CHEMICAL ENGINEERING TRANSACTIONS
VOL. 32, 2013
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

ISBN 978-88-95608-23-5; **ISSN** 1974-9791
DOI: 10.3303/CET1332161

Fungal Laccases and Enoate Reductases as Biocatalysts of Fine Chemical Transformations

Alice Romagnolo[a], Federica Spina[a], Debora Carusetta[a], Luca Nerva[a], Maria Rita Cramarossa[c], Fabio Parmeggiani[b], Luca Forti[c], Elisabetta Brenna[b], Giovanna Cristina Varese[a]

[a] Department of Life Science and Systems Biology, University of Torino, viale Mattioli 25, 10125 Torino, Italy
[b] Department of Chemistry, Materials and Chemical Engineering, Politecnico of Milano, via Mancinelli 7, 20131 Milano, Italy
[c] Department of Life Science, University of Modena and Reggio Emilia, via Campi 183, 41100 Modena, Italy

Laccase and enoate reductase are promising tools in bioconversion of several substrates of biotechnological interest. In this study, one fungus producing laccase and two fungi producing enoate reductase were used in the bioconversion of caffeic acid and a set of unsaturated compounds (α-methylcinnamaldehyde, α- methylnitrostyrene and cyclohexenone) showing interesting conversion products.

1. Introduction

Biocatalysis has been considered as a promising area of research, in particular for the development of new sustainable technologies for the production of high-value molecules. In particular biotransformations are becoming competitive compared to traditional chemical synthesis due to the high chemo and stereoselectivity, mild condition of work and low environmental impact. Enzymes are powerful tools but only few of them are available on the market and they are often not active towards some substrates of interest (Liese et al., 2006).
New biocatalysts capable of specific synthetic requirements have to be searched in different organisms to exploit natural biodiversity looking for enzymes with interesting features, in order to use them in the conversion of various substrates.
In this context, many studies have already been focused on fungi and their enzymatic pattern as potential biocatalyst agents. The fungal potential is mainly ascribable to their various and heterogeneous enzymatic pattern. Among fungal enzymes, laccases (LAC) and enoate reductases (ER) can be used as biocatalysts in several biotransformations.
LAC belong to the “multicopper oxidases” family and are widely distributed in nature; enzymes belonging to this family have been described in plants, bacteria and fungi (Lundell et al., 2010). They may oxidise phenolic compounds and, thanks to the help of specific mediators, they are also active towards non phenolic compounds. In general, they have low substrate selectivity being able to oxidise many aromatic molecules. LAC are widely used in biotechnology and were among the first enzymes to be used in large-scale approaches (Ponzoni et al., 2007) and are widely studied in sperimental approaches (Lloret et al., 2012). However few of them have already become commercially available and hence the central goal of the research is always the identification of new enzymes, with promising potential, able to enlarge the substrates spectrum.
ER are flavin-dependent oxidoreductases belonging to the “old yellow enzyme” family, they are quite ubiquitous in different organisms (plants, bacteria and fungi) but to date their physiological function has not been still identified. ER catalyses stereoselective reduction of activated C=C bonds in presence of a nicotinamide cofactor. This reaction is one of the most powerful tool to generate up to two stereogenic centers in one step reaction. Moreover, many authors demonstrated that these enzymes have the ability to

Please cite this article as: Romagnolo A., Spina F., Carusetta D., Nerva L., Cramarossa M.R., Parmeggiani F., Forti L., Brenna E., Varese G.C., 2013, Fungal laccases and enoate reductases as biocatalysts of fine chemical transformations, Chemical Engineering Transactions, 32, 961-966 DOI: 10.3303/CET1332161

reduce a wide variety of substrates such as unsaturated aldehydes, ketones and esters, nitro olefins and nitro aromatic compounds (Brenna et al., 2012).
The aim of this study was to identify fungi producing LAC and ER to be used in the biotransformation of different types of substrate with particular emphasys to caffeic acid (CA), α-methylcinnamaldehyde (α-MCA), α-methylnitrostyrene (α-MNS) and cyclohexenone (CE). All these substrates are important precursors of flavours, polymers, antioxidants, anticoagulants and pharmaceuticals.

2. Materials and methods

2.1 Chemicals

All the chemicals were purchased by Sigma-Aldrich (Milan, Italy) with the only exception of the substrates. CA was from Fluka (Sigma-Aldrich Co, USA). Alfa-MCA, α-MNS and CE were provided by Department of Chemistry, Materials and Chemical Engineering, Politecnico of Milano.
Stock solutions (500 mM) of each substrate were prepared by dissolving substrates in dimethyl sulfoxide (DMSO) and maintained at 4 °C.

2.2 Fungal strains

All strains used are preserved at *Mycotheca Universitatis Taurinensis* Collection.
Pleurotus ostreatus MUT 2976 was selected because, in previous experiments, it was able to produce high concentrations of LAC and mediate extensive oxidation reaction towards different organic molecules (Casieri et al., 2008).
Penicillium citrinum MUT 4862 and *Absidia glauca* MUT 1157 were choosen for their putative capability to produce ER.

2.3 Caffeic acid biotransformation

P. ostreatus MUT 2976 was inoculated as 3 mL fungal suspension in 50 mL Erlenmeyer flasks containing 30 mL of Culture Medium (CM: 20 g/L glucose, 20 g/L malt exctract, 2 g/L peptone). The fungal inoculum was prepared by homogenizing agar squares (1 cm^2) derived from the margins of an overgrown colony together with sterile CM (1 mL per each cm^2) using an Ultraturrax (IKA, Staufen, Germany). Flasks were maintained in continuous agitation (110 rpm). After three days of pre-growth, CA (5 mM final concentration) was added in presence or absence of $CuSO_4$ (1 mM), a known inducer of LAC production (Baldrian and Gabriel, 2002). After 48 h the biomass dry weight and the LAC activity were monitored according to Anastasi et al. (2012). LAC assay was performed at 25 °C using 2.2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) as substrate. Enzyme activities were expressed in Units per L (U/L), where one unit is defined as the amount of enzyme that oxidise 1 µmol of substrate in 1 min.
Analysis of bioconversion products were performed by LC-MS(n)-Ion Trap. Tests were performed in triplicate.

2.4 α-methylcinnamaldehyde, α-methylnitrostyrene and cyclohexenone biotransformation

The experiment was performed as described previously for CA biotransformation with few modifications.
P. citrinum MUT 4862 and *A. glauca* MUT 1157 were inoculated by means of conidia suspension (≈ 33,000 conidia/mL). After three days of pre-growth α-MCA, α-MNS and CE, in DMSO solution (1 % v/v final concentration) were separately added (5 mM final substrate concentration). After 2, 4 and 7 days, 1 mL of culture broth was collected and stored for product analysis performed with GC-MS. At the end of each experiment, the dry weight biomass was calculated. For each substrate tests were performed in triplicate.

3. Results and discussion

3.1 Caffeic acid biotransformation

In agreement with the literature, high concentrations of LAC were detected in the presence of Cu, the line CA+$CuSO_4$ displayed a LAC production about 40 times higher than CA-$CuSO_4$ (Figure 1A). However, it should be noted that the presence of Cu inhibited the production of biomass (Figure 1B). On the other hand, CA did not induce LAC production in *P. ostreatus*. These results are in accordance with the literature, which indicates Cu as one of the main LAC inducer but with toxic effect on mycelia (Baldrian and Gabriel, 2002).

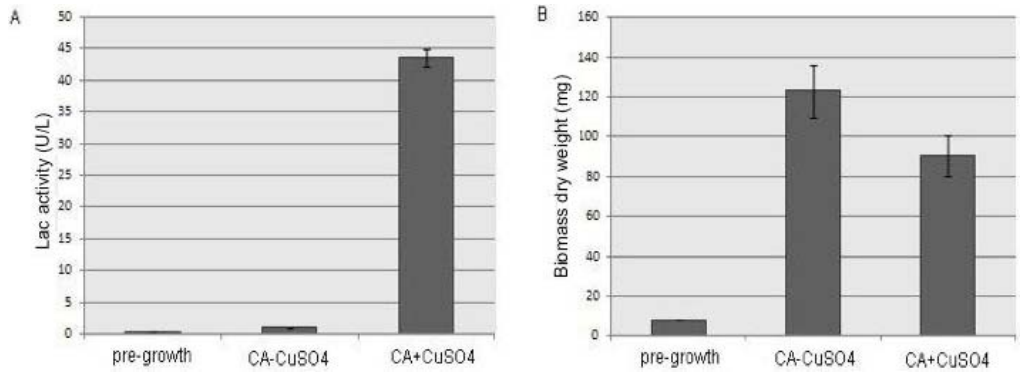

Figure 1: LAC activities (U/L) (A) and biomass dry weight measurements (mg) (B) at the beginning and after two days of culturing, in absence and presence of Cu.

Despite the differences in enzyme production, both cultural lines showed comparable conversion trends. Chromatographic analysis clearly showed that LAC mediated CA bioconversion producing at least 5 dimers and a trimer (Figure 2). The formation of this products are due to oxidation reactions mediated by LAC of *P. ostreatus* with the consequent formation of at least three active radicals (Figure 3) that may react each others producing different dimers and polymers.

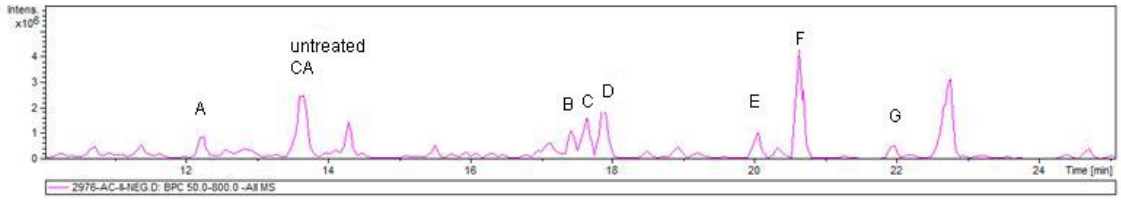

Figure 2: chromatogram showing the products of CA biotransformation by P. ostreatus.

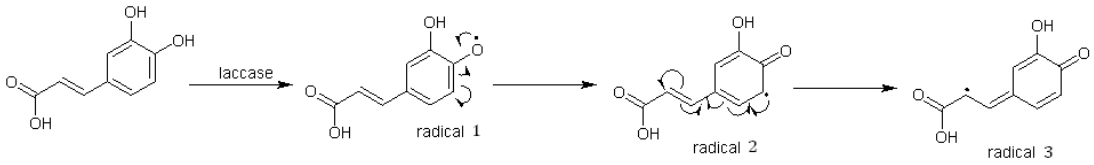

Figure 3: oxidation reaction mediated by LAC of P. ostreatus with the consequent formation of three types of radicals.

The most important class of products of the biotransformation by *P. ostreatus* are CA dimers having caffeicin-like structure (peaks A, B, D, E, F, G) i.e. arising from the addition of two OH groups of a CA molecule to the C-C bond of another molecule. In particular, LAC allowed the formation of different bonds: C-C linkage (Figure 4) or C-O linkage (Figure 5).

More in detail, products related to peak A were more polar than CA itself and then eluted faster from the reverse phase column. The formation of hydroxylated dimers can be easily explained by the coupling of radical 1 or radical 2 with radical 3, followed by addiction of a H_2O molecule to the intermediate quinones (Figure 4A; Figure 5B). Peak B is related to a dimer that arose from the coupling of two radical 2 that led the formation of a C-C linked dimer (Figure 4B). Almost identical MS spectra were observed for peaks D and E; dimer formation was due to the coupling of radical 2 and 3 and led to the formation of a dihydrobenzofuran structure (Figure 4C). Identical MS spectra were also observed for peaks F and G; dimer was formed through the coupling of radical 1 and radical 3 (Figure 5B).

Trimer derivatives were also detected (peak C); however, their structures could not be elucidated from MS/MS data.

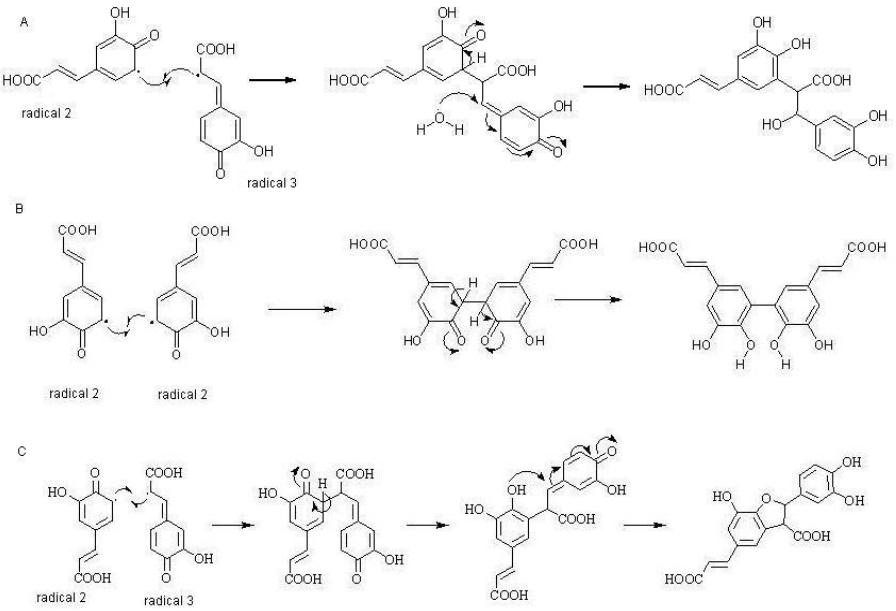

Figure 4: C-C bonds formation; α-hydroxy-β-5 dimer (A), C5-C5' linkage (B), formation of 4-O- α-β-5 dimer (C).

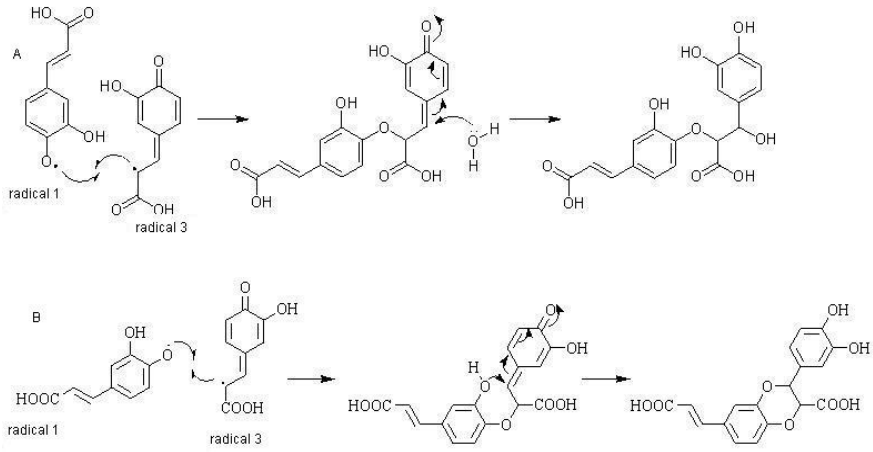

Figure 5: C-O bonds formations; coupling of radical 1 and 3 and formation of α-hydroxy-β-O-4 dimer (A) and formation of 3-O-α-β-O-4 dimer (B).

On the whole, the conversion of CA by LAC of *P. ostreatus* gave rise to five different dimers that derived from the different combination of the radicals 1, 2 and 3. These biotransformations are very interesting from a biotechnological point of view, since the main purpose of CA biotransformation is dimers formation. In fact, these molecules have attractive features, being used as anticoagulants, antioxidants, antitumors and antiparasitics (Moussouni et al., 2011).
These preliminary results showed that LAC of *P. ostreatus* are very effective in the biotransformation of CA. Future perspectives will be to evaluate the possibility to optimize the bioconversion rate of CA to dimers using the crude exctract, the partial purified enzymes and immobilized enzymes.

3.2 α-methylcinnamaldehyde, α-methylnitrostyrene and cyclohexenone biotransformation

The presence of α-MCA and α-MNS always inhibited the biomass of both *A. glauca* and *P. citrinum*; the same was true for CE towards *A. glauca.* The only exception was CE towards *P. citrinum,* which caused a

significant increase of biomass that, at the end of the experiment, was almost double (Figure 6). Thus, this substrate and/or its bioconversion products probably stimulated the growth of *P. citrinum*

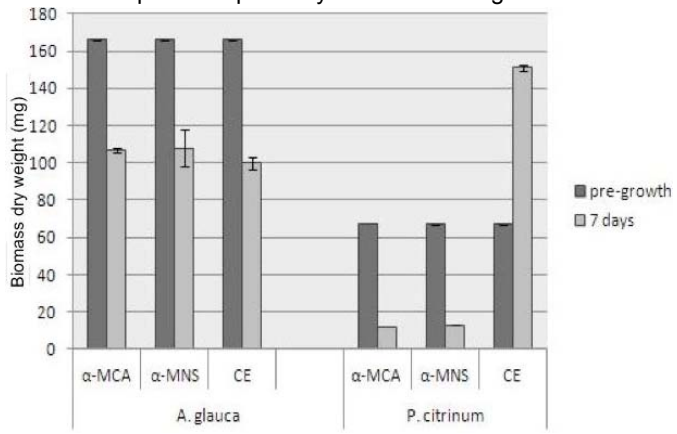

Figure 6: A. glauca and P.citrinum biomass dry weight mesurements (mg) at the beginning and after 7 days of culturing.

The results of the GC/MS analysis of the transformation products of α-MCA, α-MNS and CE by *A. glauca* and *P. citrinum* are shown in Figure 7.
Alfa-MCA was mainly converted into cinnamic alcohol (P1). The highest conversion yields were reached after 2 days: 76 % and 27 % for *A. glauca* and *P. citrinum,* respectively (Figure 7A).
Alfa-MNS was reduced into nitroalkane (P2). The conversion rate of *P. citrinum* was of about 64 % after 2 days and of about 99 % after 7 days; on the contrary, *A. glauca* displayed only 29 % of conversion at the end of the experiment (Figure 7B).

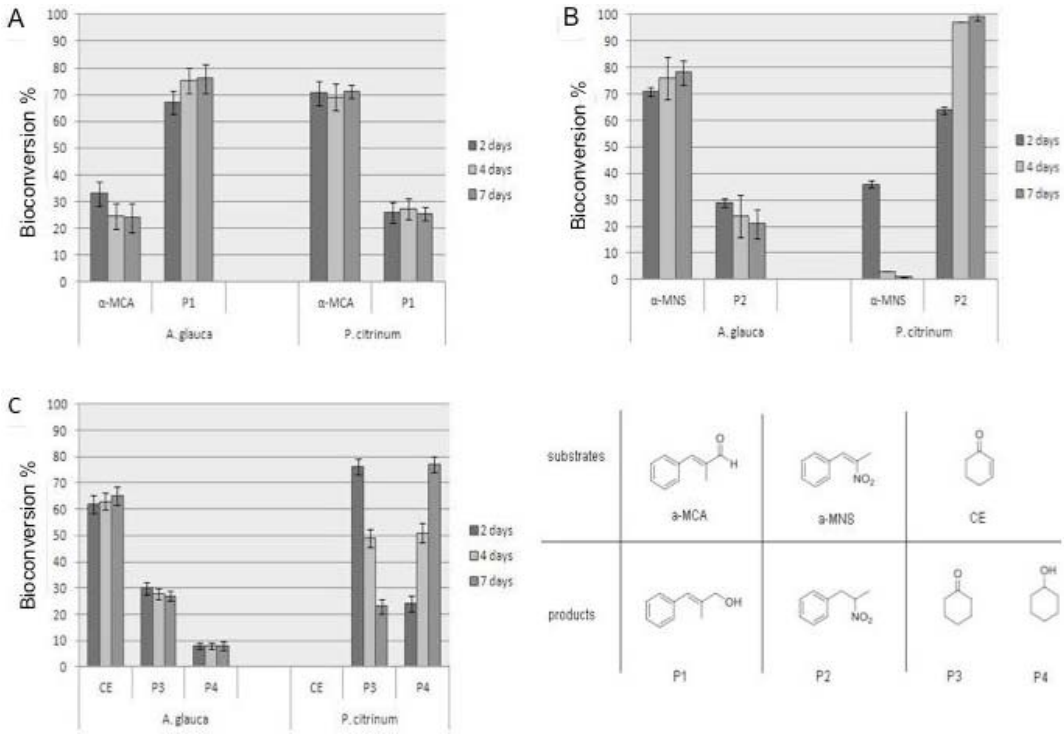

Figure 7: bioconversion of α-MCA (A), α-MNS (B), CE (C).

Cyclohexanone (P3) and cyclohexanol (P4) were the reduction products of CE. *P. citrinum* determined the highest bioconversion yields: in fact, 76 % of P3 and 24 % of P4 were produced after 2 days and, at the

end of the experiment, the final conversion yields were 77 % and 23 % for P4 and P3, respectively. On the other hand, *A. glauca* was less effective in the biotransformation of CE with a maximum yield of bioconversion after 2 days of 30 % for P3 and less than 10 % for P4 (Figure 7C).
The CE bioconversion by *P. citrinum* is very interesting, and the presence of two reduction products, suggest that the fungus mediated a two-step reaction: the reduction of the C=C double bond and of the C=O double bond. These results are very similar to the ones reported by Carballeira et al. (2004) on the bioconversion of carvone by *Diplogelasinospora grovesii, Gongronella butleri* and *Schizosaccharomyces octosporus.* Carvone is a monoterpene that presents a basic structure similar to CE with different substituent groups. The carvone conversion pathway involved two enzymes: in the first step, ER catalyses a C=C bond reduction and led to the formation of dihydrocarvone that is then reduced by an alcohol dehydrogenase (ADH) to dihydrocarveol. These kind of reactions are conceivable also for the bioconversion of CE by *P. citrinum:* since we worked with a whole-cells system, it is possible that more
These preliminary results are very interesting and show that the two selected fungi displayed reductive activity with different specificities: *A. glauca* resulted active in reducing the carbonyl group of α-MCA, whereas *P. citrinum* has proved to be effective towards the C=C reduction of α-MNS and CE. According to the best of our knowledge, this is the first time that these reactions have been observed by filamentous fungi. Next step will be to test the fungi activities on structurally related substrates, in order to investigate the effects of substituents on conversion, and, possibly, on enantioselctivity.

4. Conclusion

Overall this work represents a starting point for a subsequent step of screening that will investigate filamentous fungi role in the biotransformations of different types of substrate, in search of enzymes belonging to LAC or ER with interesting features in the bioconversion.
Future outlooks may be the purification of the enzymes and the optimization of an immobilization method that allows to employ directly the enzymes in the bioconversions, avoiding undesired side reactions and whole-cell approaches. From an industrial point of view, it would be very interesting to be able to obtain specific enzymes for biotransformation of interest that may be employed in large-scale approach.

References

Anastasi A., Spina F., Romagnolo A., Tigini V., Prigione V., Varese G.C., 2012, Integrated fungal biomass and activated sludge treatment for textile wastewaters bioremediation. Bioresource Technology 123, 106-111.

Baldrian P. and Gabriel C., 2002, Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. FEMS Microbiology Letters 206, 69-74.

Brenna E., Gatti F.G., Manfredi A., Monti D., Parmeggiani F., 2012. Enoate reductase-mediated preparation of methyl (S)-2-bromobutanoate, a useful key intermediate for the synthesis of chiral active pharmaceutical ingredients. Organic Process Research & Development 16, 262-268.

Carballeira J.D., Valmaseda M., Alvarez E., Sinisterra-Gago J.V., 2004, *Gongronella butleri*, *Schizosaccharomyces octosporus* and *Diplogelasinospora grovesii*: novel microorganisms useful for the stereoselective reduction of ketones. Enzyme and Microbial Technology 34, 611-623.

Casieri L., Varese G.C., Anastasi A., Prigione V., Svobodova K., Filipello Marchisio V., Novotny C., 2008, Decolorization and detoxication os reactive industrial dyes by immobilized fungi *Trametes pubescens* and *Pleurotus ostreatus*. Folia Microbiologica 53, 44-52.

Liese A., Seelbach K., Wandrey C., 2006, Industrial biotransformations. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Lloret L., Eibes L., Feijoo G., Moreira M.T., Lema J.M., Continuous biotransformation of estrogens by laccase in an enzymatic membrane reactor. Chemical engineering transactions 27, 31-36. DOI:10.3303/CET1227006

Lundell T.K., Makela M.R., Hilden K., 2010, Lignin-modifying enzymes in filamentous basidiomycetes-ecological, functional and phylogenetic review. Journal of Basic Microbiology 50, 5-20.

Moussouni S., Saru M.L., Ioannou E., Mansour M., Detsi A., Roussis V., Kefalas P., 2011, Crude peroxidase from onion solid waste as a tool for organic synthesis. Part II: oxidative dimerization-cyclization of methyl p-coumarate, methyl caffeate and methyl ferulate. Tetrahedron Letters 52, 1165-1168.

Ponzoni C., Beneventi E., Cramarossa M.R., Raimondi S., Trevisi G., Pagnoni U.M., Riva S., Forti L., 2007, Laccase-catalysed dimerization of hydroxystilbenes. Advanced Synthesis Catalysis 349, 1497-1506.