4th European meeting on Oxizymes
June 16-18, 2008, Helsinki, Finland

ABSTRACT BOOK
OXIZYMES IN HELSINKI
4th European meeting on Oxizymes
June 16-18, 2008, Helsinki, Finland

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The Abstract book was edited by:

Päivi Vahala, Martina Andberg, Kristiina Kruus & Liisa Viikari
Preface

The roots of the Oxizymes meetings go back to Cassis, France, where the first meeting, focusing on laccase research was held in January 2002, gathering a relatively small group of scientists. The next Oxizymes meeting was organized in Naples, Italy, in June 2004 followed by Oxizymes in Oeiras, Portugal, in 2006. Thus, the Oxizymes meetings have now been regularly held and the scope has been extended from fundamental and applied aspects of laccases to oxidative enzymes in general.

The Oxizymes meetings have been a great success not only from the scientific point of view, but also in terms of strengthening and generating networks and collaboration between academic and industrial groups. The idea of Oxizymes is to bring together colleagues and friends working on the field of oxidative enzymes to share knowledge, generate collaboration and to also constitute an incubator for many future project applications to the European Commission.

Thanks to the contributions of participants we have been able to design a programme of the Oxizymes in Helsinki that covers recent developments on oxidases, oxygenases and peroxidases. Various aspects include microbial physiology and genetics, enzymology, protein structure and structure-function studies, as well as various applications. The Oxizymes programme in Helsinki will include 35 oral presentations and about 70 posters during the two and a half days' meeting.

On behalf of the Organizing Committee we would like to express our gratitude to all persons who have contributed to the organization of this meeting, to the members of the Scientific Committee, and in particular to Ms Päivi Vahala from the secretariat. We also wish to thank all our sponsors that provide us extra-funds and finally acknowledge the support of the COST organization, VTT and the University of Helsinki.

We wish you all a fruitful meeting of scientific excellence and an enjoyable stay in Finland.

Helsinki, 4.6.2008

Liisa Viikari
Kristiina Kruus
PROGRAMME

Monday, June 16th

8.00  Registration
9.00  Opening of the meeting

SESSION 1. STRUCTURE–FUNCTION OF OXIDOREDUCTASES
SESSION CHAIR: GIOVANNI SANNIA

9.10  Insights into the functional mechanism of Melanocarpus albomyces laccase
      Nina Hakulinen, University of Joensuu, Finland

9.35  Study of the catalytic tryptophan environment in Pleurotus eryngii versatile peroxidase
      Francisco J. Ruiz-Dueñas, CIB-CSIC, Spain

10.00 Comparative analysis of 3D structures of laccase from Coriolus hirsutus at resolution
     1.85 Å and 1.2 Å
      Olga V. Koroleva, Russian Academy of Sciences, Russian Federation

10.25 Crystal structures of native and substate adducts of catechol 1,2-dioxygenase from the
     chlorophenol-utilizing gram-positive Rhodococcus opacus 1CP
      Fabrizio Briganti, Universita di Firence, Italy

10.55 – 11.20 COFFEE

SESSION 2. CHARACTERISTICS OF LACCASES
SESSION CHAIR: ÁNGEL T. MARTÍNEZ

11.20  The laccase gene family in Pleurotus ostreatus
       Giovanni Sannia, University of Naples Federico II, Italy

11.45  Copper incorporation into recombinant CotA-laccase from Bacillus subtilis
       Ligia O. Martins, Universidade Nova de Lisboa, Portugal

12.10  Unusual properties of the laccase isoenzymes from basidiomycete Steccherinum ochraceum
       strain 1833
       Ludmila Golovleva, G.K. Skryabin Institute of Biochemistry and Physiology of
       Micro-organisms, Russian Academy of Sciences, Russian Federation

12.35  A novel dimeric laccase from the aquatic ascomycetous fungus Phoma sp. UHH 5-1-03
       Dietmar Schlosser, Helmholtz Centre for Environmental Research – UFZ, Germany

13.00 – 14.15 LUNCH
**SESSION 3. MISCELLANEOUS OXIDOREDUCTASES**  
**SESSION CHAIR: LÍGIA MARTINS**

14.15 Oxizymes and vitamin C  
*Willem van Berkel, Laboratory of Biochemistry, Wageningen University, The Netherlands*

14.40 Characterization of the crosslinking ability of the *Trichoderma reesei* tyrosinase  
*Kristiina Kruus, VTT, Technical Research Centre of Finland, Finland*

15.05 Peroxygenases: extracellular mushroom enzymes which oxygenate aliphatic and aromatic carbon as well as organic heteroatoms by means of hydrogen peroxide  
*Martin Hofrichter, International Graduate school of Zittau, Germany*

15.30 A novel L-amino acid oxidase synthesized by *Pseudoalteromonas luteoviolacea*  
*Patricia Lucas-Elio, University of Murcia, Spain*

15.55 – 16.25 COFFEE

**SESSION 4. SCREENING, EVOLUTION AND PRODUCTION OF LIGNOLYTIC ENZYMES (1)**  
**SESSION CHAIR: MARTIN HOFRICHTER**

16.25 Ecology-taxonomical and physiological approaches in screening for basidiomycetes laccase producers  
*Nadezhda Psurtseva, Komarov Botanical Institute RAS, Russian Federation*

16.50 Ecology of production of ligninolytic oxidases and peroxidases in soil and litter  
*Petr Baldrian, Institute of Microbiology of the ACSR, Czech Republic*

17.15 Characterization and regulation of laccase and manganese peroxidase of the litter-decomposing fungus *Agrocybe praecox*  
*Kristiina Hildén, University of Helsinki, Finland*

19.30 – 20.30 HELSINKI CITY RECEPTION

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**Tuesday, June 17th**

**SESSION 5. SCREENING, EVOLUTION AND PRODUCTION OF LIGNOLYTIC ENZYMES (2)**  
**SESSION CHAIR: KRISTIINA KRUUS**

9.00 Phylogeny, expression and functions of fungal lignin-modifying peroxidases and laccases  
*Taina Lundell, University of Helsinki, Finland*

9.25 Prokaryotic laccases evolved by module shuffling  
*Brenda Valderrama, Universidad Nacional Autónoma de México, México*

9.50 Several approaches to regulate basidiomycete laccase and manganese peroxidase production  
*Vladimir Elisashvili, Durmishidze Institute of Biochemistry and Biotechnology, Georgia*

10.20 – 10.50 COFFEE
**SESSION 6. APPLICATIONS: FINE CHEMICALS**  
**SESSION CHAIR: WILLEM VAN BERKEL**

10.50  
Aerobic oxidation of alcohols catalyzed by laccase from *Trametes versicolor* and mediated by TEMPO  
*Isabella Arends, Delft University of Technology, The Netherlands*

11.15  
Laccase and galactose oxidase – an elegant way to glycomimetics  
*Pavla Bojarová, Centre for Biocatalysis and biotransformation, Czech Academy of Sciences, Czech Republic*

11.40  
Chemo-enzymatic synthesis of biotinylated nucleotide sugars by combination of UDP-Glc(NAc) 4’-epimerase and galactose oxidase  
*Lothar Elling, RTWH Aachen, Germany*

12.05  
Development of ‘second generation’ bioproducts from dioxygenase-mediated biocatalysis  
*Christopher Allen, The Queen’s University Belfast, Northern Ireland*

12.30  
Efficient NAD(P)⁺ regeneration for dehydrogenase reactions by a laccase/mediator system  
*Roland Ludwig, BOKU, Austria*

13.00 – 14.00  
LUNCH

14.00 – 15.15  
POSTERS

**SESSION 7. APPLICATIONS: SPECIAL PRODUCTS**  
**SESSION CHAIR: GEORG GÜBITZ**

15.15  
Bioreactor implementation of immobilized and insolubilized laccase  
*Hubert T. Cabana, University of Sherbrooke, Canada*

15.40  
Development of enzyme catalyzed electrodes for printable fuel cell application  
*Anu Koivula, VTT Technical Research Centre of Finland, Finland*

16.05  
Synthetic exploitation of laccases for the selective modification of natural compounds  
*Sergio Riva, Instituto di Chimica del Riconoscimento Molecolare, Italy*

16.30  
Estimating the redox potential of oxidoreductases through catalytic measurements  
*Marcela Ayala, Instituto de Biotecnología, México*

16.55  
PAH transformation and detoxification in soil by a consortium of white rot fungi: role of laccases and peroxidases  
*Antonella Anastasi, University of Turin, Italy*

19.00 – 24.00  
CONFERENCE DINNER
Wednesday, June 18th

**SESSION 8. APPLICATIONS: BIOPROCESSES**  
SESSION CHAIR: LIISA VIKARI

8.45 Layer-by-layer immobilised laccases in the oxy-functionalisation of lignins  
*Raffaella Perazzini, Tor Vergata University, Italy*

9.10 Grafting of functional molecules using oxidoreductases  
*Georg M. Gübitz, Graz University of Technology, Austria*

9.35 Biodegradation of humic acid by white rot fungi isolated from biosolids compost  
*Yitzhak Hadar, Faculty of Agricultural, The Hebrew University, Israel*

10.00 Toward the development of novel sustainable bioprocesses for the colour industries  
*Sophie Vanhulle, Unit of Microbiology, Belgium*

10.30 – 11.00 COFFEE

11.00 Decontamination by oxidoreductases – example of an industrial application  
*Lars Østergaard, Novozymes A/S, Bagsvaerd, Denmark*

11.25 Oxidation of tert-butyl alcohol in *Aquincola tertiariicarbonis* L108 is catalyzed by a phthalate dioxygenase-like enzyme system  
*Franziska Schäfer, Helmholtz Centre for Environmental Research – UFZ, Germany*

11.50 Electrochemical aspects of the dye degradation by redox enzymes  
*Florentina D. Munteanu, University Aurel Vlaicu of Arad, Romania*

12.30 – 13.00 CONCLUSIONS
ORAL PRESENTATIONS
**O1.1: INSIGHTS INTO THE FUNCTIONAL MECHANISM OF Melanocarpus albomyces LACCASE**

N. Hakulinen¹*, J. Kallio¹, M. Andberg², A. Koivula², K. Kruus² & J. Rouvinen¹

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²VTT Technical Research Centre of Finland, Espoo, Finland  
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*Melanocarpus albomyces* is an ascomycete fungus expressing a thermostable laccase with a neutral pH optimum. The three-dimensional structure of *M. albomyces* laccase (MaL) at 2.4 Å was solved among first complete laccase structures¹. MaL structure showed two special features: ¹dioxgen was observed inside the trinuclear site. ²The C-terminus of enzyme penetrated the tunnel leading towards the trinuclear site. Today, many crystal structures of several laccases, mostly from basidiomycete fungi, are available in PDB. So far, the plugging of the tunnel leading towards trinuclear site has not been observed in any other published laccase structures than in MaL. At the present, laccase structures show wide variety of trinuclear site geometries having one or two oxygen atoms amidst three coppers. Our recent studies have shown that the trinuclear site of laccase is sensitive to X-rays and the observed structure may depend on the data collection strategy or the intensity of the beam².

We are now studying the reaction mechanisms of laccases, both oxidation of phenolic compounds near the mononuclear site and the reduction of dioxygen at trinuclear site. We have recently solved the three-dimensional structure of recombinant *M. albomyces* laccase (rMaL) at 1.3 Å resolution³. This structure confirmed our earlier proposal regarding the dynamic behaviour of the trinuclear site and it allowed us to describe important solvent cavities of the enzyme. Very recently, crystals of rMaL have been soaked with 2,6-dimethoxyphenol and several data sets have been collected to 1.7-2.0 Å. The binding of various oxidation products will be discussed.

**References**

O1.2: STUDY OF THE CATALYTIC TRYPTOPHAN ENVIRONMENT IN *Pleurotus eryngii* VERSATILE PEROXIDASE

F. Ruiz-Dueñas1*, M. Morales1, M. Mate1, A. Romero1, M. J. Martínez1, A. Smith2 & Á. T. Martínez2

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Lignin degradation by fungal peroxidases is initiated by one-electron transfer to an exposed tryptophan radical, a reaction mediated by veratryl alcohol (VA) in lignin peroxidase (LiP). The existence of this catalytic protein radical was first described in LiP (1), but only in versatile peroxidase (VP) it has been directly detected by electronic paramagnetic resonance (EPR) (2, 3). VP differs from LiP not only in oxidation of Mn2+ at a second catalytic site (4), but also in its ability to directly oxidize different aromatic compounds and dyes that LiP can oxidize only in the presence of VA.

The catalytic tryptophan environment was compared in LiP and VP crystal structures, and six residues near VP Trp164 were modified by site-directed mutagenesis. Oxidation of Mn2+ was practically unaffected. However, several mutations modified the oxidation kinetics of the high redox-potential substrates VA and Reactive Black 5 (RB5) demonstrating that other residues contribute to substrate oxidation by the Trp164 radical. Introducing acidic residues at the tryptophan environment, as found in LiP, did not increase the efficiency of VP oxidizing VA. On the contrary, all variants harboring the R257D mutation lost their activity on RB5. Interestingly, this activity was restored when VA was added as mediator, revealing the LiP-type behavior of this variant. Moreover, combination of the A260F and R257A mutations strongly increased (20-50 folds) the apparent second-order rate constants for reduction of VP compounds I and II by VA to values similar to those found in LiP. Dissociation of the enzyme-product complex seemed to be the limiting step in the turnover of this improved variant. Non-exposed residues at the vicinity of Trp164 can also affect VP activity, as found by the M247F mutation. This was a direct effect since no modification of the surrounding residues was found in the crystal structure.

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References


Laccases belong to the family of copper-containing oxidases, which were found more than a century ago in Japanese varnish-tree *Rhus vernicifera*. Laccases (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) catalyse the oxidation of a variety of inorganic and aromatic compounds, including *o,p*-diphenols, aminophenols, polyphenols, polyamines, lignine, some inorganic ions, arylidamines, with the concomitant reduction of molecular oxygen to water. Moreover it can realize direct bioelectrocatalysis – direct electron transfer from electrode to active centre. These properties provide a wide applicability of laccase in bioremediation, biodetoxification and biosensor technology. Several structures of native fully glycosylated laccases from different sources in a “resting” form have been solved.

Here we present results of X-ray analysis of a high redox potential laccase from the fungus *Coriolus hirsutus* at resolution 1.85 Å and its T2 copper depleted derivative: Lac and T2D Lac respectively. The X-ray data were collected at EMBL (Hamburg). Laccase sequence was determined by direct PCR cloning of gene from chromosome. Amino acid sequence determined was used for refinement of 3D-structures of laccase and its derivative. The main difference with known laccase structures is that laccase studied (Lac) is presented in the form of native intermediate containing oxygen ligand in equatorial trigonal plane of T2/T3 copper cluster. Moreover in the structure of T2D Lac, peroxy ligand located between copper ions of T3 pair has been discovered.

The structural models of laccase native and “peroxide-level intermediate” should assist in the elucidation of the catalytic mechanism and electron-transfer processes in the family of blue multi-copper enzymes.
O1.4: CRYSTAL STRUCTURES OF NATIVE AND SUBSTATE ADDUCTS OF CATECHOL 1,2-DIOXYGENASE FROM THE CHLOROPHENOL-UTILIZING GRAM-POSITIVE RHODOCOCCUS OPACUS 1CP

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The first crystallographic structure of a catechol 1,2-dioxygenase from a gram-positive bacterium has been solved. The structure of catechol 1,2-dioxygenase from the Gram-positive bacterium Rhodococcus opacus (erythropolis) 1CP (Rho1,2-CTD), a Fe(III) ion containing enzyme specialized in the aerobic biodegradation of catecols, was refined at 1.94 Å resolution.

Since different kinetic properties have been observed between catechol dioxygenases isolated from gram-positive and gram-negative bacteria, the crystal structures of the adducts of Rho1,2-CTD with catechol, 3-chlorocatechol, 4-chlorocatechol, 3-methylcatechol, 4-methylcatechol and pyrogallol (benzene-1,2,3-triol) obtained under anaerobic conditions have been also elucidated. The structural differences between this two subfamilies and moreover the high structural homology of Rho1,2-CTD with the 3-chlorocatechol 1,2-dioxygenase from the same organism are discussed in term of their different substrate specificity.

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O2.1: THE LACCASE GENE FAMILY IN Pleurotus ostreatus

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Laccases (benzenediol oxygen oxidoreductase, EC 1.10.3.2) are multicopper-containing enzymes belonging to the group of blue oxidases, along with ascorbic oxidases and ceruloplasmins. Most of known laccases have fungal or plant origins, although few laccases have been identified in bacteria and insects. These enzymes are involved in many physiological functions such as morphogenesis, pathogenesis, lignin synthesis and ligninolysis. Chemically all these functions are related to the one-electron oxidation of a range of aromatic substrates such as a variety of phenolic compounds, as well as diamine and aromatic amines with the concomitant reduction of oxygen to water. Because of their high relative nonspecific oxidation capacity, laccases have been found to be useful biocatalysts for diverse biotechnological application1.

Many fungi produce several laccase isoenzymes endowed with different catalytic properties, being the physiological significance of this multiplicity still unknown. Laccase gene families have been found in different basidiomycetes fungi, indicating that they may have evolved through duplication-divergence events2.

The white-rot fungus Pleurotus ostreatus expresses multiple laccase genes encoding isoenzymes with different physico-chemical and catalytic characteristics. So far, five isoenzymes secreted by this fungus have been purified and characterized and the corresponding genes isolated3-7.

In this work three new members of the P. ostreatus laccase gene family, namely pox3, pox4 and pox5 have been isolated by PCR screening, using degenerate primers designed on the conserved copper-binding sequences8.

The expression of the three new putative laccase genes was assessed by reverse –transcriptase (RT)-PCR on total RNA extracted from the fungus grown in different cultural conditions. Mature transcripts were isolated for pox3 and pox4, while no RNA species coding for pox5 could be detected in the tested conditions. The full-length cDNAs of pox3 and pox4, code for 501-aa and 509-aa long polypeptides, respectively. Both translated sequences are preceded by a predicted 20-aa amino-terminal secretion signal peptide and all the consensus sequences involved in laccase structure and catalysis are conserved in both deduced proteins9. 3D models of the new predicted proteins have been built in order to highlight the peculiarities of these new enzymes and the structural differences with the previously characterized members of Pleurotus laccase family; regulatory sequences at the 5' terminus of the new laccase genes have been analyzed.

Further work has been focused on the characterization of the new laccase enzymes coded by pox3 and pox4 in order to shed light on their catalytic properties and on structure-function relationships of this class of enzymes. To this aim, heterologous expression of the isolated cDNAs has been performed in the heterologous hosts Kluyveromyces lactis and Saccharomyces cerevisiae. Optimization of parameters affecting the production and the setting up of a suitable purification protocol for the recombinant proteins will be both required for a deep characterization of these new enzymes.

References
O2.2: COPPER INCORPORATION INTO RECOMBINANT CotA–LACCASE FROM Bacillus subtilis

Z. Chen¹, P. Durão¹, A. T. Fernandes¹, V. Brissos¹, L. Pereira¹, S. Todorovic¹, M. M. Pereira¹, I. Bento¹, E. P. Melo²,³, P. F. Lindley¹ & L. O. Martins¹*

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We have settled a multidisciplinary research approach focused on the CotA-laccase from Bacillus subtilis as a model bacterial laccase system. Biochemical, spectroscopic and calorimetric studies have provided evidences for its robustness and potential for industrial applications as CotA is a thermoactive and intrinsically thermostable enzyme. Incomplete copper incorporation into the recombinant CotA-laccase purified from an overproducing Escherichia coli strain had always been observed during the course of our work and was identified as a major limiting factor in our studies of structure-function relationships. Recently, we have shown that protein samples produced by recombinant E. coli expressing the cotA gene, after growth in microaerobic conditions, contain a 4 copper/protein stoichiometry, ensuring that all four copper ions required for enzyme activity were incorporated into the active sites, while purified enzyme from aerobic cultures exhibit an incomplete metal incorporation (0.5:1 Cu/protein). The holoCotA produced under microaerobic conditions showed, as expected, higher catalytic rates (differences as large as 200-fold were measured) as well as, higher kinetic and thermal stability. Furthermore, our studies of copper incorporation into apo-CotA enzymes, as monitored by optical and EPR spectroscopies, point to a crucial role of copper in the folding of recombinant CotA-laccase in the cytoplasm of E. coli.

The development of appropriated recombinant E.coli culturable conditions allowed the establishment of an efficient cotA expression system, and high throughput screening systems, for directed evolution approaches, aiming in identifying variants with higher redox potential. In fact, our studies on the structure-function relationships highlighted the limitation of rational approaches to engineer the redox potential of enzyme. Laccases with high redox potential have an increased range of oxidizable substrates and improved effectiveness and versatility, preventing the requirement of expensive redox mediators for many applications.

Acknowledgements
This work was supported by FP6-NMP2-CT-2004-505899, FP6-NMP2-CT-2004-505899 and POCI/BIO/57083/2004 project grants.
New laccase isoenzymes were isolated and characterized from *S. ochraceum* 1833 strain, recently reported as a new active producer of high extracellular laccase activity. Three laccase isoenzymes (laccases I, II and III) with 57.5, 59.5 and 63 kDa molecular masses respectively were purified from the strain cultivated in glucose–peptone medium with 2 mM CuSO₄ using immobilization of the mycelium on polycaproamide tissue and 1mM 2,4-dimethylphenol as inducer. All of the three isoenzymes show maximal activity with ABTS at a low pH (≤2) and temperatures in the range from 70 to 80°C. Furthermore the isoenzymes were stable for long time of incubation at high temperature and at pH values ranging from 2 to 6. In contrast to the most of known laccases the isoenzymes from *S. ochraceum* 1833 had strongly pronounced absorption at 610 nm. All isoenzymes exhibited wide substrate specificity to non-natural test substrate ABTS and the phenolic compounds. Moreover the isoenzymes were able to decompose a number of PAHs. Among the three isoenzymes, laccase II showed a higher specificity and wider specificity for the range of investigated substrates. N-terminal amino acid sequence analysis of the purified laccase II resulted in a polypeptide sequence (VQIGPVTDLH) that showed 80% similarity with the N-terminal amino acid sequence of laccase from *Lentinula edodes*. Laccase II was successfully crystallized and 3D-structure of the enzyme was dissolved.

**Acknowledgements**

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**References**


**O2.4: A NOVEL DIMERIC LACCASE FROM THE AQUATIC ASCOMYCETOUS FUNGUS *Phoma* SP. UHH 5-1-03**

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A novel laccase produced by the aquatic ascomycetous fungus *Phoma* sp. UHH 5-1-03 was purified upon hydrophobic interaction and size exclusion chromatography (SEC) and characterized. The purified enzyme showed a pH-dependent dimerization as assessed by SEC. The monomeric form (~74 kDa, as determined upon application of molecular mass markers to SEC) dominated at pH 3 and 4. The dimeric form (~150 kDa) represented more than 90% of the total laccase at pH 5, and more than 97% at pH 7 and 8. Isoelectric focusing yielded a single band upon protein as well as activity staining, with an unusual alkaline isoelectric point > 8.3. MALDI-TOF-MS analysis of the monomer fraction obtained by SEC yielded a molecular ion (M⁺) corresponding to a molecular mass of 75.6 kDa; in good agreement with the value determined by SEC (see above).

The enzyme oxidizes typical laccase substrates (ABTS, syringaldazine, 2,6-dimethoxyphenol, guaiacol) and several synthetic dyes, but not tyrosine. Certain interesting characteristics point to a considerable biotechnological potential of the *Phoma* sp. laccase: The enzyme is still active in oxidation of the aforementioned substrates above pH 7. The laccase is remarkably stable especially in the more alkaline pH range. Furthermore, the enzyme is active at high concentrations of different organic solvents.

Only one laccase gene was found in genomic DNA of *Phoma* sp., which was completely sequenced. The gene was also found to be transcribed in cultures showing extracellular laccase activity, which were used to isolate the *Phoma* sp. laccase for purification and characterization. Based on the amino acid sequence derived from the laccase gene, a hypothetical 64.3 kDa protein with a theoretical pI of 8.1 was predicted upon use of the ProtParam tool. The difference between the predicted and experimentally determined size of the laccase can be explained by glycosylation, whereas the predicted pI fits well to the experimentally determined value. To prove encoding of the expressed protein by the identified laccase gene, laccase-specific protein bands were cut from SDS-PAGE gels and subjected to tryptic digestion. Tryptic peptides were analysed by nanoLC-ESI-MS/MS. Resulting MS/MS spectra were used to perform a search on a MASCOT server (Matrix Science, London, UK) against the cDNA sequence of the laccase translated into amino acids. Four peptides could be identified with a sum MOWSE score of 223 (p<0.05), resulting in 12% sequence coverage. Thus, the expression of the putative protein as predicted by the cDNA and genomic sequence was proven.
O3.1: OXIZYMES AND VITAMIN C

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L-Ascorbic acid (vitamin C) is an important antioxidant that is particularly abundant in plants. The mitochondrial flavoenzyme L-galactono-1,4-lactone dehydrogenase (GALDH) catalyzes the terminal step in the biosynthesis of L-ascorbic acid in plants. The enzyme uses cytochrome c as electron acceptor. GALDH is a so-called aldonolactone oxidoreductase with a conserved VAO-type FAD binding domain\(^1\). Most VAO-family members are oxidases containing covalently bound FAD. GALDH, however, contains a non-covalently bound FAD and reacts poorly with oxygen\(^3\).

We have addressed the oxygen reactivity of GALDH from *Arabidopsis thaliana*. We found that recombinant GALDH is sensitive to oxidative stress and that the substrate protects the enzyme from inactivation by hydrogen peroxide. The inactivation is due to the modification of a single cysteine residue (Cys340) that is conserved among aldonolactone oxidoreductases. Substitution of Cys340 with Ser or Ala resulted in variants that are insensitive towards oxidative stress, but show a poor affinity for L-galactono-1,4-lactone. The critical role of Cys340 in substrate binding makes the enzyme vulnerable towards inactivation by oxidation. This and the abundance of ascorbic acid in plants might explain why GALDH is not an oxidase. The production of large amounts of hydrogen peroxide in the mitochondria could stimulate enzyme inactivation and accelerate ageing.

Up to now, there are no structural rules that enable the prediction of the oxygen reactivity of flavoproteins\(^4\). Within the VAO-family there is a correlation between the covalent tethering of the FAD cofactor and oxidase activity\(^2\). A close inspection of several VAO family structures and sequences revealed another correlation with oxidase activity; nearly all oxidases contain either a Gly or Pro residue near the C4a locus of the flavin, whereas dehydrogenases have a more bulky residue at this position. Mutation of the corresponding residue in GALDH (Ala113→Gly) yielded a striking increase in oxygen reactivity. The Ala113Gly mutation most likely creates space for molecular oxygen to react with the reduced flavin.

References
O3.2: CHARACTERIZATION OF THE CROSSLINKING ABILITY OF THE Trichoderma reesei TYROSINASE

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Tyrosinases (monophenol, o-diphenol: oxygen oxidoreductase, EC 1.14.18.1) are type 3 copper proteins having a diamagnetic spin-coupled copper pair in the active centre. They catalyze the o-hydroxylation of monophenols and subsequent oxidation of o-diphenols to quinones and can thus oxidize both mono- and diphenols. Molecular oxygen is used as a terminal electron acceptor and it is reduced to water in tyrosinase-catalyzed reactions. Tyrosinases are ubiquitously distributed enzymes in nature. They are found in prokaryotic as well as in eukaryotic microbes, and in mammals, invertebrates and plants. In mammals, tyrosinases catalyze reactions in the multi-step biosynthesis of melamin and eumelamin pigments, being responsible, for instance, for skin and hair pigmentation. Furthermore they are related to browning reactions of fruit and vegetables. Tyrosinases are also capable of oxidizing protein- and peptide-bound tyrosyl residues to the corresponding quinones, which are highly reactive and can further react non-enzymatically forming inter and intramolecular crosslinks. Enzymatic crosslinking can be exploited in modification of various proteins for instance food proteins to provide alternative means for food structure modification.

We have recently reported on a discovery and overproduction of a novel tyrosinase from the filamentous fungus Trichoderma reesei. The enzyme was able to oxidize various mono- and diphenolic substrates, typical for tyrosinases. The purified tyrosinase was shown to be C-terminally processed since the determined molecular weight was significantly lower (43.2 kDa) than expected according to the putative amino acid sequence (61.2 kDa). The crosslinking abilities of the T. reesei tyrosinase as compared to the crosslinking ability of other tyrosinases from apple, potato, and the white rot fungus Pycnoporus sanguineus will be discussed in this work.
O3.3: Peroxygenases: Extracellular Mushroom Enzymes Which Oxygenate Aliphatic and Aromatic Carbon As Well As Organic Heteroatoms By Means Of Hydrogen Peroxide

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Agaric mushrooms of the families Bolbitiaceae and Coprinaceae can secrete a special type of heme biocatalyst when growing on nitrogen-rich substrates such as suspensions of soybean meal or peptone. These heme-thiolate proteins represent functional hybrids of peroxidases and cytochrome P450 monoxygenases (P450s) and are capable of catalyzing following oxygenation reactions: aromatic epoxidation/hydroxylation (e.g. of naphthalene), hydroxylation of aliphatic side chains of aromatics (e.g. Cα-hydroxylation of ethylbenzene), O-dealkylation of alkyl-aryl ethers (“etherase” activity that proceeds via instable hemiacetals), selective oxygenation of N- and S-heteroatoms (e.g. pyridine and thioanisole) as well as oxidation of aryl alcohols into the corresponding aldehydes/ketones (via instable geminal diols). In addition, mushroom peroxygenases possess classic phenol-oxidizing, brominating and catalase-like activities. The first peroxygenase of this type was discovered in Agrocybe aegerita (Black poplar mushroom) and later, similar biocatalysts have been found in two Coprinus species. They are all highly glycosylated proteins (20-40%) and use hydrogen peroxide as co-substrate that acts both as primary electron acceptor and oxygen source in O-transfers (proved by 18O-labeling experiments). Molecular studies on fungal peroxygenases are currently under investigation and indicating less than 30% similarity to any known enzyme. Due to their exceptional biochemical and molecular properties, mushroom peroxygenases could become versatile tools in bio-organic synthesis and model biocatalysts for oxygen transfer reactions accomplished by P450s.
**O3.4: A NOVEL L-AMINO ACID OXIDASE SYNTHESIZED BY**

*Pseudoalteromonas luteoviolacea*

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Two purple pigmented bacterial strains, CPMOR-1 and CPMOR-2, have been isolated from the Mediterranean Sea. Strain CPMOR-1, was isolated from the microbiota of the algae *Halopteris scoparia*, while strain CPMOR-2 was isolated from seawater samples. Both strains are aerobic, oxidase positive Gram-negative bacteria. 16S rDNA from strains CPMOR-1 and CPMOR-2 was sequenced, and the sequences were deposited in GenBank under accession numbers EU158365 and EU158366. Both, phenotypic characteristics and 16S RNA, indicate that they belong to the species *Pseudoalteromonas luteoviolacea*. The synthesis of macromolecular antibiotics is a capability described in many strains of this species\(^1\), although the nature and mechanism of action of those antibiotics has not been reported up to now.

This study shows that the macromolecular antibiotic expressed by both strains is in fact an L-amino acid oxidase (LAO) which generates hydrogen peroxide. The LAO of strain CPMOR-2 has been characterized, revealing that it is an enzyme with broad substrate specificity, similarly to the LAOs described in other bacteria such as *Cellulomonas cellulans*\(^2\) or *Rhodococcus opacus*\(^3\). However, the *P. luteoviolacea* LAO shows a novel substrate specificity since it preferentially oxidizes L-Gln, L-Met, L-Phe and L-Glu, although L-Trp, L-Ile, L-Leu, L-Arg, L-His and L-Val were also substrates at much lower rates. The novelty of this enzyme makes it of biotechnological interest.

Basidiomycetes are the most important and valuable laccase producers. Evaluation of laccase potential of basidial macromycetes \textit{ex situ} and screening for novel producers were the aim of the present study. Analysis of macromycetes for natural ligninolytic capacity using ecology-taxonomical and physiological approaches has been done. According to published molecular-generic data all Basidiomycetes were grouped into monophyletic clusters. It was shown that ligninolytic macromycetes presented in 8 clusters but most of known laccase producers were found in 3 ones: Euagaricoid – families \textit{Agaricaeae}, \textit{Bolbitiaceae}, \textit{Coprinaceae} and \textit{Tricholomataceae}; Polyporoid – \textit{Bjerkandraceae}, \textit{Polyporaceae}, \textit{Trametaceae} and \textit{Ganodermataceae}; Phlebioid cluster – \textit{Phanerochaetaeae} and \textit{Schizophyllaceae}. This analysis provided the basis for screening for producers with a high laccase potential. Over 520 cultures of about 330 aphyllophoroid and agaricoid species maintained in the LE (BIN) Basidiomycetes Culture Collection were studied. Cultures for investigation were selected out of maintained in the Collection strains of xylotrophic, litter-decomposing and other saprotrophic fungi from various taxonomic groups, as well as out of new isolates – naturally active biodestructors, obtained from different geographical regions purposefully for this research. All the strains were grown on ale-wort agar plates (ale-wort 4°B, agar 20g/l). Laccase activity was determined at 1, 2, 3 and 4 weeks of cultivation using rapid assay methods (reagents: tannic acid, syringaldazine and guaiacol). Growth ability, an important factor for cultures-producers, was expressed as a number of weeks that cultures required to cover 90 mm plates. All strains involved into the experiment were studied on their cultural characters, some cultures were fruitied and some were sequenced for taxonomical verification. The results of the screening showed that 255 out of 330 Basidiomycetes species (or over 75%) exposed positive laccase activity. High laccase was found in 108 species (about 33% of studied species). Taxonomical and ecological groups containing active species were revealed. The sufficient number of species with positive and high laccase was found in \textit{Agaricales} and \textit{Polyporales} with the best proportion of positive/high activity in \textit{Marasmiaceae}, \textit{Tricholomataceae}, \textit{Polyporaceae} and \textit{Steccherinaceae}. It was shown that Russuloid cluster (\textit{Lentinnelaceae}, \textit{Hericiaceae}, \textit{Stereaceae}, \textit{Steccherinaceae}, etc.) could be a matter of research interest as several taxa (e.g. \textit{Steccherinaceae: Antrodialla faginea}, \textit{Junghuhnia nitida}, \textit{Steccherinum murashkinskyi} and \textit{S. ochraceum}) demonstrated a great laccase potential. Distribution of laccase activity in various ecological groups was also analysed. The majority (61%) of studied species were xylotrophic, the other saprotrophics (39%) were fungi on humus, litter, dung, cones, herbs, needle, mushrooms, mosses, bark, and coal. Species with high laccase were found in all groups but not among fungi on dung, mushrooms and bark. In xylotrophic group about ½ of studied species expressed high laccase. Strains revealed high laccase by express methods like \textit{Cerrena unicolor} 2036, \textit{Steccherinum murashkinskyi} 1963, \textit{S. ochraceum} 1994, \textit{Peniophora lycii} 2142, \textit{Polyporus ciliatus} 0626 and some others were studied in detail dynamic on their physiology and laccase production. As a result of the research new taxa with high laccase potential were revealed and several novel promising laccase producers can be proposed.

This study was supported by INTAS – grant 03-51-5889, Russian Fund of Fundamental Researches – grants 06-04-49043, 08-04-01558 and 08-04-01612, and Program OBN RAN “Biological resources of Russia: the fundamental basics of rational exploitation”. **O4.1. ECOLOGY-TAXONOMICAL AND PHYSIOLOGICAL APPROACHES IN SCREENING FOR BASIDIOMYCETES LACCASE PRODUCERS**

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**O4.2. ECOLOGY OF PRODUCTION OF LIGNINOLYTIC OXIDASES AND PEROXIDASES IN SOIL AND LITTER**

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Ligninolytic oxidases and peroxidases of saprotrophic fungi are the enzymes contributing to degradation of lignin in decaying leaf litter and to the transformation of humic substances with a similar chemical structure. The aims of this work were to detect and quantify the activity of ligninolytic enzymes in the upper layers of soils, to identify their main producers and to study their localisation and factors regulating their production. These data are put into context with the production of another extracellular enzymes and the characteristics of soil microbial community and vegetation.

Activity of ligninolytic oxidases and peroxidases was measured in environmental samples from oak (*Quercus robur*) forest and from a chronosequence of primary succession on spoil heaps after brown coal mining both in the soil samples and in the samples of decomposing litter of different age. The production was put into context with the content of fungal biomass and the production of other extracellular enzymes. The production of ligninolytic enzymes of *Hypholoma fasciculare* during the colonization of litter and soil was studied in more detail in laboratory experiments with nonsterile soil or litter microcosms.

Laccase and Mn-peroxidase (MnP) but not lignin peroxidase were found in the studied soils. Activity of both enzymes decreased with the soil depth and showed a patchy pattern of horizontal distribution with “hotspots”. The seasonality of enzyme production was documented in the plots of spontaneous succession while the titres of both enzymes in hardwood forest soils was constant. The highest enzyme activity of laccase was measured in summer periods while MnP was mainly active in Autumn during a period of higher fungal biomass presence in soil. The development of vegetation and the litter quality played an essential role in the occurrence of ligninolytic enzymes in litter. During litter decomposition, laccase and Mn-peroxidase activities showed a peak of activity in later stages of litter decomposition, after the peak of beta-glycosidases but before the peak of endo-cleaving polysaccharide hydrolases.

Activity of laccase and MnP was increased during the colonization of nonsterile litter and soil by the ligninolytic fungus *Hypholoma fasciculare* although the presence of soil or litter-associated microflora can negatively affect the production of ligninolytic enzymes and mineralization of lignin or humic acids.

Laccase and MnP play important roles in the turnover of carbon in the soil environment during the transformation of lignin in the fresh biomass (fallen litter) and nutrients liberation from the recalcitrant humic material. This study shows that the occurrence of fungi, but also the seasonality, the initial quality of litter and the successional stage of litter degradation affect the production of ligninolytic enzymes both in situ and in the controlled laboratory setup.

This work was supported by the Czech Ministry of Education, Youth and Sports (LC06066 and OC08050 / COST FP0602).

References
04.3. CHARACTERIZATION AND REGULATION OF LACCASE AND MANGANESE PEROXIDASE OF THE LITTER-DECOMPOSING FUNGUS AGROCYBE PRAECOX


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Litter-decomposing fungi (LDF) are the most important organisms for lignin degradation in forest soil. Despite the ecological importance of LDF relatively little is known about the molecular characteristics of their lignin-degrading machinery. *Agrocybe praecox* is a litter-decomposing fungus that has been shown to mineralize synthetic lignin as well as degrade various aromatic substrates 1. Here we describe the regulation and characterization of laccase and MnP of *A. praecox*. During growth in birch leaf litter and Mn²⁺ supplemented liquid cultures, *A. praecox* produced both MnP and laccase. MnPs of *A. praecox* have nearly neutral pH values, whereas most MnPs from wood-inhabiting white-rot fungi have optimal activity under acidic conditions 2. The supplementation of culture media with veratryl alcohol or heavy metals (Cu, Cd, Pb) showed that only copper induced the production of laccase considerably but MnP was not produced. We have cloned and sequenced partial nucleotide sequences of *A. praecox* MnP and two different laccase encoding genes. A comparison of the putative amino acid sequence of the predicted mature region of MnP encoding gene showed highest similarity to MnP3 of white-rot fungus *Pleurotus ostreatus*. The predicted amino acid sequence of partial laccase encoding genes showed the highest similarity to *Pholiota nameko* laccase. Laccase gene expression was detected when the fungus was cultivated in both leaf and needle litter as well as in straw. The molecular characterization of MnP and laccase aims to understand better the lignin degradation pattern and the regulation of litter decay metabolism of *A. praecox*.

References

05.1: PHYLOGENY, EXPRESSION AND FUNCTIONS OF FUNGAL LIGNIN-MODIFYING PEROXIDASES AND LACCASES


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Fungal Class II extracellular, lignin-modifying heme peroxidases (LIPs, MNPs, VPs) show diverse functions as biocatalysts (degradation of lignin, conversion of xenobiotics, bleaching of coloured compounds and polymeric dyes) but minor differences in protein structure. Recently, we described structurally and phylogenetically divergent MNPs from two biotechnologically promising, white-rot in wood causing basidiomycetes Phlebia radiata and Physisporinus (Ceriporiopsis) rivulosus. In wood cultures, both species express two distinct types of MNPs (long-MNP and short-MNP) that are functionally similar but differ in protein sequence and length, reducing substrate specificity, 3-D protein structure, and in the gene intron-exon structure. However, of these two species, only P. radiata produces LIP as expressed in multiple isozymes encoded by at least three lip genes.

When focussing on the laccases produced by the two fungi, several isozymes are found at protein and gene level. Fungal laccases have been successfully adopted for multiple applications in e.g. removal of phenolic compounds from beverages and industrial waste waters. Novel thermo tolerant laccases from P. rivulosus may be utilised as biocatalysts for similar treatments. We recently demonstrated that of the two P. radiata laccases characterized so far, both are expressed on wood cultures. However, their functions may significantly diverge as depicted by their phylogenetic positioning within the multicopper oxidase (MCO) superfamily.

When put together, it is possible to find similarities between fungal species within the pattern of expression of lignin-modifying enzymes (peroxidases and laccases) in wood cultures, and their overall strategy and selectivity for degradation of the wood components and lignin. Similarities may be seen also in the protein phylogeny, in particular within the Class II heme peroxidases, and functions for conversion of wood lignin and model compounds.
O5.2: PROKARYOTIC LACCASES EVOLVED BY MODULE SHUFFLING

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Fungal laccases have received a preponderant position among industrial enzymes based on their potential as oxidants¹. However, their assimilation into novel processes and products has been hampered by their catalytic limitations, i.e. their sensitivity to halides and alkaline conditions. In this regard, the exploration of novel biological sources has unveiled the existence of related enzymes with a more diverse catalytic profile²-⁵.

In order to gain further insight into the evolutionary diversity of prokaryotic laccases we assembled a collection of 114 sequences cured by the sole restriction of preserving all residues required for copper-atom binding. The collection is notoriously divergent at the sequence level with an overall similarity below 20%. In a previous work, we concluded that the evolutionary reconstruction of fungal sequences was clearly reminiscent of a common origin⁶. In contrast, the vast heterogeneity found among prokaryotic laccases precluded the identification of a single ancestor and was more consistent with a modular evolution model¹. Two of the modules, those located in the edges of the protein sequences, are directly involved in the coordination of the T1 and trinuclear copper sites and may represent the minimal scaffold requirement for catalytic activity. Each one of these modules are clearly homologous, not only among prokaryotic sequences but also with fungal and plant counterparts, and co-evolved by mutation accumulation in a highly restricted sequence landscape. Using our modular approach for the evolutionary reconstruction, some eukaryotic sequences appeared to rise from independent origins, an information that is obscured if the highly divergent central module is included in the analysis.

The central module, naturally encompassed by the conserved amino and carboxy termini, resulted to be the more informative from an evolutionary point of view. Within the collection, the central module length varies from 77 to 792 residues and constitutes 21 to 76% of the proteins mass. According to the central module sequence conservation, the collection could be sorted in groups with no necessary taxonomic consistency. Furthermore, there was no evidence of homology among groups suggesting independent origins. In some cases, a putative ancestor for the central module could be traced by their significant similarity to extant proteins, suggesting the occurrence of multiple and independent module/domain shuffling events as the fundamental evolutionary mechanism among prokaryotic laccases and a plausible source of their functional diversity.

Acknowledgements
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References
O5.3: SEVERAL APPROACHES TO REGULATE BASIDIOMYCETES LACCASE AND MANGANESE PEROXIDASE PRODUCTION

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Various potential industrial and environmental applications require huge amounts of low cost ligninolytic enzymes. Recent literature data and our own studies indicate that there are several approaches to achieve this aim. Firstly, an extremely high biodiversity in expression of enzyme activity among fungi isolated from different ecological niches and geographical regions has been revealed. Secondly, a crucial effect of carbon source and especially of lignocellulosic substrates on the secretion and ratio of individual enzymes has been established. Thus, the substitution of banana peels with wheat bran for the submerged fermentation by *Ganoderma lucidum* increased laccase yield from 3200 to 97300 U l\(^{-1}\). Thirdly, a special attention will be paid to the regulation of laccase and manganese peroxidase production by the synthetic dyes and TNT. Several dyes and TNT supplemented to the media at appropriate concentration and time significantly accelerate laccase or/and MnP production. For example, *Cerrena unicolor* laccase activity varied from 73000 U l\(^{-1}\) in medium with Indigo carmine to 279000 U l\(^{-1}\) in medium with Poly R478. Fourthly, we will show that heat and chemical stress of fungi (with copper and TNT) may be a potent tool regulating laccase or manganese peroxidase secretion. Fifthly, co-culture of the appropriate fungi is an appropriate approach to highly increase laccase (*Pycnoporus coccineus* + *Pleurotus dryinus*, *C. unicolor* + *Phellinus robustus*) and MnP (*Pycnoporus coccineus* + *Trametes versicolor*) yields.
O6.1: AEROBIC OXIDATION OF ALCOHOLS CATALYZED BY LACCASE FROM Trametes versicolor AND MEDIATED BY TEMPO

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Nowadays, the biocatalytical production of chemical compounds is applied for the manufacture of various products including bulk organic chemicals, pharmaceuticals, and food ingredients. However scarce examples of biocatalytic oxidation are known, while on the other hand there is a stringent need to replace classic stoichiometric reagents with green catalytic alternatives. In that respect, laccase-mediator systems that catalyze selective oxidation of sugars and alcohols have drawn increasing attention 1,2. Nitroxyl radical 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) was shown to be the most effective mediator of laccase catalyzed oxidation of alcohols and it seems likely that the oxoammonium anion, which can be formed in situ, is the actual oxidant2,3. However, the precise role of the laccase, including its catalytic mechanism is not well understood, and the published conditions (productivity and amounts of mediator required) need to be improved for industrialization. Therefore, in Delft we focus on the process optimization of the laccase-TEMPO catalyzed alcohol oxidation reactions in parallel with kinetic studies on the individual steps3,4.

In our recent studies we optimized the conditions for a range of substrates: 4-methoxybenzyl alcohol gave 100% conversion to its corresponding aldehyde, veratryl alcohol - 100%, furfuryl alcohol -66%, 1-phenyl ethanol gave 34 % conversion after 4 hours. The system turns out to be highly selective for primary versus secondary aliphatic alcohols. The explanation for this selectivity can be found in the kinetics of the oxoammonium ion oxidation under the conditions of the reaction (pH 5, phosphate buffer). In addition, we have measured separately the individual steps 1-5 as proposed in Figure 1. These results, which will be presented in the lecture, underscore the proposed mechanism and point out what the rate determining step is for the different substrates.

An important result is that we have observed the direct interaction between the blue-copper –site in the enzyme and the TEMPO molecule in stopped-flow experiments (k1 is 20 mM⁻¹ s⁻¹ and k2 is 5 s⁻¹) [5].

Selective oxidation of carbohydrates is a challenging task, especially if we look for a mild, simple and high-yielding method applicable to unprotected glycosides. The use of oxidoreductases offers an elegant solution to the synthesis of N-acetylglucosaminiduronates and N-acetyl-galactosaminiduronates (carboxy moiety at C-6) in quantitative yields. Compounds carrying a charged group at C-6 are strong ligands of the activation receptors of natural killer cells, particularly of CD69 protein. During our studies on modified substrates for glycosidases, we found that some of these C-6 modifications are surprisingly well tolerated by $\beta$-N-acetylhexosaminidases. Therefore, we used the oxidized $p$-nitrophenyl glycosides as substrates in $\beta$-N-acetylhexosaminidase-catalyzed synthesis to afford immunoactive oligosaccharides.

Preparative oxidation of galacto-structures by galactose oxidase from Dactylium dendroides to respective aldehydes was performed in a reactor with bubble-free aeration, which protects the oxidase from deactivation in the gas-liquid interphase. Disaccharide 1 was prepared from $p$-nitrophenyl 2-acetamido-2-deoxy-$\alpha$-D-galacto-hexitolaldo-1,5-pyranoside by transglycosylation, followed by oxidation to a uronic acid by NaClO$_2$. Notably, this method can be used for selective oxidation of reducing oligosaccharides at C-6. $p$-Nitrophenyl $N$-acetylgalactosaminiduronate was not cleaved by any of the 36 fungal $\beta$-N-acetylhexosaminidases tested and therefore it could not be used as a substrate for the synthesis.

Oxidation by laccase from Trametes versicolor/TEMPO system was used for the glucos-series, resulting in N-acetylgalactosaminiduronates. $p$-Nitrophenyl $N$-acetylgalactosaminiduronate was a good substrate for $\beta$-N-acetylhexosaminidase from Talaromyces flavus, and it was directly used for the synthesis of disaccharide 2.

We tested the affinity of all the compounds to the activation receptors of natural killer cells: rat NKR-P1 and human CD69 proteins. The results demonstrated the high impact of the oxidation on the ligand efficiency, particularly when multiplied by oligomerization.

Acknowledgement
Support by grant projects LC06010 (MŠMT), IAA400200503 (GA AV ČR) and research concepts AV0Z50200510 and MSM0021620808 is gratefully acknowledged.
O6.3: CHEMO-ENZYMATIC SYNTHESIS OF BIOTINYLATED NUCLEOTIDE SUGARS BY COMBINATION OF UDP-Glc(NAc) 4’-EPIMERASE AND GALACTOSE OXIDASE

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The paper presents our work on the chemo-enzymatic synthesis of novel modified nucleotide sugars and their potential use in glyobiology and biomedical research. The combination of the enzymatic oxidation of UDP-Gal(NAc) (3 and 4) using galactose oxidase with the chemical modification using biotin-ε-amido-caproylhydrazide (BACH) in one-pot led to the efficient synthesis of UDP-6-biotinyl-Gal(NAc) (UDP-Gal(NAc)-biotin, 7 and 8) in 100 mg scale [1]. In order to start from the less expensive substrates, enzymatic epimerization of UDP-Glc (1) and UDP-GlcNAc (2) and the subsequent oxidation of 3 and 4 was combined with chemical biotinylation by BACH in a one-pot synthesis [2]. Analysis by CE and NMR revealed a mixture (1.0:1.4) of the biotinylated nucleotide sugars UDP-6-biotinyl-Gal, (7) and UDP-6-biotinyl-Glc (9), respectively, in a reaction started with I. Only one single product, UDP-6-biotinyl-GalNAc (8), was formed when the reaction was initiated with 2. It could be demonstrated for the first time that a UDP-Glc(NAc) 4’-epimerase (Gne from Campylobacter jejuni) and galactose oxidase from Dactylium dendroides can be used simultaneously in enzymatic catalysis. This is of particular interest since the coaction of an enzyme demanding reductive conditions and an oxygen-dependent oxidase is unexpected. So far, the galactosyltransferases hβ3Gal-T5, hβ4Gal-T1, hβ4Gal-T4, and mα3Gal-T, were identified to transfer the Gal-biotin-label onto glycoproteins and neo-glycoconjugates by utilizing their intrinsic acceptor specificity and their acceptance of 7 as donor substrate.

References

Acknowledgements
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**O6.4: DEVELOPMENT OF ‘SECOND GENERATION’ BIOPRODUCTS FROM DIOXYGENASE-MEDIATED BIOCATALYSIS.**

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The introduction of chiral centres into low-cost, green chemical precursors is a major task for White Biotechnology. This is because the stereo-specificity of enzyme-catalysed biotransformations can potentially solve the ‘chirality’ problem for the preparation of bioactive molecules required in the pharmaceutical and agrochemical industries. Significantly, aromatic compound-degrading bacteria from the environment are a source of potentially useful enzymes for this purpose. The application of bacterial arene (or ring-hydroxylating) dioxygenase enzymes in biocatalysis has been demonstrated (1); and it has been shown that these enzymes are highly versatile - with the ability to functionalise aromatic substrates in a variety of different ways. For example, these enzymes can catalyse the transformation of the aromatic nucleus of a substituted arene to yield chiral cis-dihydrodiols metabolites with high enantiopurity (often >95% e.e.). They can also catalyse the transformation of benzylic carbon atoms (e.g. in ethylbenzene) to benzylic alcohols, alkenes to alkene cis-diols (e.g. with the substrate indene) and sulfides to chiral sulfoxides (e.g. in the substrate thioanisole) (2, 3).

Recently, we have explored the full potential of these enzymes to catalyse new and diverse transformations of substrates not normally associated with these biocatalysts – including ‘second generation’ bioproducts: that are produced when a dioxygenase-derived metabolite is subject to a further round of biotransformation after isolation or further functionalisation/modification (4, 5). Further insights into the development of these second generation methods are now presented – with special focus on the transformation of environmentally-friendly ‘green’ substrates, that may be widely available in the future as the dependence on petrochemical products in organic synthesis is reduced.

**References**

A new approach for the continuous in situ regeneration of \( \text{NAD(P)}^+ \) from \( \text{NAD(P)} \text{H} \) in dehydrogenase catalyzed reactions is presented. The enzymatic regeneration system uses laccase in combination with intermediate electron carriers (redox mediators) to oxidise the nicotinamide adenine dinucleotide \( \text{NADH} \) or the phosphorylated form \( \text{NADPH} \) by concomitant reduction of oxygen to water. Several redox mediators were tested under different pH and solvent conditions and Meldola’s blue appeared as the most promising candidate so far. Laccases from \textit{Trametes pubescens}, \textit{Rhus vernicifera} and \textit{Melanocarpus albomyces} were tested with Meldola’s blue as redox mediator in two different dehydrogenase catalyzed conversions to test the application potential. In the glucose dehydrogenase catalysed oxidation of glucose space-time yields of 7.6 g L\(^{-1}\) h\(^{-1}\) gluconic acid were obtained using \( \text{NAD}^+ \) (42 mmol L\(^{-1}\) h\(^{-1}\) \( \text{NAD}^+ \) turnover) and 6.9 g L\(^{-1}\) h\(^{-1}\) when using \( \text{NADP}^+ \) (38 mmol L\(^{-1}\) h\(^{-1}\) \( \text{NADP}^+ \) turnover). For both coenzymes turnover numbers of 1000 were obtained in these 0.5-L batch experiments. In a second test reaction an alcohol dehydrogenase was applied for the deracemisation of \((\text{rac})\)-rhododendrol. Oxidative kinetic resolution was finished after 48 h by complete conversion of \((S)\)-rhododendrol to raspberry ketone and an enantiomeric excess of \(>99.9\%\) for the remaining eutomer was achieved against strong product inhibition. The obtained results suggest that this method can prove useful for other applications by providing a high \( \text{NAD(P)}\text{H} \) oxidation rate together with a high thermodynamic driving force for complete substrate conversion. The regeneration system was quite stable and simple removal of the redox mediator was achieved by absorption to a cation exchange resin. One particular merit of this \( \text{NAD(P)}^+ \) regeneration system compared to others is its low cost of operation.
O7.1: BIOSREACTOR IMPLEMENTATION OF IMMOBILIZED AND INSOLUBILIZED LACCASE

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Laccase is a widespread multicopper phenoloxidase (EC 1.10.3.2) well-known from its natural role in the fungal degradation of lignin. Thanks to its relatively low substrate specificity, laccase can oxidize various phenol-like compounds and aromatic amines. Additionally, small molecule mediators can further extend its range of substrates towards oxidizing several non-phenolic substrates indirectly via the oxidized form of these mediators. This versatility together with a relatively good stability enable laccase to be applied in biobleaching, wastewater treatment and chemosynthesis. However, for these biotechnological applications to be sustainable in an industrial setting, the re-usability of the enzyme and its separability from the reactants and products must be ensured. To this end, immobilization of laccase has been studied by several teams. Our group has sought to immobilize laccase in order to be used towards the biocatalytic elimination of established or suspected xenoestrogens including nonylphenol (NP), bisphenol A (BPA) and triclosan (TCS). In an initial approach, our group immobilized laccase from \textit{Coriolopsis polyzona} covalently on diatomaceous earth supports (Celite\textsuperscript{8}), whose surface had been activated with aminopropyltriethoxysilane and then cross-linked with glutaraldehyde. Although the enzyme/support ratio (w/w) was relatively low, the supported biocatalyst displayed improved stability against thermal inactivation and denaturation by salts and proteases. When used in a packed-bed reactor, the immobilized laccase was able to eliminate BPA from aqueous solutions under different operational conditions, including several consecutive treatment cycles, with sustained removal performance. An enhancement of the specific activity together with retention of stability was achieved by insolubilizing the same crude laccase preparation in the form of cross-linked enzyme aggregates (CLEAs). These biocatalysts were prepared by precipitating laccase with polyethylene glycol (PEG), cross-linking with glutaraldehyde and recovering active CLEAs upon removal of PEG. Using a fluidized bed reactor, laccase CLEAs were shown to be able to (semi)continuously eliminate NP, BPA and TCS from a 5 mg l\textsuperscript{−1} solution at a hydraulic retention time (HRT) of 150 min. In a further attempt towards continuous xenoestrogen removal, a perfusion basket reactor was developed, consisting of a filtration module equipped with a three-blade marine impeller and capable of retaining active CLEAs under optimal conditions of transport and shear. In this vessel, continuous elimination of NP, BPA and TCS from a 5 mg l\textsuperscript{−1} solution was possible using a HRT of 325 min. In sum, immobilized and insolubilized laccases in the appropriate bioreactor setting have strong potential in the sustainable elimination of micropollutants but also in a variety of other biotechnological applications.
O7.2: DEVELOPMENT OF ENZYME CATALYZED ELECTRODES FOR PRINTABLE FUEL CELL APPLICATIONS

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Printed electronics with integrated power source has remarkable market potential in several mass-marketed consumer products e.g. as package integrated functionalities (sensors, displays or entertaining features etc.) or as part of diagnostic devices. One of the main requirements is that the power source should be biodegradable or possible to incinerate with normal household waste. This demand is not easily met by traditional battery technology. The materials and production costs of the power source should also be reasonable, not to significantly increase the price of the product. As an alternative power source, the miniaturized biological fuel cell has the potential to be developed to meet these demands.

The main goal of our research is to develop a printable fully enzymatic biofuel cell based on the use of enzymes as catalyst on both cathode and anode electrodes. A wide variety of different oxidoreductases can be potentially applied as biocatalysts in biofuel cells converting chemical energy from renewable chemicals to electricity with high overall efficiency. We have incorporated a high redox potential laccase from Trametes hirsuta in different types of conducting inks to produce dry printed cathode layers, where ABTS is used as the redox mediator. Suitable inks from commercial sources were screened and further optimised or experimentally developed in order to obtain enzyme electrodes expressing optimised enzymatic activity as well as good electrochemical properties. A fuel cell prototype was constructed utilizing an optimized printed laccase-ABTS layer as the cathode, and a printed Zn layer as the anode. Under humidity controlled conditions, a cell voltage between 0.8 and 0.6 V could be maintained for several days under a 2.2 kΩ load, the surface area being 16 cm². In addition, a corresponding stand-alone cell could be constructed where the cell voltage was maintained for 55 hours under the same load. These results offer a good starting point for further development of mass-producible, completely enzymatic printed biofuel cells. Work is now in progress to select a suitable anode side enzyme/mediator combination, as well as the fuel to be used. One type of potential anodic enzyme is the bacterial PQQ-dependent aldose dehydrogenase.

Acknowledgements
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O7.3: SYNTHETIC EXPLOITATION OF LACCASES FOR THE SELECTIVE MODIFICATION OF NATURAL COMPOUNDS

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Laccases are oxidoreductases belonging to the multinuclear copper-containing oxidases. The overall outcome of their catalytic cycle is the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to give four radicals. Typical substrates of laccases are phenols and aliphatic or aromatic amines, the reaction products being mixtures of dimers or oligomers derived by the coupling of the reactive radical intermediates. For instance, we have recently exploited these biotransformations to isolate new dimeric derivatives of the phytoalexin resveratrol and of its analogues, as well as of the hormone β-estradiol, of the natural antibiotic totarol, and of the flavonolignan silybin. In some of these studies we have also observed a significant influence of the solvent on the reaction outcomes.

Additionally, laccases oxidation of non-phenolic groups, particularly benzyl and – more generally – primary alcohols, is also possible thanks to the ancillary action of the so-called “mediators” (i.e., TEMPO, HBT, ABTS): the oxidation step is performed by the oxidized form of a suitable mediator, generated by its interaction with the laccase. Accordingly, we have oxidized a series of sugar derivatives (mono- and disaccharides, cyclodextrins, water soluble cellulose) and of natural glycosides.

In this communication some representative examples will be presented and discuss.

References

**O7.4: ESTIMATING THE REDOX POTENTIAL OF OXIDOREDUCTASES THROUGH CATALYTIC MEASUREMENTS**

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Redox potential of the intermediates involved in the catalytic mechanism of peroxidases cannot be measured directly by voltammetry. In the literature, mainly two methods exist to estimate the redox potential of these highly oxidizing species. By spectrophotometrically measuring the concentration of the reduced and oxidized species, the Nernst equation is thus applied to obtain the standard redox potential\(^1\)\(^2\). However, these methods present some issues regarding stability of the species, spectral discrimination between the various species that may be present in the reaction, chemical identity between the artificially generated intermediates and the actual catalytic intermediates and achievement of equilibrium. According to the theory of outer-sphere, developed by Marcus, the difference between redox potential of the species involved affects the magnitude of the rate constant in electron transfer reactions. Currently, this theory is well accepted as it accurately describes many of the redox reactions involving transition metals, organic molecules, ions and biomolecules\(^3\). In this work, we attempted to use the Marcus theory to characterize five peroxidases from fungal, plant and animal sources, and extract information on the redox potential of the catalytic intermediates. Rational selection of the system reaction allowed us to estimate the redox potential of the Compound II/Fe(III) redox couple\(^4\). A number of criteria, such as saturation kinetics, chemical homology, low steric effects and irreversibility, were considered when selecting the reaction system. The estimated values correlate well with the redox potential of the Fe(III)/Fe(II) couple; although this couple is not catalytic, its redox potential has been used as an indicator of the peroxidases’ oxidative tendency for several years\(^5\).

We propose this catalytic method as an alternative to characterize peroxidases in terms of their redox potential. Given that the estimated values are obtained under truly catalytic conditions, we believe they may represent more accurately the electron transfer process involved during the reaction. This method has been applied to study other hemoproteins, such as mutants and chemically modified preparations of yeast iso-l-cytochrome c. This approach could be also applied to other oxidoreductases, such as laccases.

**Acknowledgments**

We thank R. Román for technical assistance and Conacyt 56718 for financial support.

**References**

O7.5: PAH TRANSFORMATION AND DETOXIFICATION IN SOIL BY A CONSORTIUM OF WHITE ROT FUNGI: ROLE OF LACCASES AND PEROXIDASES

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Polycyclic aromatic hydrocarbons (PAH) are persistent organic pollutants widely distributed in the ecosphere. They are composed of two or more fused benzene rings and pose serious risks to human health owing to their carcinogenic potential. Biological treatments aimed at detoxifying PAH-contaminated soils have seen a broad and rising usage over the last few years. Among microorganisms capable of degrading PAH, extensive studies have been done on white rot fungi: their complement of enzymes comprises several peroxidases and laccases that are nonspecific, non-stereoselective and effective against a broad spectrum of aromatic compounds. Although these enzymes have been extensively studied and many authors have reported on the activity of these enzymes individually, very little is known about their combined role during the degradation process.

In this work we investigated the ability of a consortium of three basidiomycetes isolated from compost to degrade PAHs in soil. Preliminary results about the possibility to use these fungi in consortium showed that the co-cultures displayed a higher degradation capability against the antraquinonic dye Poly R 478 and a synergic production of laccases. Laboratory microcosms with PAH-spiked soils were incubated with the fungal consortium pre-grown on straw to estimate: 1) the efficiency in removing naphthalene (500 ppm) and the more recalcitrant pyrene (100 ppm); 2) the changes in soil phytotoxicity due to PAHs and/or any of their metabolites monitored by conventional tests with Lepidium sativum seeds; 3) the enzymes that may be involved in the degradation process and their combined role.

The consortium was found able to efficiently colonize soil and to decrease the concentration of naphthalene by about 70% in 21 days and of pyrene by about 56% in 28 days; in the meantime, the phytotoxicity was significantly reduced. Enzymatic assays showed that laccase, manganese peroxidase, manganese-independent peroxidase and lignin peroxidase were produced during the experiment; laccase showed the higher activity values and the higher correlation with pyrene degradation. The other enzymes resulted weakly correlated to pyrene degradation. However, strong correlations were found among the different enzymes activities, namely among laccase, manganese peroxidase and lignin peroxidase, indicating that the amount of each enzyme present in the microcosms depends on the level of the other enzymes present in the system at the same time and on the characteristics of the surrounding environment. These enzymes, in fact, compete for the same substrates and the radicals formed can interact with each other or with the fungal metabolites, generating other radicals that can continue this process. Hence, also the enzymes which show low activity values could play an important role both in the initiation of the degradation reaction or in the following oxidative steps involving fragments resulting after the first attack.

This consortium could thus be regarded as an appropriate candidate for bioremediation of the environment through the detoxification of soils contaminated by PAHs. These results also demonstrate that composts, as substrates rich in lignocellulosic materials, can be important sources of microorganisms, including ligninolytic fungi, able to degrade pollutants.
Laccase, benzenediol:oxygen oxidoreductase 1.10.3.2, catalyse the oxidation of a great variety of compounds, typically phenolic systems. By this process phenoxy radicals are generated with the concomitant reduction of O$_2$ to H$_2$O. We report the immobilization of this oxidative enzyme on alumina pellets and the subsequent use of layer by layer technique for coating the particles. The free and the immobilized enzyme was used for the functionalisation of an array of grass, softwood and hardwood milled wood and residual lignins. The oxidation was studied in presence and in absence of radical mediators, such as 1-hydroxybenzotriazole (HBT) and violuric acid (VA). The reactions were followed by measuring the consumption of O$_2$ during the enzymatic treatment. Different oxidation cycles were carried out in order to evaluate the potential recycle of this enzyme.

The structure of functionalised lignins was determined by advanced NMR spectroscopic techniques such as $^{31}$P-NMR after phosphitylation for the quantitative determination of the different phenolic, aliphatic and carboxylic OH groups, and 2D-NMR for the determination of the specific modifications induced in the polymer. The antioxidant activities.
O8.2. GRAFTING OF FUNCTIONAL MOLECULES USING OXIDOREDUCTASES

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Oxidoreductase-catalyzed grafting of functional molecules presents an eco-friendly opportunity to improve properties of materials. In a novel stepwise approach, functional molecules were bound to enzymatically modified lignocellulose containing anchor groups. As anchor groups, phenolic amines were coupled to lignin moieties of lignocellulose using laccase. Coupling reactions to lignin were mimicked using the lignin model compounds 4-O-methyl guaiacylglycerol β-guaiacyl ether, guaiacylglycerol β-guaiacyl ether, dehydrodivanillyl alcohol type dibenzodioxocin and syringylglycerol β -guaiacylether. LC-MS analysis showed that phenolic amines were coupled in 1:1 ratio to the lignin model compounds. Using this approach it was possible to increase binding of functional molecules such as by up to 60 %. Another strategy involved the synthesis of alkoxysilanes carrying phenolic substituents. After addition of water, these materials polymerize binding a wide range of non-reactive surfaces (e.g. glass, metals, synthetic polymers). Phenolic moieties of these layers were oxidized by laccases allowing covalent functional coating and grafting. Finally, novel approaches in tyrosinase catalysed grafting onto lignocellulose, polyamides and proteins will be presented.
O8.3. BIODEGRADATION OF HUMIC ACID BY WHITE ROT FUNGI ISOLATED FROM BIOSOLIDS COMPOST

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One of the main environmental problems facing the world today is safe and beneficial utilization of solid organic waste materials. Composting is an effective way of harnessing microbial populations to the fast pace of the modern world. Degradation of organic matter (OM) during composting is conducted mostly by bacterial and fungal populations. In spite of the extensive research conducted on compost to date, only a small number of studies provide information on lignin or humic substances modifying fungi isolated from compost at the thermophilic stage. Two white rot fungi were isolated from biosolid compost and studied for their ability to degrade humic acids from different origins (biosolids compost, Hulla valley peat soil and leonardite). The extent of bleaching was considered to be a meaningful indicator of the HA degradation rate. A comparison of the bleaching abilities of *Trametes* sp. M23 and *Phanerochaete* sp. Y6, grown both in liquid and solid state cultures revealed large bleaching ability for both fungi. Although perlite, as a solid support simulates fungal growth under aerobic condition, the use of liquid cultures with defined media allowed us to eliminate the NaOH extraction which was needed when the fungi were grown on the solid perlite phase. Under liquid culture conditions, the samples were more homogenous and the isolation of the HA degradation products and enzymes was more straightforward. Degradation of HA was verified by physical and chemical changes measured during the process by two fungi using $^1$H NMR spectroscopy and FTICR mass spectrometry. During the process we detected oxidation, dealkylation and a decrease in the aromatic content of the HA.
08.4. TOWARD THE DEVELOPMENT OF NOVEL SUSTAINABLE BIOPROCESSES FOR THE COLOUR INDUSTRIES


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The traditional colour industry was an important activity in Europe until the end of 20th century. It suffers now displacement to the developing world due to increasing production related environmental costs as well as high labour costs in Europe. Azoïc dyes are the largest group of dyes, both in terms of tonnage production as well as the number of different structures. Unfortunately, a survey of oral acute toxicity of 4461 dyes as measured by the 50% lethal dose has revealed that azo and cationic dyes are the most toxic, and there is ample evidence of the mutagenicity of certain dyes, especially azo dyes and amino-substituted dyes such as 4-phenylazoanilin. In response to these problems, the European Communities have adopted a new directive in 2002 (DIRECTIVE 2002/61/CE, 19th July 2002) limiting the use of azoïc dyes for clothing textile, leather articles, games containing textiles or leather, yarns…. Moreover, the Scientific Committee on Cosmetic and Non-Food Products adopted, on 27th February 2002, an “Assessment Strategies for Hair Dyes” (SCCNFP/0553/02) as there is an epidemiological evidence to indicate that the regular and long term use of hair dyes in women can be associated with the development of bladder cancer. In this case the risk of using some azoïc dyes has been pointed out and highlighting that their use will be limited. Additional problems are that the chemical synthesis pathways, as well as the dyeing of fibres are non worker-friendly processes, as during dyeing processes, approximately 10 % to 40 % of the dyes are not consumed on the substrate to which they are applied, and find their ways into wastewaters. They are flushed into the environment and constitute a non-negligible risk to living organisms. When raw materials are imported from India and China, their production (under conditions which are unacceptable in Europe) increases the world-wide sum of global pollution. New European legislations and directives (White paper, directive limiting the use of azoïc dyes; water framework directive; REACH…) though very useful for health and environment reinforce the industrial difficulties. As an answer to these problems (economics, lack of innovation, toxicity to human, non environmentally-friendly, non worker-friendly processes), this presentation describes a biotechnological approach covering three parallel objectives:

- To develop **new bioremediation technology** to detoxify coloured wastewaters.
- To develop **new safe enzyme-assisted processes** for the production of existing dyes.
- To create **new molecules of dyes which are less toxic** and synthesised biotechnologically for high added value markets.

Identification of the bottlenecks of the colour industries were analysed, in order to determine the targets and precursors of the research. Collection and screening of 300 strains for wastewater treatment, and 280 strains for dye synthesis led to the selection of 15 strains studied further for the conditions for microbial production of selected enzymes. Effective elicitors, production of novel low cost renewable fermentation media and downstream processes were studied and efficient systems for the production of enzymes with a scale-up potential were developed. With the aim to obtain novel catalytic properties, enhanced stability and higher activity, molecular and genetic tools led to the obtaining of 9 recombinant enzymes, in 5 hosts systems. Evolution engineering and hybrid enzymes were studied. Whole cell process, using free or immobilized mycelium was studied on 11 strains, including bacteria, aquatic fungi and white rot fungi in order to compare their efficiency to treat model dyes, model wastewaters and/or effluents, with the efficiency of enzymatic process using free or immobilized laccase, manganese peroxidase and versatile peroxidase. Mechanism of decolourization were studied and efficiencies of those systems were compared with activated sludge and/or ozonation.

Concerning dye biosynthesis, a screening of precursors led through enzymatic bioconversion to the creation of 500 coloured molecules, amongst which a ranking led to the selection of 10 precursors and 5 enzymes. The 25 more promising dyes were tested for industrial quality (multifibre test, water colour fastness, perspiration fastness, washing fastness…) and toxicity (bacteria, human cells, fish cells, algae, Ames test…), and 10 non mutagenic dyes with proven low toxicity and improved industrial applicability were selected.

As a conclusion, this research led to i) efficient processes to produce laccase at reduced cost, ii) the development of efficient wastewater treatment able to decolourize and detoxify effluents, ii) the development of new toxicity tests to replace animal testing, iv) the development of 10 non toxic and non mutagenic new biodyes.
O8.5: DECONTAMINATION BY OXIDOREDUCTASES – EXAMPLE OF AN INDUSTRIAL APPLICATION

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A brief introduction into selected industrial oxidoreductase applications will be given based on the product portfolio of Novozymes. The continued effort to identify and develop new areas for the application of these enzymes will be illustrated by their use in the field of decontamination. The chemistry catalysed by oxidoreductases has been explored in detail and results to demonstrate their successful application as antimicrobials will be presented.
O8.6. Oxidation of tert-butyl alcohol in Aquincola tertiaricarbonis L108 is catalyzed by a phthalate dioxygenase-like enzyme system

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In the microbial degradation of methyl tert-butylether (MTBE), a relevant environmental pollutant (additive in super fuels), tert-butyl alcohol (TBA) is an important intermediate. Metabolism of TBA is assumed to be initiated by a monooxygenase but up to now respective enzymes with that specific activity were scarcely found. In order to depict more enzymes of the degradation pathway we compared proteome patterns in one- and two-dimensional gels of strain Aquincola tertiaricarbonis L108 which were grown either on lactate, hydroxyisobutyrate (2-HIBA) or TBA. Degradation of TBA is inducible. A protein of about 55 kDa was detected after growth on TBA and was assigned by mass spectrometric analysis to phthalate dioxygenase. Sequence analysis of PCR products obtained with primers derived from the amino acid sequences of peptides supported the assignment to the hydroxylase subunit of a phthalate dioxygenase-like protein by covering 96.7% of a corresponding gene from Methylibium petroleiphilum PM1. The conserved amino acid motifs -R-x₁₂-CxHRxxxLxxG-x₉-CxYHR-x₆-G- for the Rieske [2Fe-2S] binding domain and -(D/E)xxxDxxHxxxxH- for the mononuclear iron binding domain were found. Consequently, this enzyme is a novel type of a hydroxylase. A second protein of about 30 kDa was induced after growth on TBA with a lower score and attributed to a putative iron-sulfur oxidoreductase subunit. Conserved motifs -RxYSL-x₂₀₋₂₂-RGGS- for FMN binding and -GGIGxTPxxxM- for NAD binding were detected suggesting that this protein is the small subunit of the two-component oxygenase typically containing FMN/FAD. The role of the enzyme in TBA metabolism which should catalyze the hydroxylation of a methyl group in TBA to result in 2-hydroxy-2-methylpropanediol will be verified by knock-out mutants. In addition, over-expression of the two proteins will be performed in order to study the kinetics of this enzyme.
O8.7. ELECTROCHEMICAL ASPECTS OF THE DYE DEGRADATION BY REDOX ENZYMES

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The phenomenon of bioelectrocatalysis, i.e. when an enzyme catalyses electron transfer reactions between a species in solution and an electrode, is of significant importance from both practical and fundamental points of view, e.g. for the understanding of biological electron transfer reactions and for constructing new biosensor concepts.

Azo dyes, the largest chemical class of dyes with the greatest variety of colors, have been used extensively in the latest years for the textile dyeing and paper printing. The release of azo dyes into the environment is a concern due to coloration of natural waters and due to the toxicity, mutagenicity and carcinogenicity of the dyes and their biotransformation products.

The elimination of colored effluents in wastewater treatment systems is mainly based on expensive physical or chemical procedures such as adsorption, concentration, chemical transformation and incineration¹.

Several processes for enzymatic decolourization of textile dyes have been suggested.

The extracellular ligninolytic enzyme system of white-rot fungi can degrade a wide variety of recalcitrant compounds, such as xenobiotics, lignin, and various types of dyes². The major enzymes associated with the lignin-degrading ability of white-rot fungi are lignin peroxidase (EC 1.11.1.14)³, manganese peroxidase (EC 1.11.1.13)⁴ and laccase (1.10.3.2)⁵.

The ability of laccases and peroxidase to degrade an azo dye was studied.

POSTER
PRESENTATIONS
P1. STRUCTURE-FUNCTION OF OXIDOREDUCTASES

P2. CHARACTERISTICS OF LACCASES

P3. MISCELLANEOUS OXIDOREDUCTASES

P4. SCREENING, EVOLUTION AND PRODUCTION OF LIGNOLYTIC ENZYMES (1)

P5. SCREENING, EVOLUTION AND PRODUCTION OF LIGNOLYTIC ENZYMES (2)

P6. APPLICATIONS: FINE CHEMICALS

P7. SPECIAL PRODUCTS

P8. BIOPROCESSES
We are studying a ring-fission dioxygenase from the bacterium *Pseudaminobacter salicylatoxidans* which cleaves in contrast to all other known ring-fission dioxygenases the aromatic ring systems of salicylate, various substituted salicylates and also 1-hydroxy-2-naphthoate. The gene encoding the enzyme has been cloned and sequenced and it was found that the “salicylate 1,2-dioxygenase” is clearly homologous to gentisate 1,2-dioxygenases from other bacterial sources. This is also reflected by the observation that gentisate (and also 1-hydroxy-2-naphthoate) are converted by the enzyme with higher specific activities than salicylate (1). We are currently attempting to analyse the molecular basis for the unique ability of the dioxygenase to oxidatively cleave a wide range of monohydroxylated aromatics. Therefore, the enzyme has been crystallized (2) and the structure determined. This demonstrated that the salicylate 1,2-dioxygenase belongs to the cupin superfamily and allowed the identification of amino acids involved in the binding of the catalytically active Fe(II)-ions and amino acid residues which are involved in substrate binding. Thus several targets were identified which distinguished the salicylate 1,2-dioxygenase from true gentisate 1,2-dioxygenases. We have started to site-specifically mutate the relevant amino acids and identified several mutants that showed significantly changed conversion rates for salicylate, gentisate, and 1-hydroxy-2-naphthoate.

References
P1.2. SUBUNIT ROLE AND INTERACTION IN HETERODIMERIC LACCASES FROM Pleurotus ostreatus

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The subfamily of POXA3 laccase isoenzymes produced by the fungus Pleurotus ostreatus was characterized as an example of complexity and heterogeneity of fungal isoenzymatic patterns. Two isoenzymes, POXA3a and POXA3b, were previously purified, exhibiting an unusual heterodimeric structure, composed of a large (67 kDa) and a small (18 or 16 kDa) subunit\(^1\). A unique gene, homologous to other fungal laccases, encodes the large subunit of both POXA3a and POXA3b but alternative splicing produces two variants -differing for an insertion of four amino acids- for each isoenzyme. Two genes encoding POXA3a and POXA3b small subunits were identified, and the corresponding amino acid sequences show only two amino acid substitutions. The small subunits share 24% identity with an unknown protein from Coprinopsis cinereus, a white-rot fungus whose genome has been recently sequenced. 18 and 16 kDa subunits of both POXA3a and POXA3b differ for N-glycosylation at Asn150 of the 16 kDa subunit\(^2\). Therefore, the unique difference between POXA3a and POXA3b enzymes seems to be related to the occurrence of two amino acid substitutions in their small subunits.

Heterologous expression of the large POXA3 subunit alone and co-expression of both large and small subunits in Kluyveromyces lactis was performed\(^3\). Data indicate a significant increase of laccase activity related to the presence of the small subunit. Nevertheless, the heterologously expressed POXA3 large subunit is unable to be activated by the addition of the small subunit, suggesting that only a co-folding process can produce the fully active enzyme. Moreover, clones expressing POXA3 complex shows a significant higher stability of laccase activity with respect to those expressing large subunit alone, allowing to hypothesize a role of small subunit also in the stabilization of POXA3.

To investigate on the unusual structure of POXA3, we intended to reversibly denature the protein, separated the two subunits and refolded them to compare the characteristics of the complex with those of the large subunit alone. Several attempts were performed to this aim, using different denaturant agents in various conditions. Unfortunately, the complexes dissociated only in conditions that did not allow protein refolding. Therefore POXA3 large and small subunits, although not covalently bound, interact very strongly. However a different behaviour of POXA3a and POXA3b in denaturing conditions was observed. Experiments at different pHs suggest a stronger interaction between the two POXA3b subunits, in agreement with the previous data obtained using urea as denaturing agent\(^1\). As a fact at least two different denatured intermediates can be singled out for both isoenzymes: i) a partially denatured, not active form, in which subunits are still bound together, whose activity can be recovered by renaturation, ii) an irreversibly unfolded form in which separation of the subunits had occurred.

The finding that separation of subunits implies the complete denaturation of the proteins confirms the hypothesis of the role of POXA3 small subunit in complex stabilization.

References
Laccases belong to the enzyme group termed multicopper oxidases. They catalyze the coupling between the one-electron oxidation of four molecules of a broad range of substrates, such as polyphenols, methoxysubstituted phenols and aromatic amines, with the four-electron reduction of dioxygen to water. The catalytic process is made possible by the presence of four copper ions, organized in two metal sites, classified according to their spectroscopic properties: a T1 site where the electrons from the reducing substrates are accepted and a T2/T3 trinuclear cluster, composed by a T2 copper ion and a coupled binuclear T3 copper pair, where the reduction of dioxygen to water occurs.

Although this enzymes have many biotechnological uses, among these applications in pulp and paper industry and in removal of environmental pollutants from soil and water, the catalytic mechanism and in particular the steps involving the reduction of dioxygen in the trinuclear copper cluster need further clarifications.

The crystal structure of a Blue Laccase from Steccherinum ochraceum has been recently solved at 1.9 Å. The overall structural features are typical of this class of enzymes, however distances inside the trinuclear cluster were indicative of a reduction of the metal centers induced by the electrons liberated during the x-ray data collection as it has already been reported for other laccase crystal structures. UV-Vis spectra measured during the x-ray exposure at the ID142 beamline at ESFR (Grenoble, France) support these evidences. The analysis of the structural data collected on the timescale of this reduction process has been employed to understand the catalytic mechanism of redox enzymes since it permits to drive redox reactions in crystals and capture transient intermediates of the catalytic process.

In order to isolate the reduction steps in the trinuclear copper site, we collected at the X12 beamline at DESY (Hamburg, Germany) several data sets at different doses. The model refinement of the structures obtained is still under progress; however we find different molecular arrangements of dioxygen at the trinuclear copper cluster and different redox states for the T2/T3 copper ions, going from dioxygen binding (low dose) to oxygen splitting to a complete reduction of the copper ions (high dose).

References
P1.4. PROXIMAL MUTATIONS AT THE YYPE 1 COPPER SITE OF COTA-LACCASE: CHARACTERIZATION OF I494A AND L386A MUTANTS

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In this work site-directed mutagenesis has been used to replace the residues Ile494 and the Leu386 in the CotA laccase by alanine in order to change the hydrophobic environment of the T1 Cu site, namely the hydrophobic patch surrounding its His497 ligand. This latter residue is exposed to the solvent and is presumably involved in the electron transfer pathway from reduced substrates to T1 Cu. The mutation L386A appears to cause only very subtle alterations in the properties of the enzyme indicating minimal changes in the structure of the Cu centres. However, the replacement of isoleucine 494 by an alanine leads to significant changes in the enzyme. Thus, the major visible absorption band is up-shifted by 16 nm to 625 nm and exhibits an increased intensity, whilst the intensity of the shoulder at ca. 330 nm is decreased by a factor of two. Simulation of the EPR spectrum of the I494A mutant reveals differences in the T1 as well as in the T2 centre reflecting modifications of the geometry of these centres. The intensity weighted frequencies $<\nu_{\text{Cu-S}}>$, calculated from Resonance Raman spectra are 410 cm$^{-1}$ for the Wt enzyme and 396 cm$^{-1}$ for the I494A mutant, indicating an increase of the Cu-S bond length in the T1 Cu site of the mutant. A major alteration of the I494A structure near the type 1 Cu site has been further confirmed by X-ray crystallography, which shows the presence of a fifth ligand, a solvent molecule, at the type 1 Cu site leading to an approximate trigonal bipyramidal geometry. The redox potential of the L386A and I494A are shifted downwards by about 60 and 100 mV, respectively. These changes correlate well with decreased catalytic efficiency of both mutants compared with the Wt. Therefore, these results and our previous data show that changes in amino acid residues in direct contact to the metal centre (including ligands) significantly affect the properties of T1 Cu sites of laccases and suggest that modulation of redox potential without compromising the overall reactivity may be performed through changes in residues away from this immediate contact shell.

Acknowledgements
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Reference
Laccases constitute the simplest representative members of the multicopper oxidase family, being widely distributed in nature. In a similar manner to other members of this family, these enzymes contain at least two different copper centres; a mononuclear type I blue copper centre and a trinuclear cluster comprising two type 3 and one type 2 copper atoms. Functionally, these enzymes are responsible for coupling substrate oxidation with the reduction of dioxygen to water. The substrate oxidation occurs at the mononuclear centre, shuttling electrons to the trinuclear centre where dioxygen binding and reduction occurs along with the production of water molecules.

Using CotA laccase as a model system, we have proposed a putative mechanism of oxygen reduction for this type of enzyme (Bento et al. 2006). In the present work we have determined the three dimensional structure of three different mutants of glutamate 498, in an attempt to further understand several structural and functional aspects of the enzyme mechanism. Being located within hydrogen bonding distance of a water molecule in the entrance channel and directly interacting with the dioxygen moiety that binds between the two type 3 copper atoms, E498 plays an important role in the protonation events occurring during the catalytic mechanism. In addition, this study has demonstrated the relevance of this residue to the stabilisation of the dioxygen reduction site as a whole.
P1.6. LICHEN LACCASES OF SOLORINA CROCEA AND PELTIGERA APHTHOSA

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Laccase activity in water extracts from the thalli of seven lichen species of the genus *Peltigera* was found. Two lichens *Solorina crocea* and *Peltigera aphthosa* had the largest activity. ‘Large’ and ‘small’ fractions of laccase were found in the thalli of lichens *S. crocea* and *P. aphthosa*. In both lichens, ‘large’, possibly dimeric, laccases were determined as 175 and 165 kDa (based on the gel filtration data), and ‘small’ ones were of 76 and 97 kDa (according to sodium dodecyl sulfate polyacrylamide gel electrophoresis data). The ‘large’ laccases were purified to electrophoretic homogeneity. By the substrate specificity, pH optima, and thermostability, they were typical laccases. Laccase from both lichens catalyzed oxidation of different phenols, p-phenylenediamine, ABTS, syringaldazine, but did not catalyze the oxidation of tyrosine and veratryl alcohol. The pH optimum for laccase of *S. crocea* determined with ABTS and 2,6-dimethoxyphenol was 4.2 for both substrates. Laccase from *P. aphthosa* had a more acidic pH optima: 3.6 for ABTS and below 2.7 for 2,6-dimethoxyphenol. Laccase of *P. aphthosa* was more thermostable. The enzyme preserved 85% of its activity during a 1-hour incubation at 35 °C, whereas during the same period, the activity of laccase of *S. crocea* declined to 50%. The half-time inactivation at 50 °C was a few minutes for *S. crocea* and 25 min for *P. aphthosa*. Nevertheless, both *S. crocea* and *P. aphthosa* enzymes had a low thermal stability at 60 °C. Treatment of *S. crocea* and *P. aphthosa* laccases by 1.0mM NaN₃ or 10mM 1,10-phenanthroline resulted in total loss of the enzyme activity. During oxidation of 2,6-dimethoxyphenol by laccases of *S. crocea* or *P. aphthosa*, oxygen consumption was detected. Subsequent addition of superoxide dismutase and catalase to the reaction mixture did not affect the oxidation rate. This indicated the absence of superoxide radical or hydrogen peroxide formation during the reaction, and suggests that oxygen was reduced to water. The fractions of ‘small’ laccases of 45 kDa from *S. crocea* and 55 kDa from *P. aphthosa* were consisted of two enzymes.
P2.1. LACCASE-MEDIATED DIMERIZATION OF THE FLAVONOLIGNAN Silybin: USE OF OXIDASES FOR THE STUDY OF ANTIOXIDANT MECHANISMS

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Flavonolignan silybin present in the seeds of the milk thistle (Silybum marianum) is widely used in human therapy of liver dysfunctions and as a hepatoprotectant thanks to its dual function: it acts as a highly effective radical scavenger (antilipoperoxidant) and also as an antioxidant. Molecular mechanisms of antiradical action of silybin and even functional groups responsible for this activity are not well known so far. During in vitro reaction with stable radicals (e.g., DPPH) or with laccase (Trametes pubescens), silybin forms complex mixtures of oligomeric and polymeric products whose structural analysis is virtually impossible. Methylation of 7-OH in silybin yields C-O (5) and C-C (4) dimers in the ratio of ca 1: 2.5 under laccase-mediated oxidation. Using this approach, the OH groups responsible for antiradical activity of silybin (20-OH) were determined and the molecular mechanism of the E-ring antiradical activity was explained.

This is the first report on the successful preparation and structure identification of silybin dimers. We confirmed that the phenolic 20-OH is the most important silybin moiety for its radical-scavenging activity. Moreover, based on the structures of respective dimers, we were the first to determine the mechanism of their formation and to explain the mechanism of silybin ring E antioxidant (antiradical) action at the molecular level.

Acknowledgements
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P2.2. A NOVEL LACCASE FROM AN INDONESIAN WHITE-ROT FUNGUS

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Oxidoreductases, especially laccases and peroxidases, are suitable enzymes for biobleaching processes due to their high stability and the fact that they degrade many diverse phenolic compounds.

After screening a large number of white-rot fungi for their ability to degrade different industrial textile dyes, a very interesting species was found. It was identified as a member of the Agaricales; its ribosomal ITS sequence showed closest match with *Crinipellis rorert*.1

The enzyme responsible for the bleaching activity was verified as a laccase. It could be purified with two different chromatographic steps: hydrophobic interaction chromatography followed by ion exchange chromatography. The laccase was purified to homogeneity and characterised according to its stability in diverse buffer systems, at various salt concentrations and pH values. The pH and temperature optima as well as $K_M$ values for the conversion of different substrates, the partial N-terminal amino acid sequence and the molecular mass were determined. The isolated laccase is able to degrade a number of various textile dyes such as Remazol Brilliant Blue BB, Cibacron Blue P-3R, Cibacron Brown P-6R and Procion Blue H-EXL.

The purified laccase was immobilised on different polymeric particles. The results were compared with the immobilisation of the commercially available laccase from *Trametes versicolor*. The immobilised enzyme can be used for the biobleaching of textile dyes.

Reference
1. Identification Service: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands, August 2007
P2.3. LACCASES FROM THE WHITE ROT FUNGUS *PHLEBIA RADIATA*

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The white rot fungus *Phlebia radiata* 79 (ATCC 64658) is an efficient lignin-degrader which produces a versatile set of extracellular lignin-modifying enzymes (LMEs) including manganese peroxidases (MnPs), lignin peroxidases (LiPs), and laccases. From the wood-containing cultures of *P. radiata*, we identified a new laccase-encoding gene, *lac2*, which shows the highest amino acid sequence identity with *Trametes* laccases (66%). Phylogenetic analysis indicates differential evolution for the two *P. radiata* laccases, proposing that the Lac1 and Lac2 proteins are encoded by paralogous genes. The 3-D protein homology models illustrate detailed differences between the two laccases within the topology and content of the amino acid residues surrounding the Cu-I and aromatic substrate binding site. RT-PCR revealed simultaneous expression of all the known *P. radiata* LME-encoding genes on solid-state wood cultures. This gives support to the significance of expression of multiple laccase and lignin-modifying peroxidase-encoding genes as a part of the lignin-degrading machinery of the fungus. Immunoblotting with the *P. radiata* Lac1 antiserum suggested that the Lac2 protein could be associated with the fungal cell wall or hyphal extracellular material. In liquid cultures of *P. radiata* containing either complex (peptone and yeast extract) or defined (asparagine and ammonium nitrate) nitrogen sources, extracellular laccase activity was highly affected by induction with copper. The highest laccase activity in the complex nitrogen source cultures was achieved after induction with 1.5 mM CuSO₄.
P2.4. INTRASPECIFIC DIFFERENCES IN LACCASE PRODUCTION WITHIN
Pleurotus ostreatus SPECIES AT TRANSCRIPTIONAL AND TRASLATIONAL LEVEL

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Pleurotus ostreatus is one of the most studied fungi for laccase production. Several works have investigated this fungal species for oxyclative enzymes production, namely laccases, at different levels (gene, transcript and protein). However, the different methods employed and the difficulty to compare the data obtained on different strains make the intraspecific variability still unclear. To the best of our knowledge, up to now, only one strain, used in this work as a control, has been deeply characterized for laccase production at the DOCB of the Naples University Federico II.

The aim of this work is focused on the comparison of 7 strains of P. ostreatus, isolated from different geographical regions and natural growing substrates, for laccase production at transcriptional and translational level.

All the strains were grown in liquid culture on a medium with a carbon/nitrogen ratio of 10:1 and glucose and yeast extract as carbon and nitrogen source, respectively (GHY) at 28 °C in the dark and monitored daily for 18 days for the following parameters: biomass production, total secreted proteins concentration, laccase and protease activity. Moreover we screened the 7 strains for the presence/absence of known laccase-coding genes by means of genomic DNA amplification using specific primers. All the strains displayed the same array of laccase-coding-genes.

For all the strains biomass production reached approximately the same maximum value (7-9 g/l) within 18 days and the same was true for the total protein concentration (0.015 - 0.02 g/l), although the strains displayed different growth kinetic. Differences in laccase activity production have been recorded among the different strains: laccase activity values were always significantly different and some strains displayed two peaks of production while others only one peak. The strains often displayed also significant differences in the protease activity production.

In conclusion reported data indicate that the 7 tested strains of P. ostreatus show remarkable differences in their metabolism both at the level of growth rate and at that of their ability in secreting proteins, including the assayed enzymes. Since all the analysed strains have the same genomic organization, as regard as laccase-coding genes, these different physiological behaviours should be mainly induced at a transcriptomic and/or at a proteomic level. These results underline the need to investigate the mechanisms which regulate the differential expression of laccase-coding genes suggesting us to analyse the structure of the transcription-control sequences in the promoter regions. Moreover, we are performing a proteomic analysis with a quantification of production and secretion of each laccase isoenzyme of the different strains growing in liquid and solid-state conditions in presence/absence of inducers (copper plus ferulic acid and barley husk).
P2.5. *Cerrena unicolor* GROWTH AND LACCASE BIOSYNTHESIS IN A SUBMERGED CULTURE

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The production of fungal laccase is influenced by many typical culturing parameters, such as medium composition, carbon and nitrogen concentration, temperature, pH and aeration ratio. Especially in submerged culture the oxidation conditions seems to be a crucial parameter. The white-rot basidiomycete *Cerrena unicolor* is known as an extensive producer of laccase under non-induced conditions. The extracellular laccase from this source was purified, immobilised and its several application in biotechnology were also studies. However, the investigations concerning biosynthesis of laccase as well as the kinetics of the fungal growth are still limited in literature.

The aim of the present work was to describe the growth kinetics of white-rot fungus *Cerrena unicolor* and the synthesis of its secondary metabolite laccase in a 15 l stirred bioreactor (submerged culture). Glucose was used as a carbon and L-asparagine as the nitrogen source, the aeration ratio was 0.5 slpm and paddle stirrer speed of 100 rpm.

Interestingly, laccase activity appeared at 60 h before glucose and L-asparagine were used up from the medium. This phenomena was caused by a oxygen limitation conditions (dissolved oxygen concentration in the broth was 2%) which poses a stress for the organism, disturb the fungal metabolism and occurred advantageous to the enzyme formation. The production of the enzyme was not only non-growth associated but also due to biomass decay under carbon starvation conditions. The amount of biomass in the system was strictly connected with both L-asparagine and glucose concentration in the medium. The morphological form of this biomass was completely different compare with the cultures in shaken-flasks.
P2.6. **NOVEL LACCASES FROM HIGH THROUGHPUT SCREENING OF LOW EXPLORED FAMILIES OF POLYPORALES**


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The Polyporales is the most representative order of saprophytic homobasidiomycetes causing wood decay. These filamentous fungi were related for their high lignocellulolytic potential through the production of oxidoreductases, i.e. laccases. The increasing industrial interest for laccases led us to screen 33 strains belonging to 15 families of Polyporales from the CIRM-CF collection (International Centre of Microbial Resources dedicated to Filamentous Fungi, INRA, Marseilles, France).

A high-throughput method was developed to screen 10-ml fungal cultures carried out in 16-well baffled deep wells (designed by Aventis, France) incubated at 30°C with a shaking rate of 120 rpm. Laccase assay was miniaturized and adjusted for 96-well plates and automated liquid handling robot system. The test was validated with purified laccase from *Pycnoporus cinnabarinus* available in the laboratory. Laccase activity was automatically measured each day and the results were expressed in nkat/ml. The high laccase-producers were confirmed using 100-ml fungal cultures grown into 250-ml baffled flasks.

Laccase activities ranged from 0.1 nkat/ml to 76 nkat/ml, with the highest activities shown in the genera *Amauroderma, Grammothele, Aurantiporus, Abortiporus, and Funalia*. Studies are in progress in order to purify and characterize the new laccases from the highest producers of these low explored families of Polyporales.
P2.7: INVESTIGATION ON THE ROLE OF 3-HAA AND ANTIBIOTICS ON THE GROWTH AND LACCASE PRODUCTION OF SOME Pycnoporus SPECIES


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Some filamentous fungi, when cultured in presence of other strains (incompatible, belonging to another species or genus) may lead to the formation of a “barrier” with reduced growth in the confrontation zone. The apparition of this barrier is linked to the presence of cinnabarinic acid (CA). In vitro studies showed that CA was formed through laccase coupling of 3-hydroxyanthranilic acid (3-HAA) (Eggert et al., 1995). It can be hypothesized that laccase may be produced as a defence mechanism in the self-non self recognition. In addition, it has been recently demonstrated that co-culturing of different species can result in an increase of laccase production in this confrontation zone (Chi et al., 2007), this process being species specific. Could this phenomenon be linked to Quorum Sensing? Quorum Sensing (QS) systems regulate the population density and some metabolic functions through cell communication involving small diffusing autoinducer molecules (QS molecules) that are released into the environment.

Based on these facts, the effect of 3-HAA on growth and laccase activity of different strains of P. cinnabarinus, P. sanguineus, P. coccineus and P. puniceus was tested. It has recently been suggested that the principal activity of antibiotic products may be linked to QS and Horowitz et al. (1970) have shown that tyrosinase and laccase are induced in vegetatively growing cultures of Neurospora crassa by the addition of compounds such as cycloheximide. The effect of cycloheximide was also assayed. Results showed that 3-HAA can have an up or down regulating effect on the laccase activity, depending on the strain studied. The addition of 3-HAA in the cultures of P. sanguineus 41582 induces both the growth and the production of laccase. P. cinnabarinus 39533, a strain previously demonstrated as being deficient in the pattern of laccase production, presented also a growth increase, however no effect on laccase production was observed. Cycloheximide at concentrations below 7µm was also shown to enhance laccase activity by 50% in P. puniceus 41780. Despite its very slow growth rate, this strain showed a higher laccase activity per unit of biomass ratio than the one observed for P. sanguineus 41582.
P3.1. A NOVEL PEROXIDASE FROM *Cerrena unicolor*

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_Cerrena unicolor_ is a common white-rot fungus, which efficiently decays angiosperm wood. So far laccase as a lignin degrading enzyme has been well characterised from the *Cerrena* genus, including *C. maxima* and *C. unicolor*. Peroxidase activities in this genus has gained little attention, possibly because of rather low manganese peroxidase and "ligninase" activities, as reported by Elisashvili et al. After a preliminary screening we found that *C. unicolor* was able to efficiently decolourise azo dye Reactive black 5, when the fungus was grown in a nutrient sufficient liquid medium containing peptone. On that medium laccase activity was negligible. Other tested media (synthetic low-nitrogen medium, soy medium and solid state oat husk medium) resulted in low or absent peroxidase production. The dye-decolourising peroxidase from peptone medium was purified and partially characterised. The main peroxidase protein eluted as a single peak in anion exchange column. Interestingly, this enzyme appeared to possess a good Rb5-decolourising activity and it also oxidized well veratryl alcohol. In addition, the protein showed ability for Mn(II) oxidation. The molecular weight is of the enzyme is 43kDa and its pi is 3.8. Molecular characterisation of the novel peroxidase will be presented. The characteristics of this *Cerrena* peroxidase would indicate that it could represent a versatile-type peroxidase, so far described only in genera *Pleurotus* and *Bjerkandera*. Further analyses will be described in the presentation.

References

P3.2. EXPRESSION AND CHARACTERISATION OF AN ASPERGILLUS ORYZAE CATECHOL OXIDASE NOT CARRYING A C-TERMINALLY PROCESSED DOMAIN

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Tyrosinases and catechol oxidases are metalloenzymes containing active sites homologous to the oxygen-carrier proteins called haemocyanin. Tyrosinases are metalloenzymes showing monophenolase and diphenolase activity; enzymes possessing only the diphenolase activity are classified as catechol oxidase.

In order to identify novel tyrosinases with interesting properties, a homology search against the published fungal genome sequences was carried out. The analysed tyrosinase coding genes can be divided in two groups with different lengths (350-400 residues and 400-600) and differing in the presence or absence of the C-terminal domain that has been suggested to be involved in the enzyme activation.

An uncharacterised tyrosinase–like sequence from *A. oryzae* (AoTyr4, 408 aa) shows similarity with the recently identified extracellular tyrosinase 2 in *T. reesei* (23% of identity). AoTyr4 has the highly conserved histidine pattern for the copper binding sites and an N-terminal signal sequence for the secretory pathway but lacks the C-terminal domain. AoTyr4 was expressed in *T. reesei* under the strong *cbh1* promoter and the product was shown to be secreted in good quantities. The *T. reesei* transformants were screened on activity plates containing L-tyrosine where some activity was detected. However, the purified AoTyr4 showed negligible activity on tyrosine but was active on pyrocatechol, suggesting, the enzyme should be classified as a catechol oxidase.

The biochemical characterisation of the AoTyr4 enzyme will be discussed.
P3.3. Estimating the Redox Potential of Enzymes Using Marcus Theory

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In theory it is possible to estimate the redox potential certain peroxidases and phenol oxidases, from the redox data of their substrates and the kinetics of enzymatic conversation. Dye decolorization kinetics is widely used to characterize certain redox enzymes, and from this data redox potential can be estimated. This is a valuable tool for geneticist and microbiologists to access the redox ability in selecting a redox enzyme during direct evolution or any other kind of screening experiments. The advantages and limitation of the application of Marcus theory is discussed in detail.
Lactoperoxidase (LPO), belonging to the mammalian peroxidase superfamily, is a heme-containing oxidoreductase which binds ligands and/or undergoes a series of redox reactions. Depending on substrate availability, this enzyme follows the halogenation cycle and/or the peroxidase cycle and/or acts as poor (pseudo-)catalases. The mechanism of halide oxidation starts by reaction of the ferric enzyme with H₂O₂ to form compound I, which contains two oxidizing equivalents more than the resting enzyme; halides (X⁻) or thiocyanate reduce compound I directly to native enzyme by a two-electron process and hypohalous acid are formed (HOX). Alternatively, substrates (AH₂) reduce compound I to native enzyme via compound II by two successive one-electron reductions releasing free radicals (°AH).

Although a broad literature is available on LPO catalytic intermediates, no EPR spectrum of compound I species was shown and some characteristics of these compounds are still controversial. We show EPR spectra of LPO Compound I recorded in the absence of substrates and after the addition of hydrogen peroxide and rapid freezing. Using fast freeze-quench apparatus, the species formed during the first milliseconds of the catalytic cycle and its behaviour during time is shown.

Multifrequency (9.4 and 94 GHz) EPR analysis has been used to obtain information concerning the nature of Compound I.

Reaction scheme of lactoperoxidase catalytic cycles.
P3.5: SEASONAL VARIABILITY AND SPATIAL DISTRIBUTION OF LIGNINOLYTIC ENZYMES IN OAK (Quercus petraea) FOREST SOIL

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Extracellular lignocellulose-degrading enzymes are responsible for the transformation of organic mass in hardwood forest soils\textsuperscript{1,2,3}. The aims of this study were to characterize activities of ligninolytic enzymes in the upper horizon of oak (Quercus petraea) forest soil with respect to the seasonal variability and spatial distribution. The soil profile showed a gradient of pH, organic carbon and humic compounds content, microbial biomass and enzyme activities, all decreasing with soil depth. Ligninolytic enzymes showed preferential localization in the L horizon and in the upper part of the O horizon. Activity of enzymes showed a patchy pattern of distribution with “hotspots” of different size in the course of the year. Activities of laccase and Mn-peroxidase in litter (L) and humic (O) horizons of soil were independent on fungal biomass (measured as quantity of ergosterol). The differences in enzyme activities were accompanied with the differences in the microbial community composition where the relative amount of fungal biomass decreased and actinomycete biomass increased with soil depth. The results also showed that the vertical gradients occur at a small scale: the upper and lower parts of the O horizon were significantly different with respect to most enzyme activities, microbial biomass content and community composition. Seasonality was identified as an important factor affecting not the production but the activity of ligninolytic enzymes. While highest laccase activities were found in the summer period, highest Mn-peroxidase activities were regularly recorded during the Autumn season, a period the highest fungal biomass content in the soil of study.

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References
P3.6. INHIBITION OF A FUNGAL TYROSINASE FROM Trichoderma reesei BY COPPER CHELATORS

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Tyrosinase (EC 1.14.18.1) is classified as a type 3 copper protein. It catalyses the ortho-hydroxylation of monophenols and the subsequent oxidation of the diphenolic products to the resulting quinone. In nature, tyrosinases are widely distributed in microorganisms, plants and animals. Tyrosinase plays a key role in the melanin biosynthesis and in the defence systems. Tyrosinase activity is involved in various dermatological disorders in mammals, while in plants tyrosinase is responsible for the undesired enzymatic browning reactions of fruit and vegetables. Understanding the inhibition mechanisms and discovering novel inhibitors for tyrosinase activity to prevent the synthesis of melanin is thus important in particular in the food, pharmaceutical and cosmetic industry.

Inhibition of an extracellular tyrosinase from Trichoderma reesei (TrTYR2) was studied and compared to the commercial tyrosinase from Agaricus bisporus (AbT). Four potential inhibitors acting as copper chelators of tyrosinase (azide, cyanide, EDTA and kojic acid), were tested on the catecholase activity by following product formation with spectrophotometric and polarographic assays. When 15 mM L-dopa was used as substrate, kojic acid and cyanide were the strongest inhibitors tested, while EDTA did not cause severe inhibition on the L-dopa oxidation. By an addition of 10 mM azide to the reaction mixture, about 80% of TrTYR2 activity was inhibited. Interestingly, in the presence of azide, TrTYR2 was capable to consume higher amount of substrate, when compared to the reactions with L-dopa alone. When an UV/visible spectrum of the L-dopa reaction products was followed in the presence of azide, primary products were detected at 475 nm, and secondary products at 420 nm. Increasing the concentration of azide caused a delay in the formation of the secondary products as analyzed at 420 nm. It has been suggested that azide is, in addition to a copper chelator, also a powerful nucleophile. Thus, it may also affect the non enzymatic reactions subsequent to the diphenol oxidation.
P3.7. METHYLcatechol 1,2-DIOXYGENASE FROM 4-METHYLBENZOATE-DEGRADING *Rhodococcus* *opacus* 6a

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Transformation of 4-methylcatechol (4MCat) is of a great interest because the presence of alkyl substituent at C4 atom prevents further degradation of this compound via a classic β-ketoadipate pathway. *Rhodococcus opacus* 6a isolated from petroleum-contaminated soils is able to degrade a wide range of aromatic compounds. *R. opacus* completely degrades 10 mM 4-methylbenzoate (4MB) during 40 hours. 4MCat is a key intermediate of 4MB degradation by *R. opacus* 6a. It’s further conversion proceeds via ortho-cleavage. This step is found to be catalyzed by two catechol 1,2-dioxygenases (C1,2-DO) with different substrate specificity. First enzyme is homodimer with subunit molecular mass ca 33 kDa. It is active with catechol (100%), 3-methylcatechol (3MCat)(109%), and 4MCat (188%). The preferred substrate for this enzyme is catechol, as the $k_{cat}/K_m$ specificity constant for catechol is three times higher than that one for 3-MCat and 4-MCat. Nevertheless, this C1,2-DO is a pattern of enzymes with altered substrate specificity.

Another enzyme, C1,2-DOII, is homodimer, too, with subunit molecular mass ca 28 kDa. It was activite with catechol (100%), 3MCat (120%), 4MCat (233%). The activity with 4-chloro-, 3-chloro-, 3,5-dichloro-, 3-metoxy-, 4-fluorocatechols vary at the range of 18-114% of those with catechol. The highest value of the specificity constant is calculated for 4-MCat – the intermediate of 4MB conversion. According to our knowledge this is only the second report about purification and characterization of methylcatechol 1,2-dioxygenase - new type of dioxygenase [1].

Acknowledments
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References
P3.8. UNDERSTANDING THE RADICAL MECHANISM OF ACTION OF OXIDATIVE ENZYMES ON THE LIGNOCELLULOSIC SUBSTRATE USING QUANTITATIVE $^{31}$P- NMR SPIN TRAPPING

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Quantitative $^{31}$P-NMR spin trapping techniques can be used as effective tools for detection and quantification of many free radical species. Free radical react with nitrooxide phosphorous compound, 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO), to form stable radical adducts, which are suitably detected and accurately quantified using $^{31}$P-NMR in presence of phosphorus containing internal standard. This powerful system was applied to better understand the mechanism of enzymatic oxidation of phenolic and non-phenolic lignin model compounds. This mechanism consists on H-abstraction reaction involving radical species, such as phenoxy radical and ketyl radical, which are very difficult intermediates to be detected and quantified with traditional techniques (i.e. EPR). Initially, the phenoxy radicals were produced via oxidation of different phenols by K$_3$Fe(CN)$_6$ and ketyl radicals were produced via photochemistry reaction of different acetophenones. The $^{31}$P-NMR signal for the radical adducts of phenoxy radical (PhO·) and ketyl radical (PhC-CH$_2$OH) were assigned and found to be 25.2 and 29.3 ppm respectively. Moreover to confirm the presence of the adduct, their mass spectra were detected using GC-MS analyses. Subsequently, the Spin Trap system was applied to the oxidation of phenols and apocinols in presence of Peroxidases and 1-Hydroxybenzotriazole (HBT) as mediator: the 2,4,6-trichlorophenol and 2,4,6-tributylphenol were oxidized and only phenoxy radical adducts were detected; on the contrary during the oxidation of dimethoxyphenethyl alcohol were detected hydroxy, hydroperoxy and ketyl radical. These efforts showed that it is possible to detect radical species using $^{31}$P-NMR spin trapping techniques as a foundation for understanding of the mechanism of radical activity in lignin oxidation.
P3.9. Oxidoreductases secreted by an anamorph of Bjerkandera adusta isolated from a biodeteriorated compact disc

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A fungus isolated from a compact disc found in Belize was described as a Geotrichum-like fungus according to morphological characteristics such as white dry colonies, hyaline hyphae without clamp connections and cylindrical conidia. This strain was capable to colonizing *in vitro* different types of compact discs and degrading their main components (aluminium, dyes and polycarbonate). The fungus secreted an oxidase of type aryl-alcohol oxidase in a basal medium with glucose, peptone and yeast extract (28°C, 150 rpm). Mn²⁺-oxidizing peroxidase activity only was found in the cultures supplemented with MnSO₄, while lignin peroxidase and laccase were not produced under the assayed conditions. This strain was identified as an anamorph of the white-rot fungus *Bjerkandera adusta* based on the analysis of its ITS region (GenBank number: EF441742), its morphological characteristics, and the profile of ligninolytic enzymes.

The oxidoreductases detected have been purified from liquid cultures by two anion-exchange chromatography steps followed by one size-exclusion chromatography step. The oxidase is a monomeric glycoprotein of 76 kDa with a pl of 4.45. It is optimally active at pH 6.0 and 45°C and it is highly stable for 24 h in the pH range 4.0-6.0 at 25°C. The enzyme has a flavin as cofactor bound non-covalently and it shows absorption maxima at 392 and 463 nm. These properties and the N-terminal sequence related this flavoprotein with aryl-alcohol oxidases from basidiomycetes. However, the purified protein exhibits unique catalytic properties, since it oxidizes besides of typical aryl-alcohol oxidases substrates (aromatic and aliphatic polyunsaturated alcohols) the phenolic vanillyl and coniferyl alcohols like vanillyl-alcohol oxidases from ascomycetes, and shows high activity on halogenated aromatic aldehydes. Simultaneously, two peroxidase isoenzymes have been purified and substrate specificity studies are currently being performed. They have identical molecular weight (49 kDa) and N-terminal sequence but different pl (3.8 and 4.2). Analyses of their N-terminal sequences showed the highest Blast scores with manganese peroxidase 1 from *Phlebia* sp. MG60 and two lignin peroxidases from *Trametes versicolor*. Further studies will determine the role of both oxidase and peroxidase activities during the compact disc degradation and its potential for biotechnological applications.

References
P3.10. CATALYTIC ACTIVITY OF VERSATILE PEROXIDASE FROM 
BJERKANDERA FUMOSA IN DIFFERENT pH

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The extracellular ligninolytic system from white rot fungi consists mainly of oxidative enzymes: laccases (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), and described recently versatile peroxidase (VP). VP can both efficiently oxidize Mn(II) to Mn(III) (like MnP) and carry out Mn(II)-independent activity on aromatic substrates (like LiP). Until today, VP was described only in various strains of two fungal species - Pleurotus and Bjerkandera. In the case of Bjerkandera sp. BOS55, versatile peroxidase is manganese-lignin peroxidase hybrid enzyme, which is able to oxidize various phenolic and non-phenolic substrates, such as veratryl alcohol, in the absence of Mn(II) ions. VP from Bjerkandera adusta described by Pogni and coworkers, it is a structural hybrid between LiP and MnP and this hybrid combines the catalytic properties of two above peroxidases, being able to oxidize typical LiP and MnP substrates1-5.

In the frame of this work, the catalytic activity of fungal versatile peroxidase (VP) was tested over a range of different substrates (Mn(II) ions, 2,6-dimethoxyphenol (DMP), and anthraquinone and azo dyes) and different pH values. It was shown that DMP could be oxidized by VP by two strategies, either directly by the enzyme or by diffusible chelated Mn(III) as an oxidizing agent. During the reaction catalysed by VP, ionisation of groups in the enzyme-substrate complex was observed for both the inorganic (Mn(II) ions) and the organic substrate (DMP). In this scheme, both $V_{\text{max}}$ and $K_M$ are pH-dependent. Dye oxidation was characterized by the micromolar range of $K_M$ and it proceeded both in the presence and absence of Mn(II) ions. In the case of Reactive Black 5, differences in the values of $K_M$ and $V_{\text{max}}$ were observed between reactions in the presence and absence of Mn(II) ions where the former inhibited dye oxidation.

References

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P4.1. TYROSINASES FROM NOVEL BASIDIOMYCETES: IMPLICATIONS FOR HETEROGENEITY AMONG SPECIES

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Tyrosinases are copper-containing metalloenzymes that are present in microbes as well as in mammals, invertebrates and plants. They are involved in diverse biological reactions and this enzyme shows potential in several industrial applications. In fungi, the physiological role of tyrosinases is so far only poorly investigated. Typically fungal tyrosinases are cytosolic enzymes, and the enzyme has been characterized from fruiting body extracts from a few basidiomycetes and ascomycetes Neurospora crassa and Hypocrea jecorina (Trichoderma reesei). Expression of tyrosinase in vegetative mycelium has been obtained only in stress conditions. In native conditions no extracellular tyrosinase has been found in fungal culture media. However, in H. jecorina the extracellular form, as an overexpressed form in native host, has been reported, indicating that this enzyme may be secreted out of fungal cells in some conditions. This study was undertaken to screen potential tyrosinase producers from a large number of basidiomycete fungi. We screened 55 species each on three different media. In total of 165 different species-medium pairs few showed cytosolic tyrosinase activity. Highest activities were produced on soy medium. Five potential tyrosinase producing species were further investigated. These species represented genera Pycnoporus, Gymnopilus, Agrocybe, Antrodia and Phanerochaete.

The effect of copper addition to tyrosinase production was tested. Tyrosinase production was clearly elevated in the presence of 1 mM CuSO₄ in four investigated species. Tyrosinases in the crude mycelium extracts in the investigated species were present both as active and as latent forms; however, the amount of latent form of tyrosinase appeared to vary significantly between the species, from 6% to 87%. Pycnoporus tyrosinase activity was also NaCl sensitive, confirming the result by Halaouli et al.¹ In the other species NaCl did not inhibit the activity. Analysis of mycelium extracts by isoelectric focusing indicated presence of several tyrosinase isoforms in all five investigated species. We typically found isoforms with pI 4-5, but also few more neutral and a single alkaline isoform. In H. jecorina the extracellular tyrosinase has a pI of 9.5, as reported by Selinheimo et al². This presentation compares the characteristics of the cytosolic tyrosinase activities of taxonomically distant basidiomycete fungi. Preliminary data indicates also the presence of an extracellular, actively secreted tyrosinase. Data presented indicated a wide range of variability among basidiomycete tyrosinases and highlights the importance of characterization of activities from several different fungal groups in order to find tyrosinases with improved properties for industrial applications.

References
P4.2. INHIBITION OF OXIDATIVE ENZYMES IN CHRONIC ULCERS

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Reactive oxygen species (ROS) include a number of chemically reactive molecules derived from molecular oxygen which act as signalling molecules in the regulation of various cellular processes. An important source of reactive oxidizing species in vivo, especially in chronic inflammation process, such as chronic wounds are the neutrophils. Whilst ROS production by neutrophils provides a host protective role, the persistence of these cells and their proteases appears responsible for the excessive tissue damage and thus contributes to the chronicity of the ulcers.

Myeloperoxidase (MPO) is the main neutrophil enzyme. This enzyme uses hydrogen peroxide to oxidise innumerable substrates either to hypohalous acids or reactive free radicals. Its ability to oxidise chloride to hypochlorous acid (HClO) is unique among mammalian enzymes and is considered to be the dominant activity of myeloperoxidase in vivo. Although the HClO generated allows the killing of bacteria, this acid also reacts with most biological molecules, including protease inhibitors. The inactivation of protease inhibitors has a direct effect in the protease-antiprotease balance, promoting the proteolytic damage of healthy tissues.

One strategy for chronic wound healing and prevention is the development of bio-polymer coatings incorporating myeloperoxidase inhibitors. In this work the biopolymer chitosan is modified with natural polyphenols (Hamamelis virginiana L) and its inhibitory effect on the HClO production by myeloperoxidase is evaluated by means of electrochemical techniques.
P4.3. THE MEDICINAL MUSHROOM *GANODERMA LUSIDUM* 447, AS A PROMISING ORGANISM FOR LACCASE PRODUCTION

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Our study indicates that fungal enzyme activity was distinctly affected by the growth substrate in the medium. According to the data received, the substitution of defined medium with glucose with complex substrates (ethanol production residue) as a carbon source supported very high laccase production 97340 U l⁻¹ by *Ganoderma lucidum* 447.

In this study, Cu was tested to improve enzyme production by *Ganoderma lucidum* 447. The supplementation of medium with 1mM and 3mM Cu increased laccase accumulation by *Ganoderma lucidum* 447 20-50%, respectively. Furthermore, it has been shown that the stimulating effect of copper decreased when the time from the edition of the microelement was increased from the time of inoculation.

At the final step of this study laccase from *Ganoderma lucidum* 447 was isolated from culture liquid and purified. The SDS-PAGE of purified preparation revealed two laccase bands of 43 kDa and 56 kDa. The NCBI BLAST database results shown, laccase band molecular weight of 43 kDa was match to laccase 2 [*Ganoderma* sp. BS-1], and laccase band molecular weight of 56 kDa was match to laccase LCC3-1 [*Polyporus ciliatus*]. The optimum temperature of purified laccase from *G. lucidum* 447 for the oxidation of ABTS appeared to be only 30°C. Like a majority of laccases, it has a pH optimum for ABTS oxidation at 3.0 and oxidizes a range of substrates, including mono-aromatic phenolic substrate (2,6-dimethoxyphenol), a complex phenol (syringaldazine), and the nonphenolic heterocyclic compound ABTS. The investigation of *G. lucidum* 447 laccase catalytic properties showed the lowest $K_m$ values (0.0048 and 0.005mM) for syringaldazine and ABTS, respectively, indicated a higher affinity of the enzyme to these substrates. On the contrary, ferulic acid and vanillin showed the highest $K_m$ values (2.5 and 1.1481 mM), respectively.

The present study, for the first time, evaluated the potential of the medicinal mushroom, *Ganoderma lucidum* 447, to produce laccase. The substitution of glucose by complex substrates enhanced the production of laccase by *G. lucidum* 447. Purification and characterisation of laccase isoenzymes form *G. lucidum* 447.
Desiccation tolerance (DT) occurs widely in the plant kingdom and can be divided to poikolochlorophyllous (PDT) and homoiochlorophyllous (HDT) desiccation-tolerant organisms on the basis of their chlorophyll condition during dehydration. The homoiochlorophyllous desiccation tolerant plants (HDT) retain their chlorophyll content and photosynthetic apparatus during desiccation while poikilochlorophyllous desiccation tolerant (PDT) plants form the other group of DT plants where species lose their chlorophyll during desiccation that must be reformed following remoistening. The HDT strategy is more favourable in habitats where the desiccated periods are short and these short periods of desiccation and rehydration frequently alternate. In contrast to HDT plants, PDT strategy has evolved in habitats where the duration of the desiccated states takes months.

Interestingly there are no reports about the presence of cell wall redox enzymes in DT plants in spite of the fact that it is possible that these enzymes could have further roles in the removal of stress-induced harmful reactive oxygen species (ROS) and quinine radicals supposed by Beckett and Minibayeva. In this study, we tested for the presence of cell wall redox enzymes in 4 desiccation tolerant Xerophyta species: X. spekei, X. scabrida, X. dasylirioides and X. sp. Two main types of enzymes were detected, laccases and tyrosinases, although small amounts of catalase-peroxidase were also found. Identification of laccases was based on ability of Xerophyta extracts to readily metabolize substrates such as 2,2'-azino(bis-3-ethylbenzthiazoline-6-sulfonate) (ABTS) and syringaldazine in the absence of hydrogen peroxide, and sensitivity of the enzymes to cyanide and azide. Further testing showed that Xerophyta species can also readily metabolize substrates such as tyrosine and 3,4 dihydroxyphenylalanine (DOPA), substrates more usually associated with another group of multi-copper oxidases, the tyrosinases. Detergents strongly activated tyrosinase activity. Within the Xerophyta species, the activities of the enzymes were significantly correlated to each other.

Possible roles of these enzymes in Xerophyta species are discussed.

References
In order to improve industrial processes by enzymatic means, the enzymes involved must possess good catalytic efficiency as well as good stability characteristics in various conditions, including elevated temperature regimes and alkalinity. To elicitate a large variety of different oxidoreductases, three liquid media and one solid state medium were selected after preliminary tests for screening. In total, 55 basidiomycete isolates from ecologically different habitats, including white-rot polypores, litter-degrading agarics and corticoid wood degrading fungi, were screened. The assays measured especially laccase, manganese peroxidase and dye-decolorizing oxidase and peroxidase activities. Azure B (AzB) and Reactive Black 5 (Rb5) were used as dyes. The miniaturized oxidoreductase assays were adjusted for 96-well plates and automated liquid handling robot system. The assays were validated with commercially available enzymes.

MnP activities were screened using Mn(II) oxidation method with MnSO₄ as the substrate. Most of the screened media samples contained MnP activity: 159 out of 220 samples (72%) contained MnP activity at least to some extent. This may reflect the quality of the media: they were selected to favour MnP production in these fungi. Especially solid state cultivation induced the highest MnP productions. Most MnPs had optimum pH at 5. Interestingly soy and peptone media induced pH stable MnPs that could retain moderate or good activity at pH 7. Three samples possessed their maximal activity at pH 7. These MnPs were found from the genera Daedaliopsis, Climacocystis and Hyphodontia.

Laccase is a very common enzyme in wood and litter degrading fungi, but in our assay conditions ratio of laccase positive/laccase negative was rather low: from the media 56% of samples (123 investigated media samples / 220 total amount of assayed media samples) were laccase-positive. The most interesting laccases could retain well their activity at pH 7. No laccases were found that could show their pH optimum at pH 7; however, one species possessed a stable laccase that retained activity at pH 7 with 88% of the maximal activity, which occurred at pH 5.

The dyes that were utilised can be used especially for assays for lignin and versatile peroxidases. These dyes were convenient for detecting such activities as no UV-plates were necessary, and the culture media composition/colored compounds did not affect the detection. Dye bleaching activity appeared to be a characteristic for rather few species and culture conditions. From the 220 different culture liquid or extract samples only 42 possessed Rb5 or AzB dye decolourisation (19 %). 27 species, such as Cerrena, Phlebia, Pleurotus, Phanerochaete and Trametes bleached these dyes. Rb5 appeared to be a slightly more degradable dye than the diazo dye AzB for assaying peroxidases. All activities were detected at pH 3.

Novel oxidoreductases with interesting properties were found as a result of the screening. The most interesting enzymes are currently under detailed investigation.
P4.6. SYNTHETIC DYES DECOLOURIZATION AND LIGNINOLYTIC ENZYMES PRODUCTION BY THE WHITE-ROT BASIDIOMYCETES

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Ten Basidiomycetes strains possessing different sets of ligninolytic systems were cultivated in pair with only laccase producing Pycnoporus coccineus on nutrient agar in Petri dishes containing 0.1% of Amaranth or Indigo carmine. Cerrena unicolor, Fomes fomentarius, and Funalia trogii distinguished by high rate of dyes decolourization. Contrary to these fungi, P. coccineus appeared to be very poor Amaranth decolourizer proving that the presence of laccase is not sufficient for this dye decolourization. Indigo carmine was more or less decolorized by all tested fungi, including P. coccineus. Hence, laccase participates in the decolourization of Indigo carmine.

In submerged fermentation of lignocellulosic substrates tested dyes were decolourized by the selected fungi with different extent. 0.5% Indigo carmine practically completely was decolourized in 5 days of fungi cultivation. The production of laccase is sufficient for this dye decolourization. C. unicolor, Phellinus robustus, and Trametes versicolor completely decolourized Amaranth during the same time. Production by these fungi of both laccase and MnP favoured to the decolourization of Amaranth. Among the fungi studied, only P. robustus accomplished decolourization of RBBR in 5 days of submerged fermentation, possibly, owing to the especially high MnP activity. Poly R478 appeared to be a comparatively recalcitrant dye. Among fungi tested, C. unicolor expressed tremendous laccase activity in submerged fermentation of mandarin peels at presence of Amaranth (209 700 U l⁻¹) and Poly R478 (279300 U l⁻¹). This set of experiments permitted to reveal a new producer of laccase - Pseudotremella gibbosa that accumulated 20-26 U ml⁻¹ of laccase at presence of Amaranth and RBBR. In submerged cultivation of C. unicolor in synthetic medium with glycerol the effect of dyes depended on their concentration. For example, the addition of 0.1, 0.3 and 1.0 mM RBBR caused increase of laccase activity by 38, 137, and 213%, respectively, as compared with the control medium. At the same time, the concentration of 0.1 mM Phenol red was sufficient to almost 2-fold increase the laccase yield as compared with the control.
P4.7. TESTS FOR OXIDASES IN PRESERVATION OF CCBAS BASIDIOMYCETE CULTURES

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Efficient scientific work requires a reliable source of cultures, which is ensured by their safe storage. Besides survival, another principal requirement for successful maintenance of fungal strains is the ability to preserve their genetic and physiological features unchanged. Routine tests carried out in most fungal collections to estimate the success of surviving the process of preservation involve at present usually growth tests together with morphology assessment of fungal colonies, sometimes also certain enzyme production tests. In our Culture Collection of Basidiomycetes (CCBAS) we introduced among others also tests for production of laccase (ABTS test), manganese peroxidase (MBTH+DMAB test) and several other oxidases (α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-manosidase, α-fucosidase) using semiquantitative API-ZYME test. Occasionally we estimate also production of hydrogen peroxide and interrelated enzymes (H2O2-generating aryl alcohol oxidase and H2O2-requiring lignin peroxidase). The results indicate that a new perlite cryopreservation method, developed and routinely used in our laboratory, is able to preserve the crucial characteristics of fungal cultures tested. As the basis of our collection form wood-rotting basidiomycetes, we also compared the enzyme profiles of several basidiomycete strains growing on their natural wood substrates – spruce, pine and oak – with those growing on the commonly used wort agar medium. The profiles were practically identical and so this medium can be used for the respective evaluation.

Acknowledgements
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P4.8. THE COMPARISON OF OXIDATIVE ENZYME ACTIVITIES OF ECTOMYCORRHIZAL, ROOT ENDOPHYTIC, LITTER-DECOMPOSING AND WOOD-DECOMPOSING FUNGAL STRAINS

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Several ecologically different fungal groups inhabit boreal forest soil. Ectomycorrhizal fungi form symbiotic association with the roots of all boreal forest trees and they are of major importance for plant nutrient uptake and protection of roots against pathogens and drought stress. Ectomycorrhizal fungi are obtaining carbon directly from host plant. Root-endophytic fungi have been found to be common within tree roots while their ecological role and way of functioning is not entirely understood. Litter-decomposing and wood-decomposing fungi are free-living soil fungi which obtain energy from degradation of soil organic matter or wood and they have been widely studied for their production of oxidative enzymes. Also, several applications are developed using fungi belonging to these two latter functional groups.

Pure culture strains from these four ecologically different functional groups will be tested for their laccase and manganese peroxidase production on different growth media. The aim is to connect ecological functioning and niche to oxidative enzyme production. Preliminary results will be presented in the poster.
P4.9. THE HYPERTHERMOPHILIC NATURE OF THE METALLO-OXIDASE FROM AQUIFEX AEOLICUS

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The stability of the *Aquifex aeolicus* multicopper oxidase (McoA) was studied by spectroscopy and calorimetry in order to understand its thermophilic nature. The enzyme, which is an efficient metallo-oxidase of cuprous and ferrous ions [1], is hyperthermostable with thermal unfolding characterized by temperature values at the midpoint of 105, 110 and 114°C. Chemical denaturation revealed however a very low stability at room temperature (2.8 kcal/mol). This low stability was related to enzyme copper depletion that presumably occurs before the unfolding of the tertiary structure, as well as to the higher tendency for aggregation exhibited by McoA at the folded state. Indeed, by unfolding kinetics, measured with the stopped-flow technique, the stabilizing effect of copper on McoA could be quantified as 1.5 kcal/mol. Furthermore, the use of this technique revealed that folded McoA aggregates in the presence of guanidinium hydrochloride, i.e., under unfolding conditions, an uncommon phenomenon, further confirmed by light scattering and gel filtration chromatography. Our results show that the hyperthermophilic nature of McoA is based on an extremely flat dependence of stability on temperature explained by the very low heat capacity change upon unfolding. This low value is mostly related to aggregation of the folded state which competes with unfolding. The high conformational dynamics of a methionine-rich segment, probably involved in conformational changes during the catalytic mechanism, also contributes to the very low heat capacity change upon unfolding.

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References
P4.10. THE BASIDIOMYCETES OF THE Genus Pycnoporus:
RED FUNGI FOR WHITE BIOTECHNOLOGY

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In the framework of the EEC project called BIOR ENEW (NMP2-CT-2006-026456), the natural biodiversity was explored among the genus Pycnoporus for the screening of new fungal oxido-reductases (such as laccases or peroxidases) suitable for white biotechnology applications. The selected strains were collected on decayed wood from different geographical areas, in particular from tropical environments (high temperature, high humidity) such as French Guyana, French New Caledonia, Venezuela, China and Australia. Original strains from tropical environments seem to be good candidates to found laccases with new properties, especially concerning the biochemical characteristics including high redox potential, stability towards temperature and solvents, and improved optimal pH.

Forty strains from the three species P. cinnabarinus, P. coccineus and P. sanguineus showed an interesting level of activity, comprised between 19 and 630 nkat/mL. Moreover, the laccase gene(s) of these forty strains was isolated. The method used in this work consisted in cloning the nucleotide sequence comprised between the consensus sequences of the copper binding regions I and IV of laccases, with PCR degenerated primers named F2 (5’-caytggcayggrttcttcc-3’) and R8 (5’-gagrtggaagtcratgtgrc-3’). The translation of these nucleotide sequences corresponded to about 75-80% of the entire protein. The forty deduced partial proteins showed between 87.6 and 99.7 % similarity. Taking into account the level of activity and the percentage of similarity, the laccases from the strains P. coccineus BRFM938, P. sanguineus BRFM66 and P. sanguineus BRFM902 were chosen for purification and biochemical characterization. These three purified proteins showed suitable properties for white biotechnology, among them high thermal, pH, and solvent stabilities, compared to literature data1,2. They were also able to degrade, in vitro, Poly-R478, a surrogate substrate for lignin-substrate degradation3. The full laccase genes and the corresponding cDNA of the strains BRFM938, BRFM66 and BRFM902 are currently isolated by inverse PCR. These genes, combined with the laccase gene of P. cinnabarinus sss4, will be used for molecular breeding by DNA shuffling.

Moreover, the presence of Mn-peroxidase genes and/or proteins was also shown among these forty strains of P. coccineus, P. cinnabarinus and P. sanguineus.

We are also developing the genus Pycnoporus as a new model for fungal expression system. Monokaryotic cell-lines are required for genetic experiments. A monokaryotic strain of P. cinnabarinus (called strain BRFM44 and obtained after meiosis), which is deficient in laccase activity, was thus selected as host for heterologous production of basidiomycete lignolytic enzymes. A plasmid expression vector was constructed, in which the expression of the gene can be placed under the control of a constitutive gpd promoter or an inducible lac promoter5. At present, the greatest efforts are directed to the improvement of this system for heterologous expression of enzymes involved in lignin degradation.

P5.1. EXPRESSION SYSTEM OF COTA-LACCASE FOR DIRECTED EVOLUTION AND VALIDATION OF HIGH-THROUGHPUT SCREENINGS FOR THE OXIDATION OF HIGH-REDOX POTENTIAL DYES

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Substrate oxidation by laccases involves the Marcus “outer-sphere” mechanism in which the redox potential (E°) difference between the substrate and the T1 Cu site of these enzymes (together with the reorganization energy and the electronic coupling) determines the electron transfer and thus, the enzymatic oxidation rate. By plotting the decolourisation values of 12 different anthraquinonic and azo dyes by CotA-laccase and the E° of dyes (measured by cyclic voltammetry) a direct correlation could be observed in accordance with results in the literature. Thus, a high E° increases the range of oxidizable substrates and improves the effectiveness and versatility of laccases, preventing the requirement of redox mediators for many applications. Our studies on the structure-function relationships revealed that changes of amino acid residues in direct contact to the metal centre significantly affect the properties of T1 Cu sites of laccases and the enzyme overall reactivity and stability1,2. Furthermore, they highlighted the limitation of rational approaches by using site-directed mutagenesis to engineer the E°. Directed evolution has merged in the past few years as a powerful alternative to rational approaches for engineering biocatalysts. The key to the directed evolution process is the establishment of an efficient expression system and screening system that accommodates the predicted diversity generated by the mutagenesis techniques. In this study the expression levels of cotA in five different *Escherichia coli* host strains, growing in 96-well microtiter plates (MPT), were compared under different cultivation conditions. The determination of protein concentration and enzymatic activity for ABTS and 2,6 DMP were performed in culture supernatants and in lysates supernatants of different *E. coli* cultures. The lowest coefficient variation (CV) in 96-well MPT (12-15%) was found for lysates supernatants of BL21 and KRX host *E. coli* strains grown under microaerobic conditions. A high-throughput screening (HTS) for dye decolourisation using a colorimetric assay was established that would allow the identification of higher E° mutants. To validate the dye decolourisation assay, lysates supernatant of the strain KRX was used to test the decolourisation of the dyes Acid Blue 62 (E°= 987 mV vs SHE), Reactive Black 5 (E°= 922 mV vs SHE) and Direct Black 38 (E°= 835 mV vs SHE) and the reproducibility in 96-well MPT in the presence of 10 mM ABTS. A CV between 5 to 15% was achieved. Thus, the designed HTS has high reproducibility and is sensitive enough to detect the (usually low) activity levels characteristic of the first generations of directed evolution.

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References
Pyranose dehydrogenase (PDH) of *Agaricus meleagris* was purified from mycelial culture media and characterized as a monomeric glycoprotein with covalently bound flavin adenine dinucleotide. The enzyme oxidizes different aldopyranoses, preferentially sugar constituents of (hemi)cellulose such as D-glucose, D-galactose, D-xylose and cellobiose to the corresponding C-2 or C-2,3 (di)dehydro sugars, indicating a role in lignocellulose breakdown. The enzyme catalyzed the regiospecific conversion of D-galactose to 2-dehydro-D-galactose, an intermediate in a possible biotechnological process for redox isomerization of D-galactose to D-tagatose. Three genes (named *pdh1*, *pdh2* and *pdh3*) putatively encoding pyranose dehydrogenases were isolated. All three genes displayed a conserved structure and organization. The N-terminal sections encode putative signal peptides consistent with the enzymes extracellular secretion. We cultivated the fungus on various carbon sources and analyzed transcription of all three genes over a period of several weeks using real-time RT-PCR. The glyceraldehyde-3-phosphate dehydrogenase gene from *A. meleagris* served as reference. *pdh2* and *pdh3* are essentially transcribed constitutively, whereas *pdh1* expression is upregulated upon exhaustion of the carbon source; *pdh1* appears to be additionally regulated under conditions of oxygen limitation. These data are consistent with an assumed role in lignocellulose degradation.
P5.3. REGULATION OF LACCASE, TYROSINASE AND LYSINE OXIDASE EXPRESSION IN *MARINOMONAS MEDITERRANEA*

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The physiological role of oxidase activities in bacteria is greatly unknown. The determination of factors regulating their expression could offer some clues about that physiological role. Moreover, that knowledge will be also of interest in order to achieve maximal levels of expression that will facilitate the characterization of the enzymes and their biotechnological applications.

*M. mediterranea* is a Gram-negative melanogenic marine bacterium expressing different oxidase activities. It synthesizes two copper enzymes with polyphenol oxidase (PPO) activities. PpoB1 is a tyrosinase involved in melanin synthesis which shows SDS-activated tyrosine hydroxylase and DOPA oxidase enzymatic activities (TH-SDS and DO-SDS). The second PPO is PpoA, a multipotent membrane-located laccase with an unknown physiological function. In addition, *M. mediterranea* synthesizes a protein with L-lysine-ε-oxidase activity, named by our group as marinocine. This is a broad-spectrum antibacterial protein whose activity is due to the hydrogen peroxide generated as a result of the L-lysine oxidation in epsilon position. The diversity of oxidase activities expressed by *M. mediterranea* makes it a good model in the study of the regulation of those activities.

The regulation of oxidase activities expression in *M. mediterranea* has been studied by measuring the enzymatic activities in different growth conditions. In addition, transcriptional fusions of the promoter regions of the genes encoding those activities to the *lacZ* gene were generated in order to study their transcriptional regulation. In order to characterize possible regulatory regions, versions of the promoter regions with different lengths were generated. These constructions were introduced in *M. mediterranea* strain MMB-1R (wild type strain) and strain T103 (strain mutated in the regulatory protein PpoS).

The results obtained indicate that all oxidase activities studied are regulated by PpoS. Moreover, those activities are growth phase regulated at the transcriptional level. They are induced at the beginning of the stationary phase of growth in both complex and chemically defined medium. The results obtained indicate that *M. mediterranea* tyrosinase, laccase and lysine oxidase activities are regulated in response to stressful environmental conditions.

**References**

P5.4. PRODUCTION OF LACCASE BY THE COMPOST-COLONIZING ASCOMYCETE *Paecilomyces inflatus*

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The compost colonizing ascomycete *Paecilomyces inflatus*, that was earlier found to convert \(^{14}\)C-labeled lignin and humic acid into \(^{14}\)CO\(_2\) and water-soluble \(^{14}\)C- fragments, produced laccase as the only ligninolytic oxidoreductase. The highest enzyme activities, under the conditions of solid-state fermentation, were measured in authentic compost samples compared with wood, straw and bran substrates. Phenolic compounds, e.g. in residual lignin, flavonoids, and other complex aromatics (e.g. fulvic acids in compost), which are always present in extracts of plant and compost materials probably induced/stimulated the production of laccase. The highest laccase levels for *P. inflatus* were observed at neutral and slightly alkaline pH (6.5 -7.5), which resembles laccase production by other compost fungi as well as the property of laccase directly isolated from compost.

To characterize this particular laccase and its production by *P. inflatus* in more detail, enzyme production was studied in defined media amended with different supplementing ingredients. Thus, the Czapek-Dox liquid medium was supplemented with glucose, cellobiose, CM-cellulose, birchwood xylan, citrus pectin, meat peptone, yeast extract, ammonium salts, compost humic acids (CHA), vanillin, vanillic acid and Cu\(^{2+}\). It turned out that media containing xylan, cellobiose or meat peptone produced significantly higher laccase activities than the others.

Activity detected in the presence of xylan was almost 4-fold higher than that observed in media containing glucose. When glucose was used as substrate, laccase activity increased after glucose had been depleted in the medium. Meat peptone was found to be the best nitrogen source that supported both the growth and the production of laccase. Basal medium with CHA, vanillin and vanillic acid also favoured laccase production but did not have any effect on the mycelial dry weight of *P. inflatus*. Finally, extracellular laccase formation was also stimulated by the addition of Cu\(^{2+}\) to fungal cultures. The optimal concentration of Cu was found to be 75 \(\mu\)M, while higher concentrations (up to 150 \(\mu\)M Cu) were detrimental for the fungus.
P5.5. SELECTION OF NEW LACCASES BY DIRECTED EVOLUTION

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Laccases catalyze the oxidation of a wide variety of substrates and can be applied in several different sectors such as paper industry, bioremediation, organic synthesis and laundry detergents\(^1\). The white-rot fungus *Pleurotus ostreatus* is able to express multiple laccase genes encoding isoenzymes with different and particularly interesting physico-chemical characteristics: POXC, POXA1w, POXA1b, POXA3a and POXA3b\(^2\). Several *P. ostreatus* laccases have been successfully expressed in yeasts\(^3\) and the availability of established heterologous recombinant expression systems has allowed the construction of mutated, “better performing” enzymes through molecular evolution techniques\(^4\). Directed evolution has emerged as method of choice for engineering functions and properties of the analyzed enzymes. This approach mimics *in vitro* the natural process of molecular evolution that is able to generate a potentially infinite plethora of proteins with new functions and properties, such as stability to temperature and solvents, improved catalytic performance and changed substrate specificity\(^5\).

Two cDNAs encoding *P. ostreatus* laccases, POXC and POXA1b, were selected as “parent molecules” to guide the evolution of laccases with higher specific activity and different substrate specificities. Genetic variants were created by random mutagenesis through error prone PCR (EP-PCR) and DNA shuffling. After two round of mutations, four POXA1b mutants (1M9B, 1L2B, 1M10B and 3M7C) were selected for their improved activity against ABTS. Catalytic and kinetic properties of the four selected mutants were analyzed and compared with those of the wild-type enzyme. The best performing mutant, 3M7C, contains two mutations, L112F and P494T. Molecular dynamic simulations have been performed on the models of POXA1b, 1M9B, and 3M7C, and their analyses suggests that the substitution P494T is responsible both for the increased stability and for the higher activity of this mutant\(^4\).

New criteria of selection were applied in a further screening of the 2400 mutants library, using different substrates (e.g. 2,6 dimethoxyphenol, DMP), assaying the enzyme stability and activity at different operating conditions (pH, temperature). Three new mutants were selected for their improved performances and characterised from a structural and functional point of view. They showed stability at pH 5 and at 60°C higher than that of the wild-type enzyme.

A new mutant was rationally designed and constructed. This new mutant, R4mut, contains the mutations of the two parental enzymes (1M10B and 3M7C): L112F, P494T, K37Q and K51N. Thermodynamic and catalytic characterization of this mutant is in progress. This new clone will be used as template for producing a new collection of mutants.

References

P5.6. THE USE OF FLOW CYTOMETRY FOR THE MEASUREMENT OF BIOMASS IN A SOLID STATE FERMENTATION PROCESS WITH WHITE ROT FUNGI

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Solid state fermentation (SSF) – fermenting microorganisms on a mostly humid solid substrate without or nearly without any free liquid – is a fermentation technique that has a very long tradition e.g. fermentation of soy sauce or the use of Penicillium roqueforti for the production of cheese. Usually the product originating from a solid state fermentation is the microbiologically modified solid substrate. A handicap for the implementation of solid state fermentations into industrial processes beyond the traditional ones is the difficulty to control the process due to the impossibility to measure the amount of biomass directly during and after the process.

Especially filamentous fungi like white rot fungi are very interesting candidates for a SSF process, as their natural habitat is wood or litter and the products of interest are their extra-cellular excreted enzymes. So far there are miscellaneous indirect methods to measure biomass in a SSF process and a frequently applied method is the determination of ergosterol, a component of the fungal cell wall, although the concentration of ergosterol in the fungal cell wall is changing with the age of the mycelium.

We developed an alternative and rapid method for the measurement of biomass in a SSF process by measuring the number of fungal nuclei by flow cytometry and compared the calculated results of fungal biomass with the amount of biomass calculated by measurement of ergosterol in the fungal cell wall.

The white rot fungi Marasmius sp. originating from Indonesia was grown on wheat husks. The biomass and the solid substrate were disintegrated by Ultra turrax and stained with a fluorescent dyes. The number of nuclei in the samples was counted by a flow cytometer. The amount of resulting biomass was determined by checking the number of nuclei enclosed in fungal mycelium without any solid substrate. The dry weight of fungal biomass without solid substrate was compared to the amount of nuclei in sheer mycelium and correlated to the number of nuclei in mycelium grown on solid substrate. To compare our method with the measurement of ergosterol, the amount of ergosterol of mycelium grown on solid substrate under the same conditions as described above and sheer mycelium was determined and correlated to dry weight.

We found that both methods revealed a comparable amount of biomass in the SSF process, with the advantage that the measurement of nuclei is quicker and less elaborate. Moreover it displays direct information about the vitality of the mycelium.
The results of rapid assay methods have revealed over 230 macromycetes cultures from the LE (BIN) Culture Collection producing laccase on MA plates. As substrates were used syringaldazine and guaiacol. Biotechnological application of basidiomycetes strains cannot be done without laboratory research on their cultivation conditions. Mushrooms cultures of various ecological groups may have different requirements regarding medium compounds and fermentation methods. To reveal optimized cultivation conditions for growth and Lac production over 40 most Lac active strains selected by express-methods were studied on various liquid media. Studied basidiomycetes were taxonomically different belonging to Agaricaceae, Atheliaceae, Auriscalpiaceae, Corticiaceae, Marasmiaceae, Polyporaceae, Polyporiae, Steccherinaceae, Strophariaceae and Tricholomataceae. Over 30 strains were characterized by Lac activity and individual priorities for nutritional media and cultivation method. It was shown that over half cultivated strains produced high active laccase. Polyporaceae and Steccherinaceae species were the most productive. Strains of Cerrena unicolor, Hericium erinaceus, Trametes gibbosa and some of Polyporus species were characterized by considerable Lac activity and biomass production on 1.5% ale-wort and glucose-peptone (GP) medium. Cultures of Fomes fomentarius, Oudemansiella mucida, Tubaria sp., and Polyporus squamosus growing on malt extract showed Lac activity. Fomes fomentarius strains produced Lac under stationary and submerged cultivation on liquid malt extract, 1.5% ale-wort, and GP (low activity), but not on glucose-mineral LN-AS medium. Agaricoid fungi Oudemansiella mucida produced Lac on all studied liquid media, but the activity was not promising. Low Lac activity was detected also in Hypholoma species. Cultures of Steccherinum murashkinskyi and Steccherinum ochraceum produced high Lac on various media but showed difference in growth. Lac activity was not detected for studied Armillaria borealis, Conocybe vexans, Marasmius rotula and Microporus luteus strains, as well as for some strains of Polyporus ciliatus and P. varius, under used cultivation conditions while they revealed high activity in rapid assay experiments. Lack of Lac production on used liquid media requires additional study on physiology of these strains and optimization of fermentation conditions for induction of the Lac production. Cerrena unicolor, Flammulaster limulatoides, Peniophora lycii, Steccherinum murashkinskyi and S. ochraceum strains were stable in exposing laccase activity on 1.5% ale-wort (about 16 - 40 u/ml). Induction of Lac activity by 0.15 g/l CuSO₄ was observed when Trametes gibbosa (124 u/ml) and Steccherinum murashkinskyi (50-550 u/ml) cultivated on GP medium. As the result of the study several promising Lac producing strains were selected.

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P5.8. OPTIMIZATION OF LACCASE PRODUCTION BY AQUATIC ASCOMYCETOUS FUNGI

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A central composition experimental design and response surface methodology was applied to optimise laccase production of two aquatic ascomycetous fungi, Phoma sp. UHH 5-1-03 and Coniothyrium sp. Kl-S5, in shaking flasks. A complex and inexpensive plant-based medium (tomato juice) and two elicitors (Remazol Brilliant Blue R [RBBR] and CuSO₄) were tested in combination at three concentrations. The highest laccase activity reached 6322 ± 403 U/l at day 9 for Phoma sp. Coniothyrium sp. exerted a maximum laccase activity of 3035 ± 111 U/l at day 4. Optimal conditions were 30% tomato juice and 450 mg/l RBBR for both strains. A concentration of 250 µM CuSO₄ led to highest laccase activities in cultures of Coniothyrium sp. and 50 µM CuSO₄ was most effective for Phoma sp. A remarkable synergistic effect of tomato juice and RBBR on laccase production was observed in both strains. The upscaling potential of the optimal induction conditions was demonstrated in a lab-scale fermenter which resulted in maximum activities of 11026 ± 177 U/l at day 6 for Phoma sp. and 11534 ± 161 U/l at day 9 for Coniothyrium sp. With this study a promising alternative for laccase production in ascomycetes based on a cheap complex substrate in combination with two elicitors is presented.
P5.9. EFFECTIVE-COST PRODUCTION OF LACCASE: REUTILISATION OF A NATURAL ADSORBENT

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The naturally wood-colonising fungus *Trametes pubescens* has been described as one of the most promising candidates for laccase production1. Laccases (p-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are multicopper-containing enzymes that catalyse the one-electron oxidation of phenolic substrates and aromatic amines with the simultaneous four-electron reduction of molecular oxygen to water. The broad substrate specificity of laccases, together with the fact that they use molecular oxygen as the final electron acceptor instead of the hydrogen peroxide used by ligninolytic peroxidases, make laccases highly interesting for industrial and environmental applications. Therefore, taking into account the industrial importance of laccases, research focusing on obtaining high production at low cost is required.

Most studies concerning ligninolytic enzyme production by white-rot fungi have been performed in liquid cultures, which do not reflect their natural habitat (wood). Recently, Toca Herrera et al. (2007)2 have pointed out the enormous potential of solid-state fermentation (SSF) for the production of laccase, especially using agro-wastes as support-substrates.

[Figure 1. Laccase activities obtained by *T. pubescens* grown under SSF conditions using dyed and undyed SS]

In the present study, the potential of dye-adsorbed sunflower seed shells (SS) as a support-substrate for laccase production by *T. pubescens* under SSF was investigated. Thus, SS were firstly used as dye adsorbents3 and subsequently used as support-substrates for laccase production. As shown in Figure 1, dye-adsorbed SS effectively produced laccase. Although higher laccase activities were obtained with the undyed SS, the laccase activities attained with the dyed SS were high enough to consider this material as a support for laccase production. In addition, the utilisation of such a material helps to solve the problem of used adsorbent. More studies in order to optimise the culture conditions are underway in our laboratory.

P6.1. ANIONIC PEROXIDASES-INDUCED CHEMILUMINESCENCE AND THEIR USE IN CHEMILUMINESCENT ELISA

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To increase the sensitivity of enzyme immunoassay (EIA) the enhanced chemiluminescence reaction is often used. In this case horseradish peroxidase (HRP), luminol and p-iodophenol are used as enzyme label, substrate and enhancer, respectively. However, this reaction has drawback connecting with quick quenching the enzyme-induced chemiluminescence. Contrary to HRP, some anionic peroxidases such as peroxidases purified from soybeans (SbP), palm tree leaf and sweet potato tuber produce a long-term chemiluminescent signal upon a catalytic oxidation of luminol. Favorable conditions for these enzymes were observed. Since among the anionic peroxidases studied only SbP is commercially available, the given peroxidase was employed as enzyme label in indirect competitive chemiluminescent ELISA (CL-ELISA) for sulfamethoxypyridazine (SMP), one of broadly used sulfonamides, and mouse IgG. The obtained results demonstrated that SbP is the more perspective label in CL-ELISA than HRP, because in the case of SbP use the assay sensitivity was higher, whereas experimental errors – lower.
Cellobiose Dehydrogenase (CDH) is an extracellular flavocytochrome produced by wood degrading, saprophytic or plant pathogen fungi. Because of the rarely found ability to communicate directly with an electrode via its cytochrome domain (direct electron transfer, DET), CDH is thoroughly studied in bioelectrochemistry for the application in a new biosensor generation. CDH from the ascomycete *Myriococcum thermophilum* was investigated for use in a so called third generation biosensor utilizing its DET properties. To this purpose, CDH was immobilized by physical adsorption on a graphite electrode and amperometrically characterized using a flow injection analysis (FIA) system. First, the system parameters like applied potential, pH and buffer composition were optimized. With these optimized settings the detection limit, linear range and sensitivity were determined for a number of carbohydrates. The detection limit for cellobiose and lactose was in the low µM range. Additionally, *M. thermophilum* CDH readily detects glucose, a property not observed for any CDH up to now. The efficiency of the direct electron transfer between the active site of the enzyme and the electrode was compared by amperometric flow injection measurements to the mediated electron transfer (MET) using the same setup and 1,4 benzoquinone as the mediator. *M. thermophilum* CDH showed an only 8 fold higher MET than DET rate, which indicates a very efficient DET behaviour of this enzyme. Furthermore, the long term stability of the developed sensor was tested. Even by simple physical adsorption on graphite electrodes the CDH biosensor was stable for several days. It can be concluded that *M. thermophilum* CDH is suitable to construct a very stable and versatile third generation biosensor, showing high currents for a number of analytes including glucose.
P7.2. CROSS-LINKING β-CASEIN WITH TYROSINASE AND TRANSGLUTAMINASE

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In food production optimal texture, stability as well as controlled digestibility of proteins and carbohydrates and low fat contents, are the properties needed for the improvement of food quality and well-being. Recently, much of the research efforts have been focused on the modification of dairy proteins due to increasing use of dairy products by all age groups. The high proportion of β-casein in raw milk (25% of all proteins) makes it attractive target protein for enzymatic modifications.

Transglutaminase is an acyltransferase, commercially available and it has been extensively studied on its effect on different food matrices including milk products. It is known to catalyze acyl-transfer reactions between a γ-carboxyamine group of a peptide- or protein-bound glutamyl residue and a primary amino group of different substrates, leading to intra- or intermolecular protein cross-linking. Tyrosinase is an oxidative enzyme, known to catalyze the oxidation of the phenolic ring of tyrosine residues to the corresponding quinones, possibly subsequently resulting in the formation of tyrosine-tyrosine, tyrosine-cysteine and tyrosine-lysine cross-links. β-casein contains 11 lysine, 19 glutamic acid and 4 tyrosine amino acids. Therefore, it is an excellent substrate for these two different types of enzymes. The reaction catalysed by tyrosinases on proteins is not fully understood, mainly due to the lack of pure enzyme needed for the detailed reaction mechanism studies. So far much of the work has been done with commercial mushroom tyrosinase.

In this work cross-linking reactions of Trichoderma reesei tyrosinase and transglutaminase on β-casein were studied, within the optimal pH and temperature conditions. Different enzyme dosages (10, 100 and 1000 nkat/g) and times (2, 6 and 24 hours) were applied. The reactivity of tyrosinase on β-casein was tested with oxygen consumption measurement (OXY-10, Presens, Germany). The cross-linked products were analysed by SDS-PAGE and size exclusion chromatography (SEC) using TSK G6000 PWXL and Superdex 200 5/150 GL columns connected in series coupled with a multi-angle laser light scattering (MALLS) to determine the molecular size of the created products. FAN (free amino nitrogen measurement) was used to monitor the amount of free amino groups in the cross-linked products.

Through the use of SEC – MALLS, the molecular masses of the cross-linked products were determined. In the case of the reference enzyme, transglutaminase, the largest cross-linked products (~3000) are obtained using the enzyme dosage of 100 nkat/g and high reaction times. Low enzyme dosages resulted in the creation of intermediate products of a non distinct shape, making difficult the calculation of their size. In the case of tyrosinase the largest products (~2000) were obtained by using high enzyme dosage. It was noticed that the product size rises linearly with the raise in enzyme dosage and reaction time, while having well defined peaks. The results obtained by SDS-Page are in agreement with the previously mentioned results. Their accuracy is lower due to the high molecular mass obtained from the cross-linking reactions. FAN method appeared to be accurate for β-Casein while for the cross-linked samples it showed a clear decrease in the number of free amino groups that could react. However it was not feasible to differentiate between the samples possibly due to the complex and unknown structure they obtain through cross-linking. In the future, the structure and the digestibility of the cross-linked products will be studied in detail.
P7.3. LACCASE-AIDED PROTEIN MODIFICATION: EFFECTS ON THE STRUCTURAL PROPERTIES OF ACIDIFIED SODIUM CASEINATE GELS

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Effect of laccase-catalyzed modification of proteins on rheological, structural and textural properties of acidified sodium caseinate gels were studied. Laccase used was from filamentous fungus *Trametes hirsuta*. Protein modification was performed by incubating sodium caseinate solution (5% w/w) with laccase using dosages 0.5-25 nkat/g protein with or without 2.5mM of ferulic acid (FA) for 2 hours at 25°C and 45°C, after which the solutions were acidified by 1.13% (w/w) of glucono-delta-lactone (GDL) and incubated further for 22 hours at 25°C. Laccase as such was not able to crosslink casein proteins, but protein fragmentation was observed on SDS-PAGE especially when high laccase dosages were used. Incorporation of FA as an auxiliary compound was found to enhance the formation of covalent bonds significantly. Polymerization was most pronounced when 25nkat/g of enzyme was used together with FA at 45°C. Size exclusion chromatography (SEC) confirmed that degree of casein polymerisation increased as a function of enzyme dosage. SEC results also showed that FA is incorporated in protein network totally at all enzyme dosages. The microstructure of the gels was studied by CLSM. The effect of laccase on the microstructure was not very profound. High laccase dosage together with FA resulted in slightly bigger particle aggregates while high laccase dosage without FA gave a slightly more porous and coarse gel. Texture analysis showed that firmness of the caseinate gels without FA was not significantly different from each other at both cross-linking temperatures. Significant increase in both gel firmness and in the kinetics of gel formation was obtained when laccase was used together with FA. Small deformation oscillatory measurements showed that all samples had higher elastic moduli (G') than control due to formation of covalent bonds between proteins. Sample with high laccase dosage together with FA had a significantly higher G' which is very much in accordance with the texture analysis. Onset of gel formation was about 20 min. earlier for the samples with high laccase dosage (with and without FA).
P7.4: LACCASE AND NATURAL MEDIATORS IN BIOMODIFICATION OF SPECIALTY PULPS

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In the last decades laccases were widely studied as lignin oxidation promoters in presence of non-phenolic mediators and the so-called laccase-mediator systems (LMS) are the most promising enzymatic systems for pulp bleaching. Moreover, recent studies have focused on a new approach to the use of LMS for the biomodification of lignocellulosic fibres, in order to confer them new properties.

A growing concern exist about the possible toxicity and the high cost of synthetic mediators, and generate the need to search for alternative compounds easily available at the pulp and paper industry, like natural phenols.

In this work, two types of non-wood pulps were investigated: flax (Linum usitatissimum) and sisal (Agave sisalana), employed for the production of high added value products (specialty papers).

An enzymatic stage (L stage) was applied to both pulps: flax pulp was treated with Pycnoporus cinnabarinus laccase and three natural mediators, namely syringaldehyde (SA), acetosyringone (AS) and p-coumaric acid (PCA); sisal pulp was treated with Trametes villosa laccase and four natural mediators, namely synaptic acid (SINAC), ferulic acid (FERAC), coniferylaldehyde (COALD) and sinapilaldehyde (SALD). Each of them was compared to the synthetic one 1-hydroxybenzotriazole (HBT), whose potential for bleaching different pulp types has been confirmed by numerous studies.

L stage was followed by a peroxide bleaching treatment (P stage). Pulp properties, Kappa Number (an estimation of lignin content) and Brightness were measured in the initial pulp and after both stages. Moreover, effluents were analyzed in terms of COD, colour and residual activity of laccases.

In flax pulp, after P stage, all natural mediators determined a decrease in kappa number, marked in the case of SA (45% lower than the control) and comparable to the one obtained with HBT (55%). Brightness was increased significantly by all natural mediators, especially by AS and SA (23% in both cases), whose results were very similar to the ones showed by HBT (25% upper the control).

In sisal pulp, after L stage, all natural mediators determine an increase in kappa number, particularly marked in the case of COALD (58%). The increase in kappa number denotes a possible partial condensation of the mediators in the phenoxy radical form on the pulp. HBT is the only mediator which produced a decrease (13%) in kappa number. After P stage, pulp treated with HBT showed the lowest value of kappa number (15% lower than the control), no remarkable decrease was observed with all natural mediators. Brightness could be improved only by HBT in both L and P stages (4 and 13% respectively). In the L stage all natural mediators produce a decrease in this property.

Natural mediators represent a powerful alternative to synthetic mediators for flax pulp biobleaching. In the case of sisal pulp natural mediators did not result effective for bleaching, nevertheless their tendency to couple to fibres opens up new perspective for functionalisation and modification of fibres properties.

Whatever the application, natural mediators offer a new way to study environmentally friendly processes of pulp and paper production, thanks to the wide availability from plant materials and pulping liquors.
P8.1. APPLICATION OF IMMOBILIZED FUNGAL BIOMASS FOR DECOLOURIZATION AND DETOXIFICATION OF MODEL TEXTILE WASTEWATER

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The textile industry produces large quantities of highly coloured effluents, which are generally toxic and resistant to destruction by biological treatment methods. It is necessary to find an effective method of wastewater treatment capable of removing colour and toxic organic compounds from textile effluents¹, ². For the application of white rot fungi in the treatment schemes for dye-containing wastewater, the process must be designed either to retain pure culture conditions or to function under non-sterile conditions³.

The major objective of this study was to investigate the performance of white rot fungal strains immobilized on plastic mesh scourer, for biological treatment of model wastewater on the repeated-batch decolourization system. These fungi were previously shown to have potential in textile dye biodegradation⁴. During decolourization experiments a model colour wastewater imitating real industrial effluents was used. Both strains were used repeatedly using batches containing new portion of model wastewater. During decolourization process selected parameters were monitored such as absorption (within range 250-700 nm), pH, conductivity, COD and BOD values, and the toxicity using luminescence bacteria. Our results show that white rot fungi have potential in decolourization and detoxification textile dyes.

Acknowledgements
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References
In this study, we tested for the ability of eight lichen species to decolourize different types of dyes. Our earlier investigations showed that only lichens belonging to Suborder Peltigerineae possess high activities of the multicopper oxidases, laccases and tyrosinases. Therefore we selected *Pseudocyphellaria aurata*, *Peltigera rufesens*, *Collema flaccidum*, *Lobaria scrobiculata* and *Sticta cf. sublimbata* belonging to Suborder Peltigerineae and as controls from other groups *Cetraria nivalis*, *Flavocetraria islandica* and *Cladonia stellaris*. Dyes tested included the indigoid dye Acid blue 74, the anthraquinone-type dye Remazol Brilliant Blue R, the azo dye Chicago Sky Blue 6B and Acid Red 103 belong to the quinone-imine dye class. We tested the ability of both leachates and lichen thalli to decolourize the dyes. In addition, we tested the ability of 1-HBT to act as a “mediator” for laccase to enhance the decolourisation processes by increasing the enzyme activity.

Interestingly, results showed that not only species belonging to Suborder Peltigerineae but also species from other lichen group effectively decolourised dyes after 48 h. The extent of the decolourisation varied between the selected species the dyes used. The “mediator” 1-HBT greatly stimulated decolourisation but only in lichens from Suborder Peltigerineae. Therefore we conclude that multicopper oxidases definitely participate in decolourisation of dyes perform by lichens but other oxidases appear to be present as well.
We had recently developed and published some new laccase aided oxidation systems which are based on the generation of reactive oxygen species (ROS) or nitrogen species (RNS) for delignification of pulp or for other applications. We will summarize results referring to laccase mediator approaches with a new group of mediators such as carbazate compounds. These compounds used are cheap and degradable. They are applied either alone or in combination with other oxidation systems which use hydrolases, mainly special proteinases or lipases, in the presence of fatty acids and peroxide in order to form perfatty acids and other oxidants.

Due to the mostly crude character of these lipases or proteinases (produced e.g. by moulds like *Aspergillus* strains) the corresponding side activities such as xylanases etc. can further significantly improved the overall performance.

The presented results show good delignification rates up to 40% and more which can be reached during a 2-4 hours treatment at pH 4-8, at 40-60 °C and ca. 10% consistency.
P8.4. ENZYMATIC BLEACHING OF COTTON

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Much effort has been made in order to use oxidative enzymes in delignification and bleaching in the pulp and paper industry. Peroxidases and laccases produced by white rot fungi are known oxidizing enzymes participating in lignin degradation. Bleaching of cotton in the textile industry is usually performed with hydrogen peroxide at alkaline conditions and elevated temperatures. Thus, it is a water and energy consuming process, too. In our project we decided to tackle the question whether oxidizing enzymes from white rot fungi are also able to bleach cotton.

Neither peroxidase nor laccase treatment of cotton resulted in bleaching. In contrary, both enzymes even enhanced the brownish colour of native cotton despite their ability to decolourize synthetic textile dyes. The same could be observed with soluble lignin. Isolated laccases and peroxidases increased the absorption of soluble lignin. Best results with enzymatic treatment of cotton with isolated enzymes were obtained in our investigations with pectinase, xylanase and hemicellulase.

Therefore, we tried to perform bleaching with white rot fungi as whole organisms. When grown on lignin-containing agar plates, first a darkening of lignin can be observed followed by complete decolourization with ongoing growth of the mycelium. This indicates that darkening of lignin may be an early step in its degradation. Cotton was also bleached to adequate whiteness on agar plates with whole organisms. However, many white rot fungi produce too many cellulases resulting in destruction of cotton fibres. Best results with no observed destruction of fabric were obtained with a Bjerkandera adusta strain.

Bleaching of cotton fabric also occurred in liquid culture even when the direct contact between fungus and fabric is prevented by a membrane. This shows that the fungus secretes enzymes and possibly mediators with bleaching activity. Cellulase, xylanase, pectinase, and mannanase were detected as polymeric glycoside hydrolyzing enzymes. Manganese peroxidase and lignin peroxidase account for oxidizing enzymes. Furthermore, the presence of cellobiose dehydrogenase and an esterase was detected in a concentrated sample.
P8.5. USE OF FREE AND IMMOBILISED NOVEL LACCASES AND PEROXIDASES FOR BLEACHING OF TEXTILE DYES


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During the textile dying process 30 to 40% of dyes do not bind to the cotton fiber and remain in the waste water. The costs for cleaning coloured waste water with different physical, chemical, electrochemical or biological methods are high. The use of bleaching enzymes like laccases and peroxidases could help to reduce the amount of water needed in dying processes and could also help to remove non-bound dyes still present from the staining process. Enzymatic processes are considered to be environmentally friendly and can also be less cost intensive.

We performed a screening for dye decolourisation by white rot fungi. Because of the similar phenolic structure of lignin and industrial dyes, white rot fungi can degrade them. 25 different fungal strains were investigated for their ability to bleach 45 of yellow, orange, red, blue and black dyes with monoazo, disazo, oxazine, phthalocyanine and anthraquinone structures used in the technical dying process. Bleaching experiments were carried out on agar plates and in liquid cultures. Whereas nearly all fungi can degrade blue and black dyes, only some fungi can also decompose yellow, orange and red dyes. The decolourised solutions resulting from bleaching experiments in liquid cultures were used for two different toxicity assays. Until now, no toxic degradation products were found. Now we are purifying and characterising the most effective laccases and peroxidases from different white rot fungi involved in the decolourisation process.

Enzymes for bioremediation need to show a high stability and it must be possible to run the process at low costs. For this purpose, the multiple use of the enzymes is necessary which can be achieved by the employing immobilised enzymes. Polymeric particles and porous microgels were choosen as carriers which can be produced in a number of different sizes, morphologies and with surface layers modified by different functional groups. We investigated the immobilisation of laccase on different particles with epoxy and amino carriers. Separation of the enzyme can be realised by a magnetic core inside the particles.
Textile industry is an example for a type of industry that consumes large volumes of water and chemicals for processing textile materials. Especially in the industrial process of cotton dyeing or printing the amount of dyestuff which is covalently bounded to the cotton fibers can vary from 98% to only 50% depending on the type of dyestuff used. The remaining hydrolyzed dyestuff must be washed from the fabric to avoid bleeding from the finished products. What makes the treatment of textile wastewater very difficult is the diversity of textile dyestuffs used in the industrial production of textile products.

Two white rot fungi were immobilized on two different types of brown-coal products. Consecutively the decolorization of artificial textile wastewater made from 4 different types of reactive textile dyes, 3 different types of blue dyes and one yellow dye, was observed.

A *Marasmius* species from Indonesia was compared to *Trametes hirsuta* in solid state fermentation in RITA®-Systems (temporary lift up immersion) on lignite granules and on lignitic xylite for growth, extracellular enzyme production and textile dye degradation.

Lignitic xylite and lignite granules were observed for their ability to absorb the different textile dyes. Lignitic xylite shows a better ability to absorb the dyestuff but its potential to uphold the load is poor whereas lignite granules present a lower absorption capacity. Both fungal species grew poorly on xylite but much better on lignite granules.

The production of extracellular enzymes of both fungal species was quite different. *Marasmius sp.* produced up to 67 U/L laccase on lignite granules but 10 U/L on xylite, no other extracellular enzymes could be detected; *T. hirsuta* produced 1343 U/L laccase when immobilized on granules and 12 U/L unspecific peroxidase in maximum whereas on xylite 898 U/L laccase and 14 U/L unspecific peroxidase could be detected.

A successful decolorization of the textile dyes was mainly depending on the type of color and to a lesser extent on the amount and the continuous presence of enzymes in the fermenter.

Lignite granules proved as a feasible material to combine the effects of absorption and enzymatic degradation of textile dyes.
P8.7. BLEACHING OF TEXTILE DYES IN WASTEWATERS: EFFECT OF LACCASES WITH AND WITHOUT MEDIATORS

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Laccases belong to the so-called blue-multicopper oxidase family. Functionally, they couple the monoelectronic oxidations of a wide variety of substrates with the reduction of molecular oxygen to water. The search for new, more efficient and environmentally sound processes for the textile, pulp and paper industries has increased interest in these “green” catalysts, which work with air and produce water as the only by-product.

Suitable low molecular –weight compounds, called mediators, can be used in combination with laccases to indirectly oxidise non-phenolic (and thus “unnatural”) substrates, including industrial synthetic dyes and several other aromatic hydrocarbons. In this context, the research presented here has been focused on the spectroscopic characterization of the reactive radical intermediates formed in laccase-mediator systems. The insight into the interactions of laccase with different substrates can be in fact of great help in designing more effective enzyme-based catalysts for a variety of industrial applications with particular emphasis in bioremediation processes.

In addition, the bleaching activity of laccases from White Rot Fungi (WRT) have been tested both in the presence and in the absence of synthetic and natural redox mediators against selected single textile dyes, as well as towards solutions mimicking a variety of real wastewaters. In almost all the cases tested, the presence of the mediator enhanced the reaction rate and the percentage of dye decolourization, making the enzymatic system extremely suitable for industrial applications.
The colouration of fibres is a complex phenomenon that requires the application of a wide range of dyes and auxiliaries, aggressive pH and elevated temperature. Oxidative enzymes such as laccases can be used to produce dyes \textit{in situ} based on colourless dye precursors, e.g. 2,5-diaminobenzenesulfonic acid (2,5-DABSA) and catechol. Laccase oxidises catechol converting it into reactive quinone species, which react subsequently non-enzymatically with amines forming 1,4-Michael-type adducts. This work demonstrates the evolution of the laccase-assisted dyeing concept for cellulose substrates. The major difficulty in cellulose dyeing consists in the fact that the oxidatively-generated dye does not have affinity towards the fibres. The lack of chemical bond formation between the laccase-generated dyes and cellulose was compensated by optimisation of the concentration of the dye precursors, yielding up to 70% dye fixation due to the formation of an insoluble coloured product. Further improvement of the method consisted on the covalently immobilisation of 2,5-DABSA on the fabric surface. The aromatic amine was introduced to a previously tosylated fabric through nucleophilic displacement of the tosyl groups. This aminated cellulose allowed for covalent fixation (up to 95%) of the \textit{in situ} generated from catechol and amine dye upon oxidation with laccase. The structure of the enzymatically generated dye was studied by means of HPLC-MS and FTIR techniques and electrochemical evidences of the dye fixation mechanism were also obtained.
Skin lightening products have become increasingly popular in the past few years. Their applications include lightening or whitening of the skin as well as treatment of pigmentary disorders such as chloasma, freckles, pregnancy marks and age spots. In addition, decolourization of hair as preliminary step to dyeing is a most common practice in hair cosmetics.

The major determinants of the colour of skin, hairs and eyes in man and other mammals are melanins. These are heterogeneous polymers made up of 5,6-dihydroxyindoles units possibly at different oxidation states. This structural feature is similar to lignin or coal, in which polymers are composed of indolic or phenolic subunits. White-rot fungi produce various extracellular oxidases and peroxidases, capable of oxidizing a broad spectrum of structurally different substrates, including lignin or coal as well as highly toxic compounds and azo-dye. Such capabilities of lignin-degrading enzymes suggest their use in decolourisation of melanins. Previous studies were carried out using fungal melanins, and only a report has appeared on the bleaching of a synthetic melanin, but the preparation procedure and the substrate were not provided. Very recently, Mohorcic and co-workers published cosmetic applications of a bleaching enzyme of fungal origin.

We report here the results of a study in which the two white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their lignin-degrading enzymes were tested for efficiency in decolourization of melanin from *Sepia officinalis*, a widely accepted standard of natural melanins. The former fungus proved more effective than the latter in melanin decolourisation. In liquid cultures, the added melanin was initially adsorbed onto the fungal mycelium, and then the colour was smoothly discharged over ten to fifteen days from either the medium or the mycelium. Decolourization was due to extensive melanin degradation, as shown by HPLC identification in the culture broth of PTCA (pyrrole-2,3,5-tricarboxylic acid), the typical product of chemical degradation of eumelanin. Yields of PTCA, obtained by the action of the fungus after 10 days of incubation, was at least 80-90% of those obtained after chemical degradation of the same melanin under oxidative alkaline conditions. The effects of melanin concentration on fungal efficiency of decolourization was also evaluated. Synthetic melamins prepared by oxidation of the main biosynthetic precursors 5,6-dihydroxyindole and its 2-carboxy derivative were also efficiently degraded by the fungus.

Work is in progress to identify the enzyme(s) involved in melanin degradation and its cellular localization.

References:
P8.10. FUNGAL LACCASE, CELLOBIOSE DEHYDROGENASE, AND CHEMICAL MEDIATORS: COMBINED ACTIONS FOR THE DECOLORIZATION OF DIFFERENT CLASSES OF TEXTILE DYES

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Dyes belonging to the mono-, di-, tri- and poly-azo as well as anthraquinonic and mono-azo Cr-complexed classes, chosen among the most utilized in textile applications, were employed for a comparative enzymatic decolorization study using the extracellular crude culture extracts from the white rot fungus Funalia (Trametes) trogii grown on different culture media and activators able to trigger different levels of expression of oxidizing enzymes: laccase and cellobiose dehydrogenase. Laccase containing extracts were capable to decolorize some dyes from all the different classes analyzed, whereas the recalcitrant dyes were subjected to the combined action of laccase and the chemical mediator HBT, or laccase plus cellobiose dehydrogenase. Correlations among the decolorization degree of the various dyes and their electronic and structural diversities are rationalized and discussed.

CDH oxidize oligosaccharides like cellobiose, cellotriose, or lactose to the corresponding lactones.

The reduced enzyme is reoxidized by different electron acceptors including Fe(III) and oxygen by a Fenton type reaction produce highly reactive hydroxyl radicals. The attack of hydroxyl radicals generated by CDH can yield to the conversion of nonphenolic structures to phenolic ones, thus rendering the molecule easily oxidized by laccases or peroxidases.

The utilization of cellobiose dehydrogenase in support to the activity of laccase for the decolorization of azo textile dyes resulted in substantial increases in decolorization for all the refractory dyes proving to be a valid alternative to more expensive and less environmentally friendly chemical treatments of textile dyes wastes.

References
P8.11. COMBINED ACTION OF A BACTERIAL MONOOXYGENASE AND A FUNGAL LACCASE FOR THE BIODEGRADATION OF MONO- AND POLY-AROMATIC HYDROCARBONS

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One of the key factors in efficient bioremediation processes is the capacity to simultaneously degrade a broad spectrum of toxic pollutants, but this goal is very difficult to achieve utilizing single microorganisms, because all of them are specialized for the utilization of a limited number of compounds. For this reason the combined action of a wide substrate range Toluene o-xylene MonoOxygenase (ToMO) from Pseudomonas sp. OX1, able to convert many aromatic compounds into mono- and di-hydroxylated derivatives, and fungal laccases from Pleurotus ostreatus, which oxidize these hydroxylated products yielding polymers with reduced toxicity, is studied.

This strategy permits to overcome many of the substrate specificity problems and dead end toxic products formation generally encountered in complex bacterial biodegradation pathways. In particular, toluene and naphthalene degradations were tested as representative of mono- and poly-aromatic pollutants and the combined action was optimized in micellar and microemulsioned systems, able to increase the bioavailability of the hydrophobic aromatic pollutants and consequently the conversion rates. This approach allows efficient hydroxylations of hydrophobic substrates thus favoring the further action of fungal oxidases and a protocol is optimized for the complete degradation of different initial concentrations of substrates¹.

References
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An important factor determining the use of enzymes in a technological process is their expense. Since enzymes are catalytic molecules, they are not directly consumed by the processes in which they are used. Immobilisation provides easy recovery and reuse of the enzymes, and easy separation of the products. It also allows for continuous processes to be practicable, with a considerable saving in enzyme, labour and overhead costs. A wide variety of insoluble materials may be used to immobilise the enzymes.

Wetlands has designed customisable lab scale and pilot scale systems in order to exploit immobilised enzymes. These can be used for waste water treatment or for synthesis (e.g. dye synthesis) with little modifications. Different technologies are available in a single plant with few adjustments; and can be processed for instance in a packed or fluidised bed configuration.

The lab scale and pilot scale plants are fully automated with temperature and pH control, online spectrophotometer, conductivity, dissolved oxygen concentration measurement by Wetlands ingeniously built optical oxygen biosensor, and data logging. Wetlands devices can be employed for research work as well as for small to medium scale treatment/production.

Promising results have been obtained in waste treatment (decolouration and detoxification of dye industry effluents) and synthesis (new dyes) within the framework of the FP6 SOPHIED project.
P8.13. BIOTRANSFORMATION AND DETOXIFICATION OF THE AZO DYE SUDAN ORANGE G WITH BACTERIAL COTA-LACCASE

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The present study reports on the biotransformation of the azo dye Sudan Orange G by the oxidative bacterial enzyme CotA-laccase from Bacillus subtilis. In the absence of redox mediators over 98% of Sudan Orange G is decolorized within 7 hours. However, the presence of catalytic amounts of 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) leads to a two-fold increase in the rate of biotransformation. Enzyme inhibition at high SOG concentrations was observed and a Ki value of 474 µM calculated. The redox potential of the SOG dye depends on pH as shown by cyclic voltammetry; E0 decreases with increasing pH values up to ≅ 7 and then remains constant. These results are consistent with the determined pKa values of 6.9 and 11.7, of the two oxidizable groups of SOG, and are in agreement with the bell-shape pH profile of the enzyme with an optimum of 8. Seven biotransformation products were identified using high-performance liquid chromatography and mass spectrometry. Furthermore, a mechanistic pathway for the azo dye conversion by CotA-laccase is proposed. Taken together, these approaches revealed that the enzymatic oxidation of the Sudan Orange G results in the production of oligomers and, possibly polymers, through radical coupling reactions. This correlates with the presence of low aqueous soluble products and the final brown color of enzymatic reactions. A bioassay based on inhibitory effects over the growth of Saccharomyces cerevisiae shows that the enzymatic bioremediation process reduces 3-fold the toxicity of Sudan Orange G.

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P8.14. COVALENT IMMOBILIZATION OF LACCASE ONTO SILICEOUS MESOSTRUCTURED CELLULAR FOAMS TO OBTAIN VERY EFFECTIVE BIOCATALYSTS

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Laccases belong to a group of oxidative enzymes called blue copper oxygenases, in which four copper atoms are bound in the redox sites. They act via a free radical mechanism, using molecular oxygen, in relatively non-specific reactions with various organics: alkenes, methoxy-phenols, aminophenols, aryl amines, polyphenols and polyamines. Laccases are rather stable and readily accessible from numerous sources, e.g. fungi. To pave the way for biocatalysts reuse and recycling and also to improve their stability, the enzymes should be immobilized on solid supports. The aim of this work was to investigate the properties of laccase covalently bonded with the siliceous mesostructured cellular foams (MCFs). The latter materials were most recently shown to have huge potentials to afford very efficient biocatalysts1.

EXPERIMENTAL The wood-rotting fungus Cerrena unicolor (Bull.ex.Fr.) Murr, No. 139 was produced and prepurified as described earlier2. Laccase activity was measured in pH 5.3, 30 ºC and using 2,2’-azino-bis(3-ethylbenzotiazoline-6-sulfonate) as substrate3. One activity unit was defined as the amount of the enzyme that increases of absorbance of products (420 nm) by 1.0 per min. Preparation of MCF-based biocatalysts was carried out as described previously1.

RESULTS Immobilization of enzymes on various siliceous materials clearly indicated that MCFs are particularly attractive due to their unique texture with ultra large cage-like mesopores, up to 40 nm in size. That allows to host most enzymes and decreases mass transport limitation for substrates and products. Among MCFs functionalised with oxirane or amino groups those grafted with 2-aminoethyl-3-aminopropyltrimethoxysilane and then activated with glutaraldehyde appeared to afford the best biocatalyst. Its activity was 2181 U per 1 mL of the carrier and it was 78 times higher than that of laccase immobilized on acrylic carriers and about 25-27 times larger than of cellulose- or conventional silica gel -based preparations.

For the best preparation thermal and pH stability, and activity profiles were determined. Laccase immobilized on MCFs was stable up to 40 ºC whereas temperature for a native enzyme should be less than 30 ºC. Moreover, immobilized preparation was stable in a very wide pH range from 3.6 to 6.0 and it was fairly stable during storage without any stabilizers at 4 ºC. After one year the preparation showed 40 % of its initial activity. The influence of agitation rate and temperature on the initial reaction rate indicated that the studied reaction are kinetically controlled. To examine in more detail the kinetic properties, the values of K_m and k_cat in Michaelis-Menten equation were determined from batch experiments. The value of k_cat /K_m was only 2.5 times lower than the value for a native enzyme. Finally, comparison of reactivity of the immobilized and native enzyme in the batch system led us to conclude that the biocatalyst obtained is very well suited to the batch mode operation. As MCFs are powders with a mean particle size of 18-50 µm, the downstream separation of reaction products from biocatalyst has to be realised by centrifugation or microfiltration. Practical comparison of these methods indicated that using a 0.22 µm pore diameter membrane less than 10 % of the carrier loss was observed after 16 runs. For the time being, native and immobilized laccase were tested in dyes decolorization. The rate and magnitude of dyes decolorization by the biocatalysts prepared was similar to that observed for a native enzyme. But those developed can easily be recycled and it is of considerable practical importance.

References

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P8.15. A SEARCH OF A SUITABLE CARRIER FOR EFFECTIVE IMMOBILIZATION OF MUSHROOM TYROSINASE

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Polyphenol oxidases, also known as tyrosinases, are oxidizing enzymes that use molecular oxygen in the site-specific hydroxylation and oxidation of phenols and catechols. Tyrosinase contains two coordinated copper ions at reaction site which act cooperatively in two separate or sequential catalytic reactions: hydroxylation of phenols and oxidation of catechols to o-quinones. The quinines formed undergo further, non-enzymatic reactions resulting in polymerization up to di- tri-polymeric phenolics, exhibiting biological activity. An additional feature of tyrosinase catalyzed reactions is a suicide inactivation by quinone products. Control of this inactivation by physical separation of the enzyme from products can be facilitated by the attachment of enzymes into insoluble carriers. This paves the way for biocatalyst recycling and may additionally improve enzyme stability. The aim of this work was to find: effective method of tyrosinase immobilization (adsorption or covalent attachment), appropriate anchor groups (-NH₂, -COOH, -OH, glycidyl residue), suitable activation procedure (GA - glutaraldehyde, DVS - divinyl sulfone, CDI – carbodiimide) and carrier matrices: copolymer of butyl acrylate and ethylene glycol dimethacrylate (Acrylic), cellulose-based Granocel (G), conventional silica gel pellets (Z) or powdered mesostructured siliceous cellular foams (MCF).

Tyrosinase from mushrooms was extracted and prepurified using the method described in¹. Cresolase and catecholase activity was measured in pH 7.0, 30 °C and 1.0 mM tyrosine or L-DOPA as substrates. One activity unit (U) was defined as the amount of the enzyme that increases of absorbance of coloured products (475 nm – tyrosine and L-DOPA) by 0.001 per min. Preparation and functionalisation of Acrylic, G, Z and MCFs carriers was presented previously², 3.

Activities of tyrosinase adsorbed on carriers did not exceed 228 U/ml and these preparations could totally deactivate after 1 month storage. Covalent attachment proved more efficient. Comparison of the best preparations among groups of carriers tested showed the enzyme preference to hydrophilic matrix with OH-groups activated with hydrophobic spacer whereas more hydrophobic ones had to posses amine groups activated with GA. In Z-carriers almost no activity was observed. The results clearly indicate that MCFs are particularly efficient due to their texture with ultra large pores, up to 40 nm, that allow to host most enzymes with lower mass transport limitation of substrates and products. Among MCF carriers, only those with amine groups allowed to obtain very high activities, although MCF-APM catalysts had very low storage stability. Interestingly, relatively poor storage stability in cresolase activity is accompanied by exceptional catecholase activity of the stored samples of MCF-APT and MCF-ATM. The most active and stable preparation from each group of carriers was tested for thermal inactivation. It was found that after incubation at 55 °C, native enzyme lost 50 % of initial activity after 2.2 min whereas tyrosinase immobilized on Acrylic and DEAE_G after 33 min and preparation based on MCF-APT after 20 min. Potentials of new catalysts are now being tested in the oxidation of substrates such as: phenol; 2-chlorophenol; 2,4-dichlorophenol, 4-aminophenol, p-phenylenediamine, tannic acid and pyrocatechin.

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P.8.16: IMMOBILIZATION OF LACCASE FROM Trametes versicolor ON BIOPOLYMER CHITOSAN USING EDC AS A CONJUGATING AGENT

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Laccase are oxidative enzymes actively being studied for bio-applications in the pulp and paper industry, environmental remediation and chemical synthesis. Because of its huge industrial application of this enzyme, immobilization techniques were developed to improve the stability and reusability of this biocatalyst. Chitosan was selected as a solid support because of its high amino group which easily link with enzyme, soluble in mildly acidic aqueous solutions but insoluble near and above its pKa value and exhibits interesting functions as a heavy metal adsorbent, antimicrobial agent, biodegradable, abundant in nature and its production is of low cost and ecologically interesting. The dissolved chitosan is a cationic polyelectrolyte that can be conjugated with proteins to make functional biomaterials with novel physiochemical and biological properties. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] is perhaps the most popular carbodiimide used in conjugating biological substances. Its water solubility allows for direct addition to the reaction without prior organic solvent dissolution. It facilitates the formation of amide bonds between a carboxylic acid residue and an amine residue by cross-linking since the amide linkage does not add a spacer molecule.

In this study the renewable biopolymer chitosan was conjugated with commercially available laccase from T. Versicolor. Different immobilization strategies were tested such as the variation of the molar excess of EDC present in solution comparatively to laccase. To do so, we used 1, 5, 10, 50, 100 and 200-fold molar excess of EDC in the laccase-chitosan conjugation solution. The laccase-chitosan conjugation by the addition of EDC resulted in a solid biocatalyst with a laccase activity of ± 30 U/g of chitosan. Furthermore, the efficiency of laccase activity recuperation from the biocatalyst (per gram of chitosan) increased when increase in EDC concentration in solution. The increase was 5-13 folds (1-200 molar excess) higher than the control where no EDC was used in conjugation solution. Similarly, the overall efficiency of laccase conjugation relative to the initial free laccase activity (laccase activity recovered from biocatalyst divided by initial free laccase activity and multiplied by hundred) increased when increase in EDC concentration. Preliminary results indicate that the immobilization of laccase on biopolymer chitosan using EDC appears to be a promising procedure for the preparation of solid biocatalyst for several industrial applications.
P8.17: BIODEGRADATION OF TETRABROMOBISPHENOL A AND BROMINATED PHENOLS BY TRAMETES VERSICOLOR

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Tetrabromobisphenol A (TBBPA) is a bulk brominated flame retardant used to improve fire safety of electrical and electronic equipment. 2,4,6-Tribromophenol (TBP) is also produced industrially as reactive flame retardant intermediate and pesticide component. Together with bromo- and dibromophenols, it also originates from the combustion of leaded fuels. Bromophenols are also found as natural products in marine environments. The presence of these compounds in the environment raised concerns, as they are known or suspected of having multiple adverse effects on living organisms (endocrine disruption effects, toxicity to aquatic life etc.).

Little is known about microorganisms useful as potential degraders of the brominated aromatic compounds. Among eukaryotic organisms, white rot fungi belong to the best xenobiotic degraders. There are well-known for their remarkable biodegradation ability towards synthetic dyes1. Biodegradation activity of these fungi and their laccases towards bisphenol A was also reported2.

In the present work, we examined the biodegradation potential of Trametes versicolor CCBAS 612 towards the structurally similar TBBPA, as well as TBP and mono- and dibrominated phenols. All examined compounds (see Fig. 1) were efficiently degraded by this organism. A significant removal of the compounds from the cultivation medium was observed already after 1 day, and the residual concentrations after 7 days largely did not exceed 20% of the initial ones that were 1 mM except for TBP and TBBPA (0.2 mM). It is generally accepted that ligninolytic enzymes play a key role in the breakdown of xenobiotic compounds by white rot fungi, but other factors like hydrogen peroxide may also affect the degradation process. Therefore, the activities of laccase and manganese peroxidase, as well as the hydrogen peroxide concentrations were monitored during the process [2]. The utility of application of the purified laccase from Trametes versicolor, instead of whole-cell cultures, for brominated compound degradation is also discussed.

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