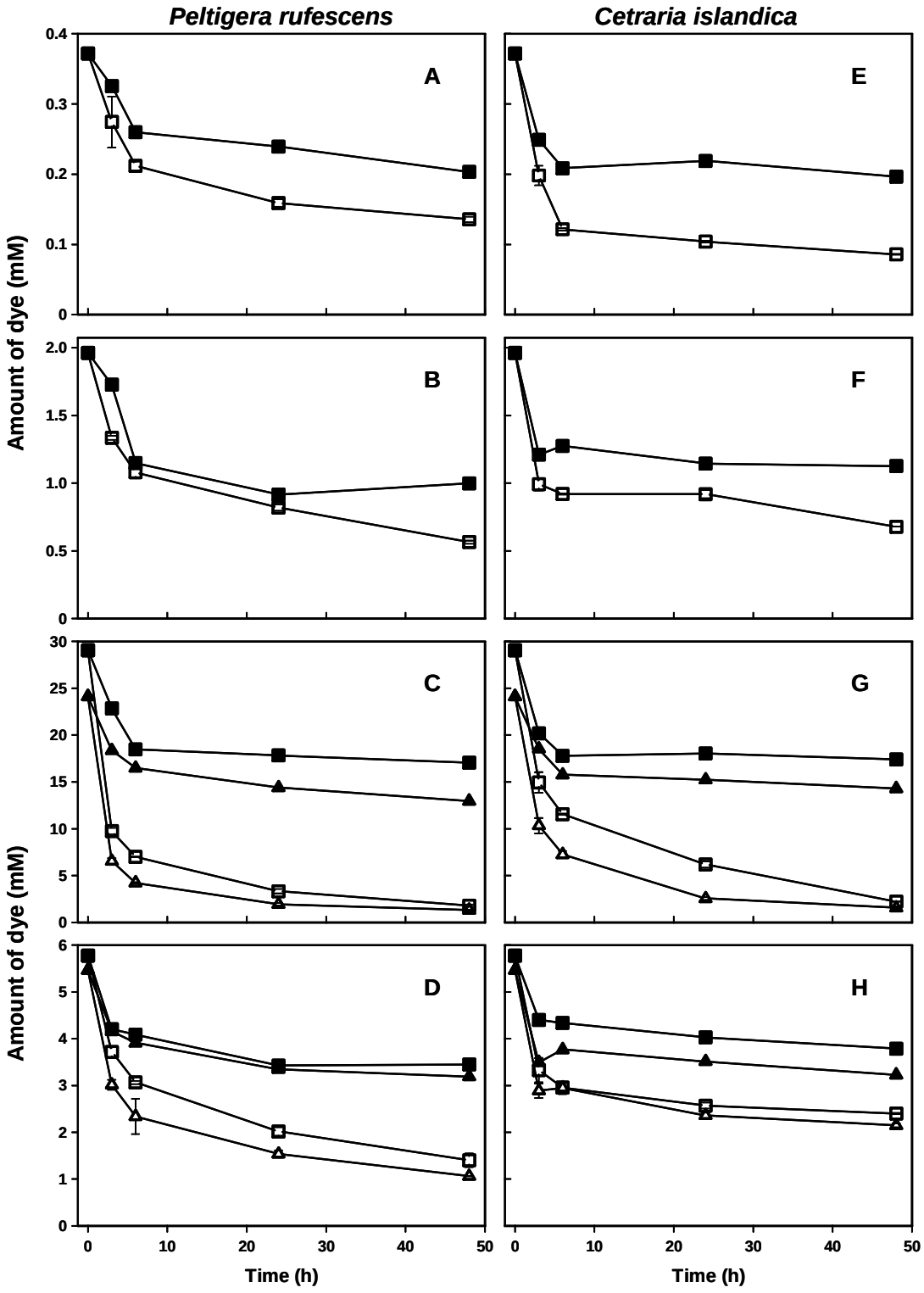



**Figure 6.3.** Decolourization of dyes by discs of *Peltigera rufescens* and *Cetraria islandica*. Four different dyes were used: Remazol Brilliant Blue R (A, E), Acid Blue 74 (B, F), Chicago Sky Blue 6B (C, G) and Acid Red 103 (D, H). Amount of dye in controls (dyes

without lichen discs) are indicated by solid square without the mediator 1-HBT (■) and with 1-HBT it was followed by solid triangle (▲). In lichen leachates they are showed by open square without the mediator 1-HBT (□) and with 1-HBT it was demonstrated by open triangle (△). Points are indicated by the average of three replicates while error bars show the standard deviation.

All lichens tested could decolourize synthetic dyes to some extent, but the most efficient overall was the lichen *Collema flaccidum* (Figure 6.4). The dye RBBR was the most efficiently decolourized dye, being decolourized by 30-70% after 48 h followed by Acid Blue 74 (around 20-65% after the same period) in all tested species. The decolourization of Acid Red 103 and Chicago Sky Blue 6B were investigated without and with 1-HBT. The quinone-imine dye Acid Red 103 was significantly decolourized by all lichen discs tested within 24 h in the presence and absence of 1-HBT (Figure 6.4). The greatest proportion of dye decolorized was almost 90% and was observed after 48 h of incubation in case of lichen discs from *Lobaria scrobiculata* and *Sticta cf. sublimbata*. Chicago Sky Blue 6B was highly decolourized by *Collema flaccidum* (higher than 80%) and the extent of the dye degradation did not change using the redox mediator 1-HBT. In case of *Cladonia stellaris*, 1-HBT rather inhibited than increased the degradation rate of Chicago Sky Blue 6B. In all the other lichen species tested significant increases were found in the decolourization of Chicago Sky Blue 6B in the presence of a mediator (Figure 6.4).

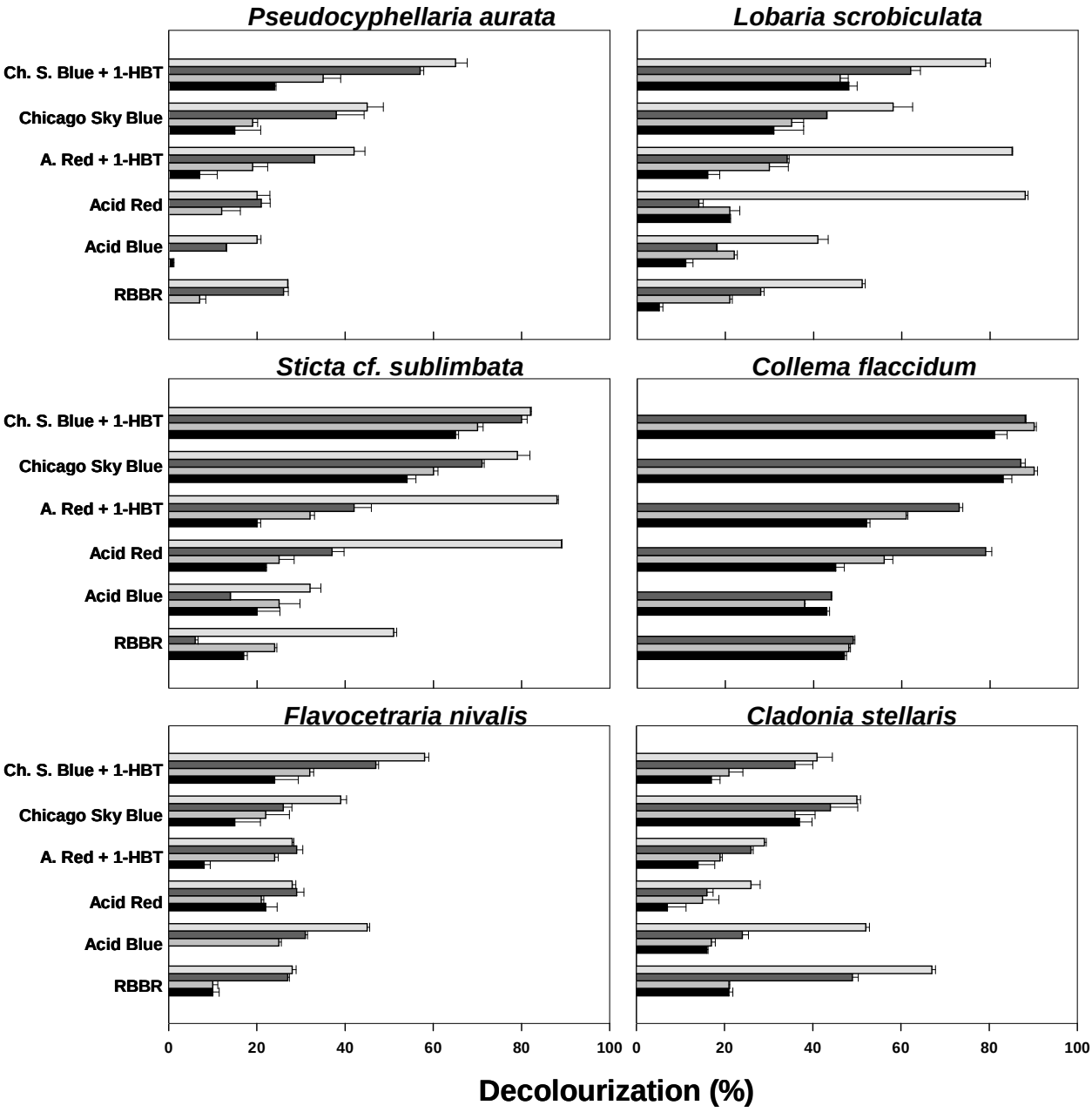



**Figure 6.4.** Decolourization percentage of different synthetic dyes by cell wall redox enzymes including laccases from a range of lichens discs. Dyes and mediator are indicated on y-axis. Bars show the time of the decolourization processes; the black bar corresponds to 3 h of degradation, then successive bars to 6, 24 and 48 h of degradation. Bars represent the average of three replicates while the error bars indicate the standard deviation.

## 6.3. Discussion

*General discussion*

The textile industry and the dye manufacturing industry both produce high volumes of effluents containing a mixture of different type of dyes (Revankar and Lele, 2007). Dyes are very stable compounds, and are therefore difficult to remove by conventional biological processes such as activated sludge treatment (Wong and Yuen, 1996). Ligninolytic enzymes derived from ligninolytic organisms are capable of catalyzing the oxidation of various types of dyes such as azo, heterocyclic, reactive and polymeric dyes (Novotny *et al*., 2004a). The potential of lignolytic enzymes from a range of fungi to decolourize have been reviewed several times (Wesenberg *et al*., 2003; Kaushik and Malik, 2009). These enzymes, including laccases, have many advantages that make them suitable for degrade a wide variety of compounds such as dyes; they are produced extracellularly and can bind non-specifically to their substrate making it possible to use them in a broad range of applications (Kaushik and Malik, 2009). These authors suggested that variations in the substrate specificity of the ligninolytic enzymes determine the overall efficiency of decolourization of different dyes by the same enzymes. In addition to laccase, lignin and manganese peroxidase, it has been reported that cellobiose dehydrogenase (CDH) has also an important role in the decolourization of azo and anthraquinonic dyes due to its ability to indirectly generate free hydroxyl radicals in a Fenton type reaction (Vanhulle *et al*., 2007; Ciullini *et al*., 2008). The enzyme acts *in vitro* synergism with laccases. However, we have never detected CDH in lichens. Most of the studies deal with the decolourization of dyes by fungi recommended that the processes are more effective in the presence of redox mediators (Nagai *et al*., 2002; Baldrian, 2004; Couto, 2007), and are affected by culture and effluent conditions (derived from textile industry) such as pH, temperature, initial dye concentration, dye class, media components, shaking etc. (Kaushik and Malik, 2009). Dyes having higher redox potential than laccase cannot be directly degraded by laccases, and therefore a redox mediator (such as 1-HBT, Figure 6.5) is needed to perform the oxidation process.

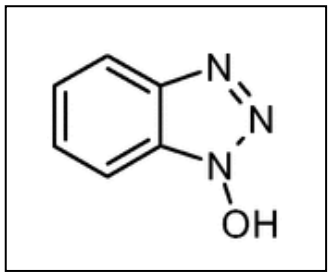



**Figure 6.5.** The structure of 1-hydroxybenzotriazol (1-HBT).

In the first step, laccases oxidize the redox mediators through either a one-electron oxidation of substrate (Bourbonnais *et al*., 1998) or withdrawal of an H-atom from the substrates (Fabbrini *et al*., 2002). The latter process is operated in case of 1-HBT (Figure 6.6). The resulting cation radicals posses higher redox potential than laccase and co-oxidize the substrates/dyes (see Figure 1.8 in Chapter 1, Claus *et al*., 2002; Ciullini *et al*., 2008).

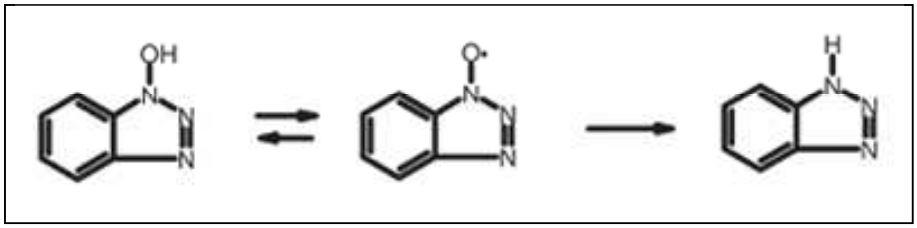



**Figure 6.6.** HBT-radical pathway (taken from Wells *et al*., 2006).

The laccase/HBT system is generally more effective than laccase alone, since the free-radical HBT species formed by the action of laccase on reduced HBT is a stronger oxidant than laccase itself (Ciullini *et al*., 2008).

Various types of dyes (0.01% final concentration) were used at pH 7.0, and dye solutions were shaken during the experiments (see in Materials and Methods, Chapter 2). The optimum pH for dye decolourization of laccases and other oxidative enzymes lies in the acidic range where the enzymes show very high activity (pH 4-5, Kapdan *et al*., 2000; Parshetti *et al*., 2007; Asgher *et al*., 2008). However, Yesilada *et al*. (2002) and Ciullini *et al*. (2008) found better decolourization rate in the pH values ranging from 6 to 11. Nevertheless, most wastewaters from textile industries are characterized by a neutral to alkaline pH (around 7-11) (Manu and Chaudhari, 2002). It is well known that initial dye concentration has strong effect on the decolourization process. Many studies indicate that the rate of decolourization was inversely related to the concentration of dye in solution (Kapdan *et al*., 2000; Parshetti *et al*., 2007). The rate of decolourization is also affected by shaking the effluent. It has been reported that shaking

conditions (100-150 rpm) are better for higher colour removal and for faster and complete adsorption because of better oxygen transfer and nutrient distribution as compared to the stationary cultures (Jarosz-Wilkolazka *et al*., 2002; Yesilada *et al*., 2002; Parshetti *et al*., 2007). However, in contrast to the favourable effect of shaking on colour removal in decolourization processes higher enzymatic activities can be observed in static conditions (Novotny *et al*., 2004b; Kaushik and Malik, 2009). The chemical structures of the dyes (Figure 6.7), especially their electron distribution and charge density, determine the extent to which they can decolourize, and the rate of their decolourization (Pasti-Grigsby *et al*., 1992; Ciullini *et al*., 2008). The effect of the chemical structure of dyes will be discussed below.

*Decolourization of synthetic dyes by lichen leachates and discs*

In the present study, dye decolourization rates of extracellular leachates and discs were tested in both Peligeralean species (*Pseudocyphellaria aurata, Collema flaccidum, Lobaria scrobiculata, Peltigera rufescens* and *Sticta cf. Sublimbata*) and also some non-Peltigeralean lichens (*Cetraria islandica, Flavocetraria nivalis* and *Cladonia stellaris*). Leachates from the Peligeralean, but not the non-Peltigeralean species would have contained laccases and tyrosinases. Interestingly, none of the lichen leachates could decolourize the dye RBBR, Acid Blue 74, Acid Red 103 and Fast Green FCF. However, Chicago Sky Blue 6B was significantly decolourized by *Sticta cf. sublimbata* but not by other lichen leachates (Figure 6.2). This is very interesting because *Peltigera rufescens* leachates had much higher laccase activity compared to *Sticta*. Possibly *Sticta* has both laccases and other extracellular enzymes/oxidases that can decolourize synthetic dyes. The mediator 1-HBT did not increase the decolourization.

Decolourization of synthetic dyes by lichen discs gave higher rates of degradation, possibly because they contained natural mediators present in lichens and/or contain other extracellular oxidases. All lichens could to some extent decolourize dyes tested, but their efficiency varied (Figure 6.3 and 6.4). Interestingly lichens from non-suborder Peltigerineae such as the lichen *Cetraria islandica, Flavocetraria nivalis* and *Cladonia stellaris* could also degrade synthetic dyes (Figure 6.3 and 6.4). In the case of lichens from suborder Peltigerineae it seems most likely that decolourization can be attributed to laccase with or without mediators. The application of laccases to biotechnology is tightly linked to their ability to produce free radicals during their oxidation reactions (reaction 1; Morozova *et al*., 2007a). Autoxidation of, for example, semiquinones produced by laccases (e.g. Guillen *et al*., 2000) will lead to extracellular $O_2\cdot^-$ production.

$$4QH_2 + O_2 \rightarrow 4Q\cdot^- + 2H_2O + 4H^+ \qquad (1)$$

We presume that in non-Peltigeralean lichens other membrane or cell wall oxidases may participate in dye decolourization processes in similar way to laccases through the production of free radicals. In the cell wall and plasma membrane not only laccases but other oxidases such as the plasma membrane bound enzyme NAD(P)H oxidase and oxalate oxidase can also form extracellular superoxide (reaction 2; Bolwell *et al*., 1998; Guillen *et al*., 2000; Martinez *et al*., 2000; Delannoy *et al*., 2003; Luthje *et al*., 2009; Oracz *et al*., 2009).

$$^3O_2 + e^- \rightarrow O_2\cdot^- \qquad (2)$$

NAD(P)H oxidases generate superoxide by the reduction of ground state oxygen using NADH or NADPH as an electron donor (Lamb and Dixon, 1997; reaction 3).

$$NADPH + 2O_2 \rightarrow NADP^+ + 2\ O_2\cdot^- + H^+ \qquad (3)$$

$O_2\cdot^-$ is less reactive compared to OH, having a half life of 2-4 μs, and a low cellular concentration ($< 10^{-11}$ M). It cannot react directly with membrane lipids to cause peroxidation, and cannot cross biological membranes (Vranová *et al*., 2002). Most $O_2\cdot^-$ formed in biochemical systems reacts with itself non-enzymatically or enzymatically (catalysed by superoxide dismutase) to form $H_2O_2$ (reaction 4).

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + {}^3O_2 \qquad (4)$$

In addition to $O_2\cdot^-$ dismutation, $H_2O_2$ can be formed by oxidases such as glycolate oxidase, glucose oxidase, urate oxidase, oxalate oxidase (the so-called "germin-like" oxidase), and amino acid oxidases (Halliwell, 1987; Halliwell and Gutteridge, 1999; Rea *et al*., 2002) in which the enzyme transfer two electrons onto each $O_2$ molecule to form $H_2O_2$ (reaction 5).

$$^3O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \qquad (5)$$

$H_2O_2$ then can be involved in the Haber-Weiss and/or Fenton reaction producing the hydroxyl radical (OH·; see Chapter 1.2.4). The hydroxyl radical is a strong oxidant that can destroy organic compounds. Oxidation of hydroxyl radical can for example breakdown azo bonds (the most active bonds of azo dye molecules) that will result in the decolourization of dyes.

Recently, Liers *et al*. (2010) found secreted "dye-decolorizing peroxidases" (DyPs) in the jelly fungus *Auricularia auricula-judae*. The function of this peroxidase differ from other peroxidases found in fungi (lignin peroxidase, manganese peroxidase) especially in terms of structure, sequence etc. The special feature of dye-decolourizing peroxidases is the ability to oxidize synthetic high-redox potential dyes. Liers *et al*. (2010) found a certain ligninolytic

activity by the oxidation of nonphenolic lignin model compounds. We have tested some species for peroxidase activity but no activity was found (see Figure 4.3, 4.5 and Table 4.5 in Chapter 4). Future work could test the possibility that DyPs occur in lichens.

*Effect of dye structures on decolourization*

The results showed that the individual dye structures influenced the extent of decolourization (Figure 6.7; Couto, 2007). The dye that was most effectively decolourized by lichen discs and leachates was Chicago Sky Blue 6B (Figure 6.2, 6.3/C, G and 6.4), and rates were higher in the presence of the mediator 1-HBT. Jarosz-Wilkolazka *et al*. (2002) reported that azo dyes such as Chicago Sky Blue 6B can not readily degraded by microorganisms because azo and sulfo groups do not occur naturally, thus sulfonated azo dyes are recalcitrant to biodegradation and can only be degraded when specific changes occur in their molecular structure (Paszczynski and Crawford, 1995). Galindo and Kalt (1999) suggested that the larger the number of sulfonate groups (such as in Chicago Sky Blue 6B) the less sensitive the dye molecules are to oxidation. However, in spite of the presence of at least two sulfonate groups that are powerful electron withdrawing substituents the most likely explanation of the results is that azo dyes contain –OH groups in *o*- or *p*-positions with respect to the azo bond, and these have been reported to be preferentially oxidized by ligninolytic enzymes such as laccase (Kandelbauer *et al*., 2004).

Degradation of RBBR occurred much more slowly especially during the initial period of the reaction (Figure 6.3/A, E and 6.4), possibly indicating a requirement for sequential oxidation at two sites in the molecule to decolourize of this dye and/or demand stronger laccase activity or longer reaction times (Nagai *et al*., 2002). However, some workers suggest that the anthraquinone type dye of RBBR is more resistant to degradation possibly because of its fused aromatic structures (Banat *et al*., 1996; Robinson *et al*., 2001; Kaushik and Malik, 2009). Therefore, most studies report that decolourization of RBBR require redox mediators (Soares *et al*., 2001). However, Champagne and Ramsay (2005) found that purified laccase decolourized RBBR five to ten times faster than the azo dyes, results confirmed by Ciullini *et al*. (2008). Nevertheless, many studies suggest that not only laccase but partially purified lignin peroxidase and RBBR oxygenase have the ability to decolorize the dye RBBR (Ollikka *et al*., 1993; Vyas and Molitoris, 1995; Novotny *et al*., 1997; Young and Yu, 1997).

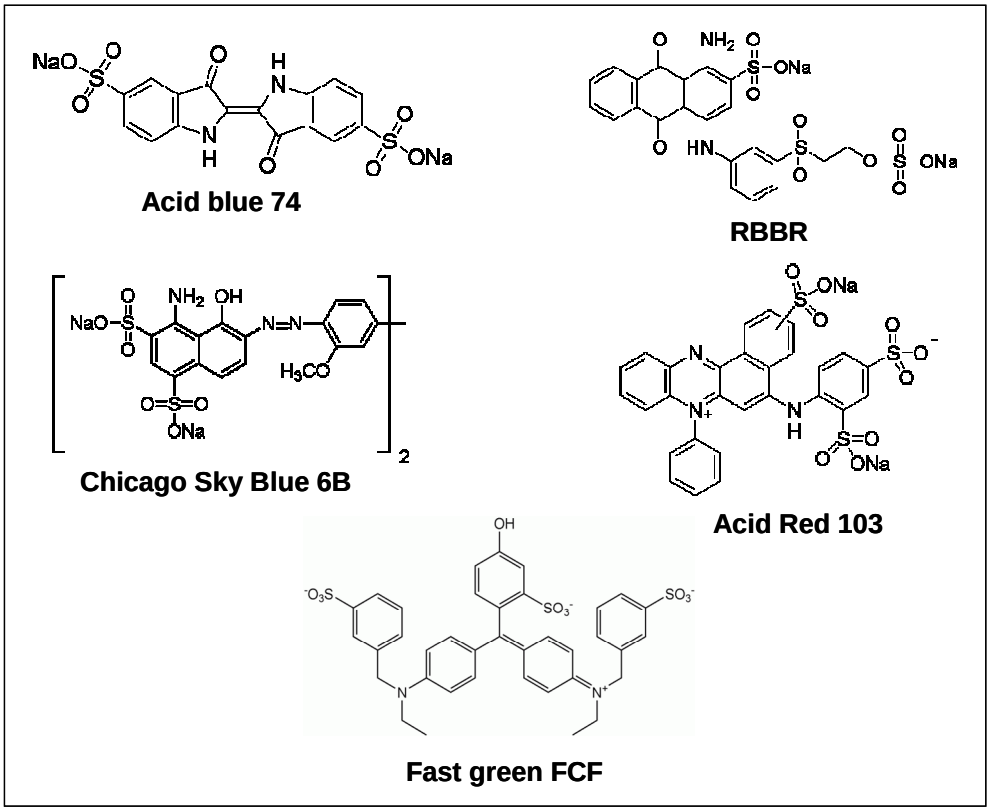



**Figure 6.7.** Molecular structure of the dyes used.

Decolourization of Acid Blue 74 (the common name is Indigo Carmine) by lichen discs was less efficient after 6 h compared to other dyes, but similar to RBBR (Figure 6.3 and 6.4). One possible explanation is that both molecules possess similar fused aromatic structures. While decolourization proceeded for up to 6 h, after 6 h the amount of decolourization did not increase in *Peltigera rufescens* and *Cetraria islandica* (Figure 6.3). This could have been the result of the presence of some inhibiting sub-products generated in the dye degradation process (Couto, 2007). It seems that the dye Indigo Carmine is sensitive to oxidation (Galindo *et al*., 2001; Champagne and Ramsay, 2010).

Acid Red 103 (also called as Azocarmine B) is a typical anionic dye that was highly decolourized by all lichens tested (Figure 6.3 and 6.4). Lichen laccases from suborder Peltigerineae showed higher degradation rate compared to lichen discs from other suborder. Interestingly, there is only one report on the degradation of Acid Red 103 by ligninolytic enzymes but not by laccase in literature (Shaffiqu *et al*., 2002). The authors found that Azocarmine B was decolourized by approximately 30% in 4 h by the moderately low redox potential horseradish peroxidases at pH 6.5.

However, it is beyond the scope of the present work to provide mechanistic interpretations of the observed results as this would require analysis and identification of the reaction products.

## 6.4. Conclusions

Kaushik and Malik (2009) summarized the application of various fungi in dye removal and found that fungi from the group of Basidiomycota which produce lignin degrading enzymes such as laccase, manganese peroxidase etc. mostly degrade textile dyes while dye removal by other fungi (e.g. Ascomycota's) is by "biosorption". Biodegradation involves the breakdown of dye into various by-products by enzymes (energy dependent process) while biosorption includes a process where solutes bind to the biomass which do not involve metabolic energy or transport (Kaushik and Malik, 2009). However, in many fungi it is likely that adsorption plays a part in the overall process, which facilitates the chromophores getting closer into contact with the degradative enzymes. These enzymes are often largely associated with the cell surface, and after initial adsorption, oxidative degradation will occur (Evans *et al*., 1994; Knapp *et al*., 2001). Non-Peltigeralean lichens became pigmented during dye decolourization suggesting that biosorption may be part of the dye removal process in these lichen-forming fungi as in Ascomycota. Interestingly, Peltigeralean lichens were not pigmented using different type of dyes indicating that lichens from suborder Peltigerineae might decolourize dyes by enzymes. Sometimes it was hard to observe whether some lichens became pigmented or not because of their structure and colour (e.g. *Collema*).

The mycobiont of lichens have many advantages that make lichens suitable for decolourization processes; fungal mycelia can produce extracellular enzymes (such as laccases), and this is probably advantageous in tolerating high concentrations of toxicants and have significant physical and enzymatic contact with the environment (Kaushik and Malik, 2009). Based on these facts and our results we suppose that lichens are probably suitable organisms for treatment of textile effluent and dye removal but lichens are extremely slow growing and therefore it almost impossible there would ever be enough biomass production to decolourization processes. Treatment of dyes can be attributed to lichens only if lichen genes can be incorporated into fast growing fungi such as yeast and these modified organisms will be used to participate in the decolourization processes. Many studies demonstrated that laccases have been expressed in yeast successfully and showed similar properties as in the organism from which they were originally isolated (Record *et al*., 2002; Jolivalt *et al*., 2005).

# CHAPTER 7

## GENERAL CONCLUSIONS

*Peltigera rufescens*

(photo was taken by E-Timdal,

http://www.nhm.uio.no/botanisk/lav/Photo_Gallery/PG_index.html).

Lichens are special organisms belonging to the Ascomycetes, an association of a fungus and green algae and/or cyanobacteria. These life forms grow on stone, bark, soil or leaves, mostly in very extreme environments where they are exposed to many biotic and abiotic stresses and need to adapt to them using a variety of morphological and chemical adaptations for surviving stressful conditions and for fast recovering of metabolic activity (Kranner *et al*., 2008). One of these mechanisms could involve cell wall redox enzymes, including laccases. Beckett *et al*. (2003) found that some lichens show high rates of extracellular redox activity. The present study focussed on two important cell wall redox enzymes in lichenized ascomycetes with high rates of redox activity specifically laccases and tyrosinases. The study suggested that in lichens, significant laccase and tyrosinase activity occurs mainly in Peltigerineae. It is interesting why these redox enzymes occur only in lichens from suborder Peltigerineae. As phenoloxidases are important enzymes of secondary metabolism in plants and fungi, Zavarzina and Zavarzin suggested (2006) that laccases and tyrosinases may play an important role in the phenolic metabolism of lichens. However, lichens from suborder Peltigerineae are characterized by low level of phenolics. It is also interesting that while many lichens can accumulate high concentrations of secondary metabolites lichens from this suborder contain these metabolites at low concentrations (Hawksworth, 1982). Secondary metabolites have antibiotic and antifungal properties and despite of the low level of metabolites in the Peltigerineae they still can resist pathogen attack. Possibly high rates of extracellular redox activity have a role in defence of lichens against pathogens (Beckett *et al*., 2003). Members of Peltigerineae have higher growth rate compared to other species and mostly occur in wetter, more productive habitats (Palmqvist *et al*., 2002). Possibly, high rates of laccases and tyrosinases in the Peltigerineae give selective advantage to these lichens by providing enough nutrients (such as sugars derive from cellulose breakdown, see below) to support fast growth and to more efficiently exploit their environment.

*Physiological significance of laccases in lichen biology*

Based on our current knowledge on the roles of laccases in other organisms (Thurston, 1994), in lichens these redox enzymes are most likely to be involved in pigment synthesis, depolymerization of organic compounds, particularly lignin, depending on initial substrate molecular weight and environmental conditions and possibly pathogen defence. Laccases are one of the main enzymes involved in delignification by white rot and other fungi, including some ascomycetes (Thurston, 1994; Leonowicz *et al*., 2001). Acting alone or with lignin peroxidase and

manganese peroxidases, laccases produce ROS that participate in reactions that remove the lignin from wood and plant litter (ten Have and Teunissen, 2001; Hammel *et al*., 2002). However, most recent findings of Liers and Beckett (unpublished, personal communication[1]) showed that partially purified lichen laccases have low redox potential, and can metabolize normal laccase substrates, but more slowly than white rot fungi. Furthermore, they cannot metabolize model lignin compounds therefore almost certainly do not participate in delignification. Nevertheless, even if lichen polyphenol oxidases do not participate in delignification on theoretical grounds it is possible that they may still contribute to organic matter turnover by participating in cellulose breakdown (Beckett and Zavarzina, unpublished). In free-living fungi, in addition to polyphenol oxidases, cellulases and secreted peroxidases are involved in organic matter turnover (Baldrian and Valaskova, 2008). In lichens only low rates of peroxidases were found (see Chapter 4). Cellulases were not studied in this project but it was found earlier in *Evernia prunastri* and *Peltigera canina* (Yagüe *et al*., 1984; Yagüe and Estevez, 1988; de los Ríos *et al*., 1997). Study of Liers and Beckett (unpublished) suggested that extracellular enzymes of lichens (laccases and tyrosinases) may participate in ROS-generating reactions and together with cellulases the enzymes are involved in a type of soft rot decay, namely in cellulose breakdown.

A further possibility for species such as *Pseudocyphellaria aurata* that grows on trees is that laccases may assist attaching lichens to their lignin-rich substratum. Another possible role for laccases in lichens is pathogen defence. The stimulation of laccase activity that occurs after wounding and desiccation (see Figure 3.5) may offer protection at a time when they are vulnerable to pathogen attack. Flowering plants defend themselves against pathogens using extracellular ROS production (Bolwell, 1999; Blee *et al*., 2001). Beckett *et al*. (2003) suggested two defense strategies occurring in lichens. The first strategy appears in lichens from suborder Peltigerineae that produce reactive oxygen species by the involvement of laccases. Interestingly, as mentioned above, members of Peltigerineae contain low concentrations of secondary metabolites (Huneck and Yoshimura, 1996), particularly compared with other lichens which apparently deter pathogens by accumulating high concentrations of secondary metabolites, the second strategy (Rundel, 1978; Gauslaa, 2005).

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*Role of tyrosinases in lichen biology*

In free-living fungi, the roles of tyrosinases remain unclear, but their low redox potentials suggest that this enzyme does not participate in degradative processes, but rather is involved exclusively in polymerization (Ghosh and Mukherjee, 1998). Many studies reported that tyrosinase can certainly catalyse melanization in fungi (van Gelder *et al*., 1997; Seo *et al*., 2003; Halaouli *et al*., 2006). While melanins are present in Peltigeralean lichens such as *Lobaria pulmonaria* (Gauslaa and Solhaug, 2001) where they appear to protect the photobiont from excessive radiation, they are also present in non-Peltigeralean lichens with very low laccase and tyrosinase activities such as *Cetraria islandica* (Nybakken *et al*., 2004). It is assumed that, as for fungi (Butler and Day, 1998), various mechanisms of melanin synthesis exist in lichens, and presumably some use other oxidases. In lichens strong evidence exists that melanin acts as screens for UV light (Gauslaa and Solhaug, 2001; Stepanenko *et al*., 2002; Solhaug *et al*., 2003). A further role of these enzymes could be to remove harmful quinone radicals and phenols in the soil or bark on which lichens grow. These compounds are produced as by-products of delignification (Rimmer, 2006). Interestingly, Krastanov (2000) recommended the use of co-immobilized laccases and tyrosinases in polyclar columns used to remove phenolic xenobiotics, because their activities on different substrates complement each other. Stimulation of the activity of these enzymes following stress is consistent with a role in removal of stress-induced toxic compounds. Future progress in understanding the roles of these enzymes in lichens will depend on the elucidation of these reactions. Recently, van Alstyne *et al.* (2006) showed that DOPA in green algae can deter herbivores, and possibly tyrosinase-catalysed DOPA formation may have the same role in lichens. Certainly, members of the Peltigerineae do not contain the normal secondary metabolites possessed by lichens outside of this suborder (Huneck and Yoshimura, 1996). However, the widespread occurrence of tyrosinases in lichens in the Peltigerineae suggests that they play an important role in the biology of these lichens.

*Potential roles of laccases and tyrosinases in the environment*

As it is discussed earlier, laccases and tyrosinases can be readily secreted therefore it is supposed these redox enzymes play an important role in the environment. It has long been recognized that lichens have an important enzymatic role in soil formation due to their weathering action on rocks (Chen *et al*., 2000; Zavarzina and Zavarzin, 2006; Zavarzina *et al*., 2011) and also serve as considerable source of mortmass for humification (Zavarzina *et al*., 2011). The recent

finding of laccases and tyrosinases in lichens confirmed that lichens may play an important role in humification as not only the source of the organic compounds for humification, but also by the production of redox enzymes that can catalyze the synthesis and degradation of humic substances (HS) depending on conditions (Zavarzina *et al*., 2011). Zavarzina *et al*. (2011) suggested that laccases of lichens may participate in humus formation via polymerization of phenols while tyrosinases take part in humification processes through the synthesis of humic acid via melanization. However, recent study indicated that laccases appear most active in breaking down humic substances (HS, Zavarzina, unpublished data, personal communication of R.P. Beckett[2]). HS comprises up to 90% of soil organic matter and has a residence time of $10^2$-$10^3$ years. It is therefore a long-time sink for atmospheric $CO_2$. The importance of HS appears in the biosphere as the largest carbon reservoir estimated at c. 1500 Pg of $C_{org}$ (Batjes, 1996). Stolbovoi (2006) reported that about 1/3 of world soil organic carbon reserves can be found in boreal forests soils and almost half of this amount is accumulated in the soils of Russia exactly where many Peltigeralean lichens occur. Laccases leached from lichens may thus participate in global carbon cycling, particularly in nutrient-poor boreal and subarctic woodlands, where members of the suborder Peltigerineae are abundant.

*Future plans*

Future work needs to sequence the proteins and construct cladograms to determine the phylogenetic position of lichen laccases and tyrosinases among those produced by other fungi. Determining the physiological significance of extracellular ROS production will be harder. A role in pathogen defence seems likely, and it would be interesting to test whether fungal parasites of *Peltigera* can induce an “oxidative burst” similar to that observed during rehydration following desiccation. The possible interaction of laccases and tyrosinases with cellulases is also needed to investigate. Future work should also assess the role of laccases and tyrosinase in decomposition, humification and melanin biosynthesis. The possible biotechnological application of these lichen enzymes should be investigated, and could include the textile, food, paper and cosmetic industries.

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# CHAPTER 8

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