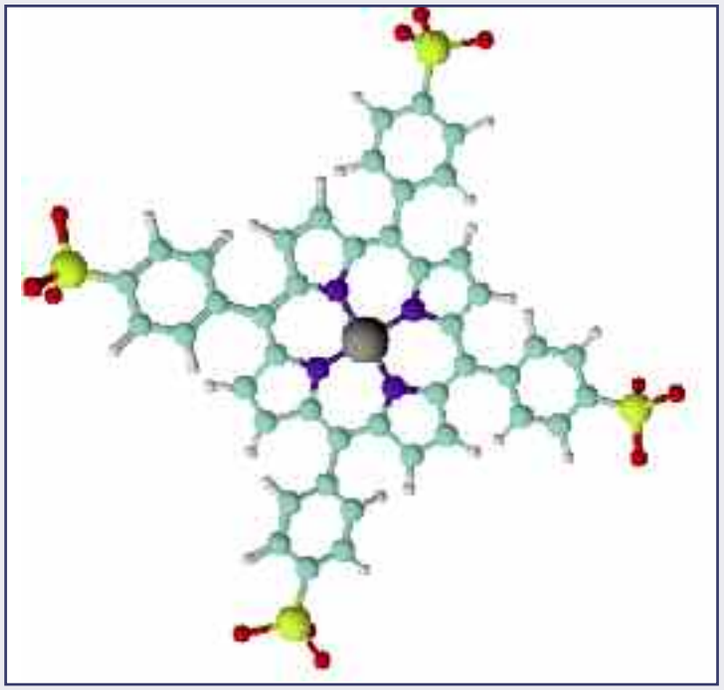

Paolo Zucca[a], Enrico Sanjust[a,b]
[a] Sezione di Chimica Biologica e Biotecnologie Biochimiche Dipartimento di Scienze e Tecnologie Biomediche (DISTEB) Università di Cagliari
[b] Consorzio per lo Sviluppo dei Sistemi a Grande Interfase (CSGI) Sesto Fiorentino (FI)
pzucca@unica.it

BIOINSPIRED ROUTES TOWARDS DELIGNIFICATION

Classical approaches towards delignification seem to be inadequate, particularly in the perspective of a sustainable green process. As immobilized metalloporphines can strictly emulate ligninolytic peroxidases active site, their use in delignification processes is presented and future trends are outlined.

Environmental persistence and polluting effect are not peculiar of non-natural chemicals. Lignocellulosic materials are widespread wastes, totally deriving from natural sources. They represent, however, highly recalcitrant pollutants, released in the environment from various sources in considerable amounts: every year ~200x10^9 tons ot plant biomass are produced [1]. Moreover, these materials represent not only polluting wastes, but also potential sources of important chemicals (cellulose, bioethanol, vanillin, catechol and other aromatic compounds) [2, 3].

Three major components can be identified in lignocelluloses: cellulose, hemicelluloses and lignin. The first two components are relatively easily to be hydrolyzed and therefore degraded [2]. On the contrary, lignin represents the most durable constituent: accordingly, its oxidative degradation represents the real challenge in order to achieve complete exploitation of cellulose, both in pulp/paper industry and in bioethanol production.

Lignin chemical stability arises from its randomly radical polymerization, that leads to the formation of a compact structure, in which several types of bonds can be found (Fig. 1): none of them hydrolyzable [2]. This makes delignification a challenging task from a chemical point of view: particularly considering the industrial demand of an inexpensive process, possibly avoiding damages to more reactive cellulose.

Classical approaches towards delignification

Many chemicals are currently employed in delignification processes, such as Cl_2, ClO_2, O_2, O_3, NaOH, H_2O_2, also combined with metallic salts [4]. These reactions are usually performed in sequence under high temperature/pressure conditions, with intermediate washes and reductive treatments. However, it is difficult to achieve an effective lignin removal without affecting cellulose fibers structure. Besides, such processes are featured by extreme operational conditions, high

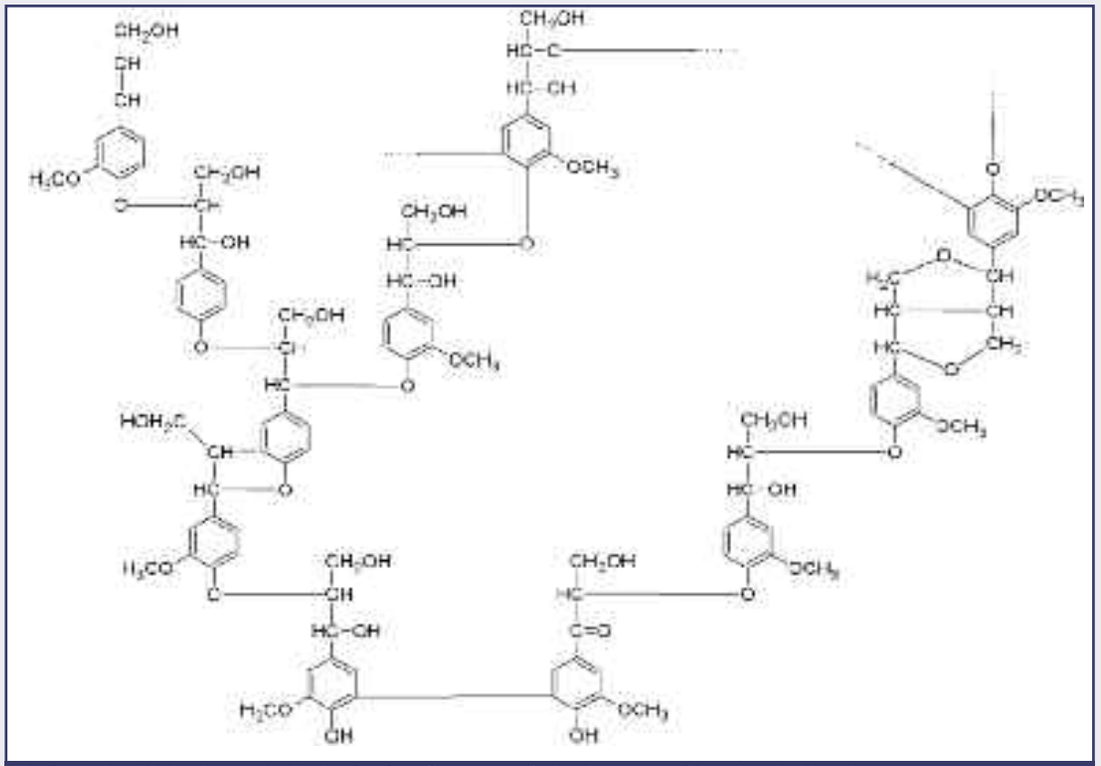

Fig. 1 - A simplified scheme of a lignin structure (from spruce) showing some of the chemical bonds, commonly found in lignins

costs, large water demand and hazardous wastes release (containing for example furans or polychlorinated dioxins) [5, 6]: this poses a serious concern as clearly stated by many international organizations [7, 8].

In order to achieve a more sustainable delignification process, milder operational conditions and more environmentally friendly oxidants should be considered. Accordingly, biocatalytic approaches have been proposed.

In particular, huge interest has arisen towards White Rot Fungi, the only organisms among whole Nature able to completely degrade lignin [2]. These Basidiomycetes secrete two fundamental class of ligninolytic enzymes: laccases and peroxidases.

Laccases (Lc) are multicopper phenol-oxidases that operate monoelectron oxidation of their substrates, reducing molecular oxygen to water [9]. Lc substrates are usually low MW phenols, whose oxidation products (phenoxy radicals) could act as redox mediators in lignin oxidation. When Lc alone acts on lignin, only partial oxidation without substantial solubilization take place, unless a suitable redox mediator is present.

Ligninolytic peroxidases (LPs) are three hemo-enzymes (lignin peroxidase LiP, manganese peroxidase MnP, and versatile peroxidase VP) with very similar structures that differ only for substrate specificity [10]. Each enzyme is characterized by a protoporphyrin IX prosthetic group, containing a ferric ion, which is coordinated in proximal position by imidazole-*N* of a histidine residue (Fig. 2a).

In the presence of H_2O_2, Fe(III)- protoporphyrin IX is first oxidized to Compound I, a two-electron-oxidized intermediate (Fig. 2b). This π-cation radical is then reduced to native form (*via* Compound II intermediate) in two monoelectronic steps: in each one a substrate molecule reduces oxidized form of the enzyme by losing one electron.

Even if LPs are able to attack directly lignin, redox mediators of the reaction are mandatory to achieve reasonable delignification rates. MnP oxidizes Mn^{2+} to Mn^{3+}, which acts as a redox mediator. Instead, aromatic compounds (both phenolic and non-phenolic) are effective substrates of LiP: in particular it shows best affinity for 3,4-dimethoxybenzyl alcohol (veratryl alcohol, VA), another redox mediator. VP is able to oxidize both LiP and MnP substrates.

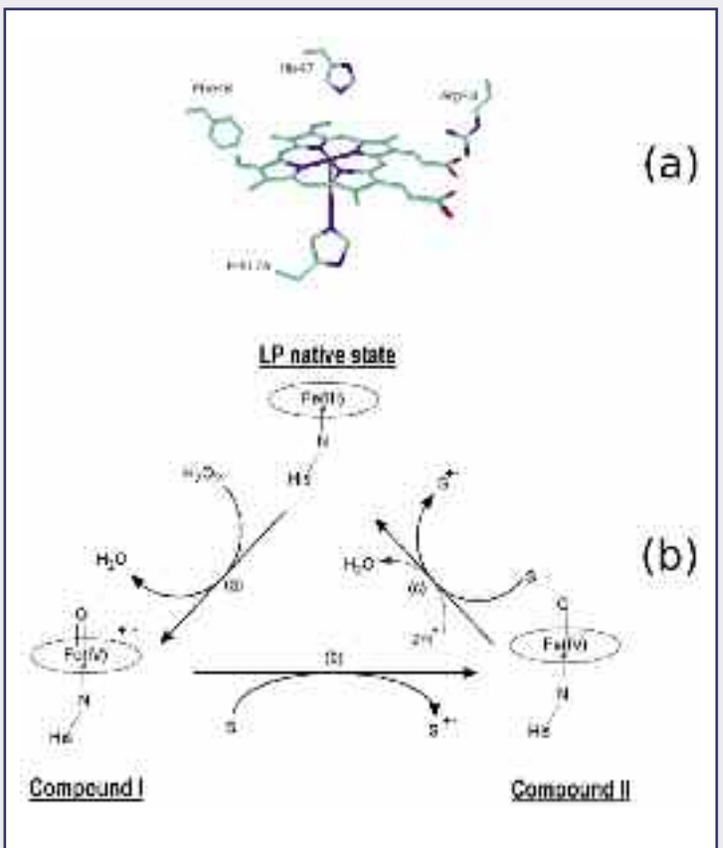

Fig. 2 - Schematic representation of ligninolytic peroxidases active site (a) and catalytic cycle (b)

Up to now, industrial applications of these enzymes are, however, prevented by high purification costs, low stability and need of other cellular components in order to complete lignin degradation. Redox mediators, in fact, must be almost continuously supplied in reaction media, as they are highly reactive species unavoidably undergoing irreversible degradation. Moreover, many other proteins seem to be involved in delignification process. It has been demonstrated that *in vivo* lignin oxidation requires the synergistic action of other enzymes such as cellobiose: quinone oxidoreductase, glucose oxidase, aryl alcohol oxidase, glyoxalate oxidase [11, 12]: only in this way reactive oxygen species (ROS) are produced (through a quinone redox cycle) and reaction becomes efficient. In this perspective, it is not surprising that purified peroxidases or laccases singly do not delignify intact lignocellulose *in vitro* [13, 14].

The only feasible way could be the use of whole fungal cells in delignification process, which could constantly supply redox mediators and all the other indispensable enzyme activities. But also this approach does not seem to be affordable, due to very slow fungal metabolism and large biomass contamination (by the fungal hyphae) of reaction products (and related expensive separation).

An innovative way: "bioinspired" catalysts

In order to overcome these drawbacks, new generations of biomimetic catalysts have been developed, with the common aim of emulating LPs activity, under mild operational conditions, with the most inexpensiveness of the process.

Several biomimetic methods have been proposed involving metalloporphines, metallophthalocyanines, polyoxometalates and iron(III) tetraamido macrocycles [15-18]. Among them, only metalloporphines strictly resemble LPs cofactor (compare Fig. 2a) and seem to be suitable for real emulation of their active sites.

Metal complexes of porphines and porphyrins (mainly Mn and Fe) have proven able to catalyze many oxidation and oxygenation reactions with several monoxygen donors (iodosylbenzene, NaClO, H_2O_2, peroxyacids etc.). These studies have been mainly addressed to the emulation of cytochrome P450 in hydrocarbons oxygenation, but also to oxidation of amines, *N*-demethylation of secondary aromatic amines or oxidative chlorinations [15, 19], while LPs emulation has been quite less investigated.

For these purposes, the use of natural metalloporphyrins has been prevented by their insolubility in common solvents and low stability in oxidizing environment: luckily, a broad range of synthetic analogues (mainly unsubstituted in the β positions, therefore being more correctly called metalloporphines) are available since some decades (Fig. 3).

The synthesis of the ancestor of this family of molecules was firstly described in 1935, when Rothemund boiled benzaldehyde and pyrrole in refluxing propionic acid to easily afford 5,10,15,20-tetraphenylporphine (**1** in Fig. 3). Only more recently catalytic activity of the complexes Fe- and Mn-**1** have been studied, but they were very unstable catalysts being degraded by their own substrates (iodosylbenzene and NaOCl) [15]. However, the way was open and Mn- and Fe-**1** deserved the label "first generation catalysts".

In a short time, it became clear that increasing electron-withdrawing power of *meso*-substituents would lead to higher stability and activity of the catalyst. This could be achieved through the presence of halogen atoms as substituents in the *meso* phenyl groups. Those were the so-called "second generation catalysts". In particular the insertion of two chlorine atoms in the 2,6 position of phenyls (**2**) had positive

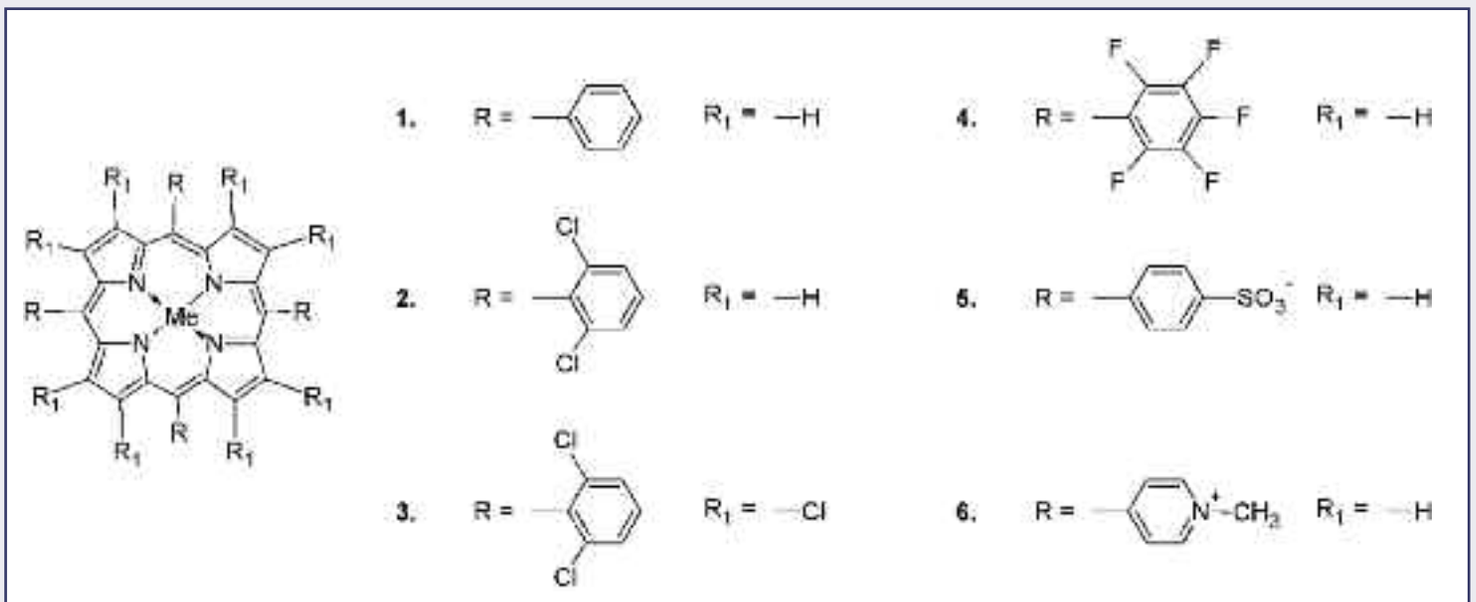

Fig. 3 - Chemical structures of most studied metalloporphines. Legend: **1** 5,10,15,20-tetrakisphenylporphine; **2** 5,10,15,20-tetrakis(2,6-dichlorophenyl)porphine; **3** β-octacholoro-5,10,15,20-tetrakis(2,6-dichlorophenyl)porphine; **4** 5,10,15,20-tetrakis(pentafluorophenyl)porphine; **5** 5,10,15,20-tetrakis(4-sulfonatophenyl)porphine; **6** 5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphine

effects: Fe and Mn-**2** were capable of high conversion of the substrates without significant loss of activity, and this led to a dramatic improvement of performances.

Also β-octa-halogenated porphines (like **3**) were synthesized: however, catalytic studies were quite conflicting and not very promising.

More efficient seemed to be perhalogenated porphines in the phenyl positions: in particular metal complexes of 5,10,15,20-tetrakis(pentafluorophenyl)porphine (**4**) and their derivatives excel for stability and catalytic efficiency.

Metal complexes of **4** are, though, not water soluble: that could be overcome by sulfonating **4** β-positions, or by completely changing *meso* electron-withdrawing pendants. Both use of 4-sulfonatophenyl (**5**) and 4-methyl-pyridinio (**6**) substituents allowed the synthesis of stable, efficient and less expensive catalysts.

Metal complexes of **4**, **5** and **6** could be easily synthesized under laboratory scale, but they are also commercially available and represent promising base for development of pre-industrial delignification processes.

Metalloporphines emulate ligninolytic peroxidases reactions

First investigations about biomimetic degradation of lignin and lignin model compounds were performed with natural heme using *t*-buthylhydroperoxide (*t*BuOOH) as the oxidant [20]. Catalyst insolubility and instability under oxidizing conditions suggested however employment of next generation porphines.

Fe-β-sulfonated-**2**, and both Fe- and Mn-**5** showed better stability during aqueous oxidation of VA and other lignin model compounds [21, 22], with a product selectivity very similar to LiP.

In those experiments $KHSO_5$, *t*BuOOH or *m*CPBA were employed as the oxidants: however, the perfect oxidant from an industrial point of view should be inexpensive, totally miscibile with H_2O (no organic solvent should be needed in a "green" process), and its degradation products should not be harmful. From this perspective, the best "clean" oxidant can only be H_2O_2, as its decomposition produces only O_2 and H_2O.

H_2O_2 has been proposed as oxidant by Artaud and coworkers in 1993 [23]: several Fe metalloporphines (such as **2, 4** and β-sulfonated-**4**) were investigated though in a partially organic reaction mixture. Those catalysts led to significant yields with many lignin model compounds.

The oxidation of real lignins has been showed few years later. Kurek and coworkers [24] noticed effective oxidative degradation of spruce lignin in presence of Fe-β-sulfonated-**4** and H_2O_2. The reaction was managed at very mild temperature (22 °C) but in large presence of organic solvent (a mixture 9:1 dioxane:H_2O).

Crestini and coworkers in 1999 [25] were able to completely eliminate organic solvent during their screening of some hydrosoluble metalloporphines (the most interesting being Mn-**5** and Mn-**6**). Residual kraft lignin was oxidized in presence of H_2O_2 at a slightly higher temperature (50 °C) but under very mild pH conditions (pH 6 in citrate buffer).

Catalyst immobilization: a compulsory goal

Those studies were very promising, but did not allow an immediate application of metalloporphines in delignification processes.

From an economical point of view, catalyst recovery after reaction is essential, in order to completely exploit its activity. Besides, complete toxicological studies of porphines have not yet been completed: so they cannot be released, but must be (perhaps easily) removed from any waste.

Moreover, when metalloporphines work free in solution, they are less stable as side reactions can occur, such as μ-oxo dimers (catalytically inactive) formation or homolytic cleavage of O-O bond to yield Fe^{IV}-OH and •OH [26].

In these perspectives, immobilization of the catalysts on a solid support should be considered as a mandatory aim.

Many approaches have been developed to immobilize metalloporphines: adsorption, ion-exchange and covalent bond formation in particular. In all cases catalysts seem to retain much of their activity. Just few studies, however, are reported about LPs-like activity of those immobilized metalloporphines.

Labat and Meunier [21] emulated LiP activity by immobilizing Mn- and Fe-**5** on ion-exchange resin Amberlite IRA 900: catalyst stability was noticeably enhanced, and repeated use gave excellent results.

More recently Crestini and coworkers [27] used supported Mn-**6** on a smectite clay to achieve oxidation of lignin and lignin model compounds. Operational conditions were extremely mild and only temperature was quite high (ranging 60-90 °C), but no organic solvent was

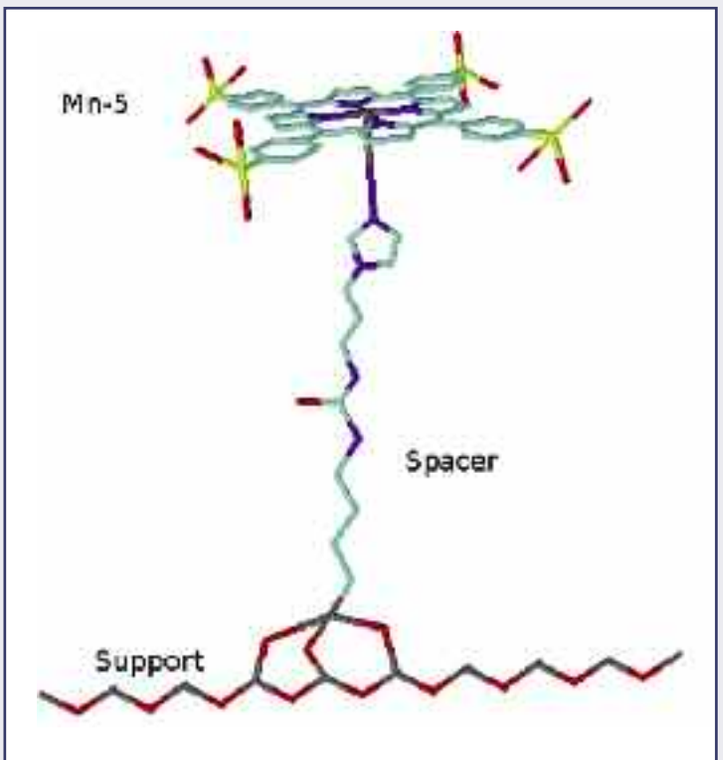

Fig. 4 - Schematic representation of catalytic sites described in [30]. Coordinative bond between Mn-5 and supported imidazole guarantees a real LiP emulation

necessary saving dioxane to solubilize reagents. Also in this case porphine stability seemed to be improved: repeated use of the catalyst was efficient, leading to quite high conversion rate.

Metal axial ligand greatly affects catalyst activity

Not even in those cases, however, real LPs emulation was achieved.
In these enzymes, fifth coordination position of heme iron is occupied by imidazole-*N* of proximal histidine (Fig. 2a): in this perspective closer emulation of their active site requires the presence of an appropriate axial ligand, perhaps imidazole.
Moreover, fundamental effects of axial ligand for porphine catalytic efficiency have been exhaustively demonstrated [15]. Using free bulk ligands, stabilization of high valence metal oxo species has been observed: this can be a problem though, when ligand/porphine interaction is too strong. It can lead to bis-ligated form which can interfere with oxygen donor/metal proper interaction, which is certainly more favoured with mono-ligated species. Moreover, if stabilization of high valence metal species is too strong, reduction of oxidized metalloporphine species could be slower. Besides, electron-deficient ligands are also able to increase redox potential of oxidized metalloporphines species, speeding up catalytic cycle completion.
Good ligands have also proven able to affect porphines chemio- and enantioselectivity and to facilitate heterolytic cleavage of O-O bond. This prevents formation of •OH, the main responsible of undesired cellulose damage during delignification processes.
Several possible ligands have been proposed: pyridine and alkyl substituted derivatives, *N*-amine oxides, and the more bioinspired imidazole.
Among them only the latter allows a real emulation of LPs active site (Fig. 2a). Moreover, its positive catalytic effects have been clearly demonstrated [28].
However, all those studies have been performed using free ligands.
The need of a good axial ligand for metalloporphine can be, however, combined with that of a suitable immobilization of the catalyst. This can be achieved properly by grafting imidazole residues on solid supports, and immobilizing metalloporphines on these through a coordinative bond.
Such generation of catalysts can be referred as real LPs emulators.
This approach, moreover, avoids the continuous supply of ligands in a hypothetical multicyclic use of the catalyst: it means an economical saving of the process. Besides, if the support grafting is properly operated, only mono-ligated metalloporphines can be obtained.

Real ligninolytic peroxidases emulation

Metalloporphine immobilization on an imidazole-grafted support has been already described [29].
In that study 3-imidazolyl-propyl trimethoxysilane was used to functionalize silica gel surface, on which Fe-**6** was then supported. The adduct was not characterized about its LiP-like activity: however, it showed a poor performance in hydroxylation and epoxidation of hydrocarbons.
This can be partially explained with the very short and hydrophobic spacer between metalloporphine and support. In order to completely emulate enzyme activity, a longer, hydrophilic and flexible spacer should be considered.
This has been achieved in 2007 [30]. Comparison between Fig. 2a and Fig. 4 clearly shows strong similarities between LPs and biomimetic catalytic sites. (3-isocyanatepropyl)triethoxysilane and *N*-(3-aminopropyl)imidazole reacted to yield an imidazolyl silane used to functionalize a silica gel. The product was used to coordinatively support Mn-**5**. The highly hydrophilic support obtained was featured by a long and flexible spacer.
Many soluble lignin model compounds were easily oxidized by this catalyst under extremely mild operational conditions (pH, temperature, low [H_2O_2], no organic solvents), without any metalloporphine leakage: a real green process has been obtained. Deep oxidative action suggested strong similarities with LiP catalysis.
Although quite less bioinspired, also pyridine is a promising coordinative ligand that can be used in metalloporphine immobilization. Pyridine, in fact, is quite more electron-withdrawing compared to imidazole: this could lead to higher redox potential of coordinated metalloporphine.
Some investigations have already been attempted [16], but pyridine seems to be weaker than imidazole as a metalloporphine ligand.

Conclusions

Emulation of ligninolytic peroxidases is a challenging task with many promising industrial applications.
This should be based on robust and catalytically efficient metalloporphines properly immobilized on solid supports that also provide axial

ligands. The reported studies showed that this structural emulation of peroxidases also leads to functional emulation.
These results are economically and environmentally crucial as they imply mild operational conditions, strictly resembling natural delignification pathway. In other words, a really green process.
The usefulness of these catalysts can be underlined by their activity towards other important polluting chemicals (i.e. olive mill wastewaters and textile dyes [31]): this can further extend their practical applications, and deserves more investigations.

However, in the perspective of lignin removal process, those heterogenous catalysts should face the necessity of redox mediation, in order to overcome mass transfer issues. Crestini and coworkers [27] already found 1-hydroxybenzotriazole as efficient mediator in immobilized metalloporphines oxidation of lignin. But, in order to maintain the process as "green" as possible, also redox mediator should be harmless and sustainable: in this perspective, the immediate aim of scientific community should be not stricly LiP, but rather MnP emulation. As only Mn^{2+} could represent the cleanest redox mediator.

Paolo Zucca is a 26 years old PhD student in Biochemistry and Molecular Biology (Cagliari University). He received Industrial Biotechnology Degree in 2004, Applied and Experimental Biology MSc in 2006 and Specialistic Master in Food Safety in 2007 (Cagliari University). From 2005 he is young researcher at Biological Chemistry Section (Cagliari University).

Enrico Sanjust was born in 1957 and received his Degree in Chemistry in 1980; he gained his PhD in Biotechnology in 1994. He is associate professor at Cagliari University since 2000. His main research interests are in the field of biomimetic catalysis and applied enzymology, with particular focus on fungal phenol oxidases.

References

[1] Y. Lin, S. Tanaka, *Appl. Microbiol. Biotechnol.*, 2006, **69,** 627.
[2] K.-E.L. Eriksson, R.A. Blanchette, P. Ander, Microbial and Enzymatic Degradation of Wood and Wood Components, Springer-Verlag, Berlin, 1990.
[3] H.M. Biorsvik, F. Minisci, *Org. Process Res. Dev.*, 1999, **3,** 341.
[4] G.A. Smook, Handbook for Pulp & Paper Technologists, Angus Wilde Publications Inc., Vancouver, 1997.
[5] M. Gavrilescu *et al., Eng. Life Sci.*, 2008, **8,** 99.
[6] L.M. Hewitt *et al., Environ. Toxicol. Chem.*, 2008, **27,** 682.
[7] EPA, Handbook on Pollution Prevention Opportunities for Bleached Kraft Pulp and Paper Mills, Washington DC USA, 1993.
[8] European Commission, Reference Document on Best Available Techniques in the Pulp and Paper Industry, 2001.
[9] H.D. Youn *et al., FEMS Microbiol. Lett.*, 1995, **132,** 183.
[10] A.T. Martinez, *Enzyme Microb. Technol.*, 2002, **30,** 425.
[11] A. Leonowicz *et al., Fungal Genet. Biol.*, 1999, **27,** 175.
[12] F. Guillen *et al., Arch. Biochem. Biophys.*, 2000, **383,** 142.
[13] H. Galliano *et al., Enzyme Microb. Technol.*, 1991, **13,** 478.
[14] K.E. Hammel, D. Cullen, *Curr. Opin. Plant Biol.*, 2008, **11,** 349.
[15] A.M.A. Rocha-Gonsalves, M.M. Pereira, *J. Mol. Catal. A: Chem.*, 1996, **113,** 209.
[16] B. Meunier, in Biomimetic Oxidations Catalyzed by Transition Metal Complexes, B. Meunier (Ed.), Imperial College Press, London, 2000, 171.
[17] C.P. Horwitz *et al., ACS Symp. Ser.*, 2006, **921,** 156.
[18] A.R. Gaspar *et al., Green Chemistry*, 2007, **9,** 717.
[19] D. Mansuy, *C.R. Chimie*, 2007, **10,** 392.
[20] A. Paszczynski *et al., Appl. Environ. Microbiol.*, 1988, **54,** 62.
[21] G. Labat, B. Meunier, *J. Org. Chem.*, 1989, **54,** 5008.
[22] F. Cui *et al., J. Biotechnol.*, 1993, **30,** 15.
[23] I. Artaud *et al., J. Org. Chem.*, 1993, **58,** 3373.
[24] B. Kurek *et al., J. Agric. Food Chem.*, 1996, **44,** 1953.
[25] C. Crestini *et al., Bioorg. Med. Chem.*, 1999, **7,** 1897.
[26] B. Meunier, Metalloporphyrins catalyzed oxidations, in F. Montanari, L. Casella, (Eds.), Kluwer Academics Publishers, Dordrecth, 1994, 11.
[27] C. Crestini *et al., Eur. J. Inorg. Chem.*, 2004, **22,** 4477.
[28] M. Moghadam *et al., J. Mol. Catal. A: Chem.*, 2004, **217,** 9.
[29] Y. Iamamoto *et al., J. Mol. Catal. A: Chem.*, 1995, **99,** 187.
[30] P. Zucca *et al., J. Mol. Catal. A: Chem.*, 2007, **278,** 220.
[31] P. Zucca *et al., J. Mol. Catal. A: Chem.*, 2008, **288,** 97.

RIASSUNTO

Approcci biomimetici di delignificazione

La rimozione della lignina costituisce una sfida industriale ancora aperta. Gli attuali approcci (compreso quello biocatalitico) non eccellono per economicità e sostenibilità del processo. Le perossidasi ligninolitiche tuttavia costituiscono ottimi modelli per la sintesi di catalizzatori biomimetici in grado di operare una delignificazione più ecocompatibile. In questa review sono discussi i tentativi finora effettuati e le future prospettive verso un processo sostenibile.