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Dottorato di ricerca in Biotecnologie vegetali

XXIII Ciclo

BIO/13

Recombinant production, functional characterization and biotechnological application of fungal oxidoreductases

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Supervisors:

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UNIVERSITÀ DEGLI STUDI DELLA Tuscia

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ABSTRACT

The oxido-reductases are enzymes able to catalyze the electron removal from oxidizer to reducer. Laccase and tyrosinase, multicopper enzymes oxidizing phenolic compounds and aromatic diamines by using molecular oxygen as the electron acceptor, belong to the oxido-reductases group. They are enzymes commonly found in plants, fungi, bacteria and insects. Laccase substrates have large versatility and this makes these enzymes highly interesting for various biotechnological and industrial applications.

Though laccases and tyrosinases can be directly purified from natural producers, protein recombinant expression in a suitable host organism is the most profitable procedure to obtain, industrially, these enzymes in high quantities and with high purity levels.

The presence of oxidative enzyme activities has been also associated to microbial detoxification of olive mill wastewaters (OMWs) after their treatment with selected basidio- and asco-mycetes.

The objectives of this study were to characterize genes coding for oxidoreductases in Basidiomycetes, in particular the *ERY3* and *ERY4* laccase genes of *Pleurotus eryingii* and the *PPO2* gene coding for the *Agaricus bisporus* tyrosinase, and to select non-conventional yeast able to produce new oxidoreductase enzymes.

The *ERY3* gene, coding for a *P. eryngii* laccase, was isolated, cloned and transformed in *Saccharomyces cerevisiae* cells. The production of recombinant laccase was assayed with a colorimetric test in presence of ABTS, on SDS-PAGE and Western blot analysis.

The recombinant yeasts cells were immobilized in calcium alginate beads and the immobilization parameters (inoculum, sodium alginate and $CaCl_2$ concentration) were optimized.

The *ERY4* laccase gene of *P. eryngii* was expressed in *S. cerevisiae* and the recombinant laccase resulted to be not biologically active. This gene was thus modified in order to study the role of its N- and C-terminal regions in determining enzyme catalytic properties. *ERY4* gene was subjected to a mutational analysis following these approaches: i) C-terminal progressive deletion to study the role of specific amino acid residues at the C-terminus of Ery4 protein, ii) site-directed mutagenesis of the C-terminal region, iii) chimerical laccases derived from the substitution of both its terminal regions with the corresponding regions of *ERY3* gene. The fourteen recombinant genes were separately expressed in *S. cerevisiae*.

Almost all mutant recombinant isoforms showed that they possess enzymatic activity and affinities for different substrates.

Genes encoding the laccase isoforms which showed the best enzymatic performances on different substrates, were expressed in *S. cerevisiae* by displaying the recombinant enzyme on yeast cell surface. The biochemical characterization, immunodetection and Western blot analysis of displayed proteins were performed. To our knowledge, this is the first example of a functional laccase immobilized on yeast surface.

The *PPO2* tyrosinase gene of *Agaricus bisporus* was expressed in *S. cerevisiae* cells and the produced protein was purified by using affinity chromatography and biochemically characterized. To our knowledge, this is the first time that an *A. bisporus* tyrosinase was expressed in heterologous host *S. cerevisiae* and in biologically active form.

In order to select, from biotechnologically relevant habitat, microorganisms producing oxidoreductases, the effluents from 5 different local olive mills were collected. Three hundred isolates were identified according to their rDNA sequence. Twelve on the 300 identified isolates, belonging to *Candida membranifaciens*, *C. tropicalis*, *Geotricum candidum*, *Pichia fermentans*, *P. holstii* and *S. cerevisiae* species, demonstrated that they were able to use OMW as unique nutrient source for their growth. Phenolic compound concentration and antimicrobial activity was rapidly and significantly decreased by the *G. candidum* strains. The physiological properties of the described *G. candidum* isolates confirmed the potential of these yeasts to produce a wide range of extracellular enzymes. One of the selected *G. candidum* strains was chosen as a model to set up an immobilized system of viable cells for mill waste bioremediation. Concentration of *G. candidum* as free cells in the medium, the COD values, the concentration of phenolic compounds, the medium decolourization and the antimicrobial activity were measured at estabished interval times for both immobilized and free *G. candidum* biomasses. Indeed, the COD removal and phenolics degradation by calcium alginate entrapped cells were higher than those of free cells, because the immobilization dramatically reduces the extracellular protease(s) activity, thus increasing the stability of microbial secreted oxidases.

RIASSUNTO

L'avvento delle biotecnologie ha consentito di produrre grandi quantitativi di enzimi ad elevati livelli di purezza da utilizzare in diversi processi industriali. Questa potenzialità ha permesso di aumentare l'efficienza delle reazioni, ridurre i costi di produzione ed i prodotti reflui di lavorazione. Le ossidasi, in particolare laccasi e tirosinasi, sono enzimi ubiquitari che trovano applicazioni biotecnologiche in ambito biomedico, farmaceutico, cosmetico, industriale ed ambientale.

Scopo di questo progetto di ricerca è l'isolamento, la caratterizzazione funzionale di laccasi e tirosinasi di origine fungina, l'espressione di tali proteine in forma ricombinante e lo sviluppo di loro potenziali applicazioni in ambito biotecnologico ed industriale.

Per quanto riguarda le laccasi da basidiomiceti, il gene *ERY3* codificante un'isoforma di laccasi, è stato isolato dal fungo basidiomicete *Pleurotus eryngii*, ed è stato espresso in forma eterologa e funzionalmente attiva nel lievito *Saccharomyces cerevisiae*. Inoltre, è stata ottimizzata una procedura di immobilizzazione delle cellule di lievito ricombinanti in sferette di calcio alginato.

Il gene *ERY4* di *P. eryngii,* codificante una laccasi, è stato espresso in forma ricombinante in *S. cerevisiae*, ma la proteina prodotta si è dimostrata non biologicamente attiva. Il gene *ERY4* è stato sottoposto ad un'analisi mutazionale secondo i tre seguenti approcci: i) delezione progressiva della parte C-terminale della proteina codificata, ii) mutagenesi puntiforme della medesima regione, iii) sostituzione nelle porzioni N e C terminale della laccasi Ery4 con le equivalenti regioni dell'isoforma Ery3. I geni ricombinanti sono stati espressi in *S. cerevisiae*. Le laccasi ricombinanti prodotte hanno mostrato di possedere, nella quasi totalità, attività enzimatica e peculiari affinità per una serie di differenti substrati.

Due delle isoforme mutanti prodotte sono state immobilizzate sulla superficie esterna delle cellule di lievito mediante fusione con proteine endogene di parete. Le proteine immobilizzate sono state caratterizzate da un punto di vista biochimico e visualizzate correttamente sulla superficie esterna delle cellule ricombinanti di lievito.

La tirosinasi Ppo2 di *Agaricus bisporus* è stata prodotta nel lievito *S. cerevisiae*, purificata mediante cromatografia di affinità e caratterizzata biochimicamente. Secondo quanto riportato in letteratura, questa è la prima espressione di una tirosinasi di origine fungina nel lievito *S. cerevisae* in forma biologicamente attiva.

Allo scopo di isolare microorganismi produttori di ossido reduttasi da nicchie ecologiche di interese biotecnologico, sono stati prelevati dei campioni di acque reflue di lavorazione (OMW) da cinque differenti oleifici del Salento (Puglia). E' stata isolata la popolazione microbica (lieviti e muffe) e un campione della stessa è stato identificato molecolarmente. I lieviti sono stati selezionati sulla base della loro capacità di usare le OMW come unica fonte di nutrimento. Un ceppo di *Geotricum candidum*, utilizzato per fermentare un campione di OMW, ha mostrato la capacità di ridurre significativamente la concentrazione dei composti fenolici e l'attività antimicrobica dello stesso. Il ceppo di *G. candidum* è stato utilizzato per la messa a punto di un sistema di immobilizzazione di cellule vive per la riduzione dell'impatto ambientale dei reflui oleari. La rimozione della COD e la degradazione dei fenoli risulta più elevata per le cellule immobilizzate rispetto alle stesse in forma libera, perché l'immobilizzazione riduce notevolmente la presenza di proteasi extracellulari, aumentando così la stabilità delle ossidasi microbiche.

Introduction

1. Introduction

Agenda 21, the work programme adopted by the 1992 United Nations Conference on Environment and Development, asserted that biotechnology "promises to make a significant contribution in enabling the development of, for example, better health care, enhanced food security through sustainable agricultural practises, improved supplies of potable water, more efficient industrial development processes for transforming raw materials, support for sustainable methods of aforestation and reforestation, and detoxification of hazardous wastes".

Retting of flax was the first biotechnological application in textile processing. More than 2000 years ago microorganisms grown on flax were used to achieve partial decortication in the extraction of lines fibres from flax stems. One of the earliest examples of industrial enzymes use was the production of whiskey. Over the years, enzymes have also been used in beauty and oral care products, textiles, food and for the fermentation of cheese, beer and wine.

The last century saw the replacement of plant-derived products with petroleum derivatives and this remarkable transformation helped humanity to overcome some of the natural limitations of relying on natural processes. The present century promises to open new avenues for increasing the use of renewable resources in the global economy, and, although the future is unpredictable, it is highly probable that biotechnology will play a visible and significant role.

Nowadays, industrial biotechnology covers two distinct areas. The first area is the use of renewable raw materials (biomass) to replace raw materials derived from fossil fuels. The second is the use of biological systems such as cells and enzymes (used as reagents or catalysts) to replace conventional, non-biological methods.

Enzymes catalyze chemical reactions with great and rate enhancements, as these reactions are the basis of the metabolism of all living organisms. As a result, they offer great potential for cleaner industrial production, and provide incredible opportunities for industry to carry out elegant, efficient and economical biocatalytic conversions. In other words, biocatalysts generate few by-products and can start from relatively less purified feedstocks. The food, feed, agriculture, paper, leather and textile industries are well suited for enzyme technology because products as well as raw material consist of (bio) molecules, which can be produced, degraded or modified by enzymatic processes. Many enzymes are commercially available, and numerous industrial applications have been described (Kirk et

al., 2002). Over 500 products covering 50 applications, from detergents to beer making, utilize enzymes produced through large-scale fermentation of microorganisms. The list of enzyme applications grows, bringing with it a list of significant social and environmental benefits. The estimated worldwide industrial enzyme market in 2000 was approximately $1.5 bilion (with North America and Europe accounting for 35% and 31% of, respectively) and is traditionally divided into three segments. The largest, at 65% of sales, is that of technical enzymes, the second largest segment, at 25% of the market, is that of food enzymes, finally, feed enzymes contributes approximately to 10% of the market.

Although the advantages of replacing chemical treatments with enzymes are often compelling from a societal and environmental viewpoint, enzymes must compete economically with often entrenched and extremely inexpensive traditional chemical processes. Commercial enzyme products were originally developed as simple fermentation broths of naturally occurring organisms. Often the resulting products were complex mixtures of secreted enzymes produced at relatively low yields (Cherry and Fidantsef, 2003). Today, over 90% of industrial enzymes are produced as recombinant protein to maximize product purity and economy of production. Microorganisms such as moulds, yeasts or bacteria, are being improved to work as "cell factories" to efficiently produce industrial enzymes targeted at specific tasks (Radman et al., 2003). Furthermore, the latest developments within modern biotechnology, introducing protein engineering and directed evolution, have further revolutionized the development of industrial enzymes (Böttcher and Bornscheuer, 2010). These advances have made it possible to provide tailor-made enzymes displaying new activities and adapted to new process conditions, enabling a further expansion of their industrial use.

A versatile class of enzymes, with a relative small market, but with a remarkable biotechonological and industrial importance, are the oxidases. The oxidases catalyze reactions involving direct activation of oxygen, and within this group, the multi-copper oxidases, as laccase and tyrosinase (or polyphenol oxidase), catalyze the four-electron reduction of oxygen to water with the concomitant one-electron oxidation of a substrate, e.g. phenolic compounds and aromatic diamines.

In this introduction, biocatalytic reactions involving two major groups of phenol oxidases, laccases and tyrosinases are explained and the strategies developed to obtain novel phenol oxidases from natural sources, in particular microorganisms, are exposed. These two classes are members of a large group of copper-containing oxidases which are well-know but have only fairly recently been structurally elucidated (McGuirl and Dooley, 1999). The

two groups can readily be biochemically distinguished from each other, because laccases are capable of catalyzing the oxidation of *p*-diphenols as well as *o*-diphenols, while tyrosinase can only react with *o*-diphenols (Carunchio et al., 2001).

1.1 Laccase

1.1.1 Properties of laccase

1.1.1.1 Origin and distribution

Laccases (benzenediol: oxygen oxidoreductase, p-phenol oxidase EC 1.10.3.2) are enzymes belonging to the group of polyphenol oxidases and contain copper atoms in their catalytic centre. They were first discovered by Yoshida (1883) in plants starting from the observation that the latex of a Chinese or Japanese lacquer tree (*Rhus* sp.) was rapidly hardened in the presence of air. The enzyme was named laccase about 10 years later after isolation and purification of the responsible catalyst (Bertrand, 1894). Other reports are those of Wosilait et al. (1954) on the presence of a laccase in leaves of *Aesculus parviflora* and in green shoots of tea (Gregory and Bendall, 1966). Five distinct laccases have been identified in the xylem tissue of *Populus euramericana* (Ranocha et al., 1999), and other isoforms in maple (*Acer pseudoplatanus*) (LaFayette et al., 1995), tobacco (*Nicotiana tabacum*) (Kiefer-Meyer et al., 1996), poplar (*Populus trichocarpa*) (Ranocha et al., 1999), and yellow poplar (*Liridendron tulipifer*) (LaFayette et al., 1999). In *Pinus taeda* tissue eight laccases were identified and all of them were expressed predominantly in xylem tissue (Sato et al., 2001).

Fungal laccases were also discovered during the 19th century (Bertrand, 1896; Laborde, 1896). Laccases are thought to be nearly ubiquitous among fungi, actually the presence of laccases has been documented in virtually every examined fungus. Laccases are produced in multiple isoforms depending on the fungal species and environmental conditions.

Some phenoloxidases showing “laccase properties” have been purified from larval and adult cuticles of insects such as *Drosophila virilise* (Yamazaki, 1969), the silk moth *Bombyx mori* (Yamazaki, 1972), *Sarcophaga bullata* (Barret, 1987), *Manduca sexta* (Thomas et al., 1989), *D. melanogaster* (Sugumaran et al., 1992) and the malaria mosquito *Anopheles gambiae* (Dittmer et al., 2004).

Laccase activity was also found in the haemocytes of the red swamp crayfish (Cardenas and Dankert, 2000).

Corresponding genes have been found in Gram-negative and Gram-positive bacteria, including species living in extreme habitats, e.g. in *Oceanobacillus iheyensis* or *Aquifex*

aeolicus and in the archaebacterium *Pyrobaculum aerophilum* (Alexandre and Zhulin, 2000; Claus, 2003).

1.1.1.2 Modular structure and catalytic mechanism of laccase

Laccases have been isolated as monomeric, dimeric or even tetrameric proteins in their active holoenzyme form, showing generally an acid pI, and usually containing four copper atoms per monomer, bound to three redox sites. Based on spectroscopic analysis, which reflects geometric and electronic features, copper centers are differentiated as type 1 (T1), or blue copper center, type 2 (T2) or normal copper, and type 3 (T3) or coupled binuclear copper center (Fig. 1) (Solomon et al., 1996). Type 1 copper has a trigonal coordination, with two histidines and a cysteine as conserver ligands (HYS-CYS-HYS motive). Type 1 copper is responsible for laccases blue colour, because of the cysteine-copper covalent bond that produces an intense electronic absorption band nearly 600 nm. The occurrence of laccase enzyme which lack the typical absorption around 600nm has been reported. For example, a "white laccase" (containing 1Cu, 1Fe, 2Zn atoms) has been purified from *P. ostreatus* (Palmieri et al., 1997), while "yellow laccases" (containing copper but in an altered oxidation state) have also been reported (Leontievsky et al., 1997). As a result of type 1 copper high *redox* potential (nearly 790 mV), it represents the site where substrates to oxidize take place.

Type 2 copper shows no absorption in the visible spectrum, and reveals paramagnetic properties in EPR (Electron Paramagnetic Resounance*)* studies. It is strategically positioned close to the type 3 copper, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm (oxidized form) and by the absence of an EPR signal as a result of the antiferromagnetic coupling of the copper pair. The type 3 copper centre is also the common features of another protein superfamily, including tyrosinases and haemocyanins (Decker and Terwilliger, 2000). Type 2 and type 3 copper centers form a trinuclear center where oxygen reduction and water release occur. Type 2 copper is coordinated by two and type 3 copper atoms by six histidines (Fig. 1).

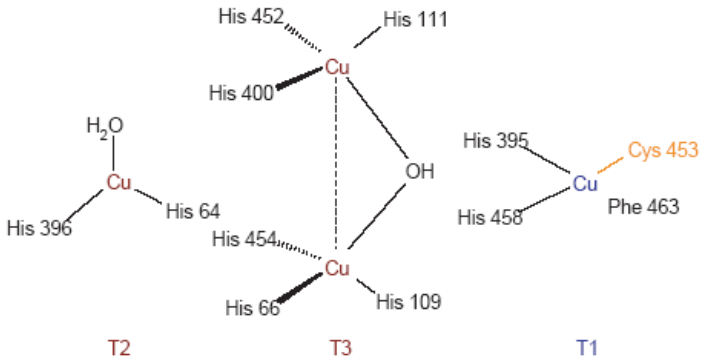

Fig. 1 Model of the laccase trinuclear oxygen

Laccases are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases able to oxidise diphenols and related substances and to use molecular oxygen as an electron acceptor. In contrast to most enzymes, which are generally very substrate specific, laccases act on a suprising broad range of substrates, including inorganic/organic metal complexes, ferrocyanide, anilines, benzenethiols, as well as other redox inorganic, organic or biological compounds, as long as their redox potentials are not too high (Xu et al., 1996). When oxidized by laccase, the substrate donates an electron to the T1 copper, whereas the reduction of oxygen to water takes place in the trinuclear copper center (T2/T3) which is located about 12 Å away from T1. One catalytic cycle involves the transfer of altogether four electrons, which are carried from T1 to the T2/T3 cluster through a conserved "Hys-Cys-Hys motive" (Fig. 2).

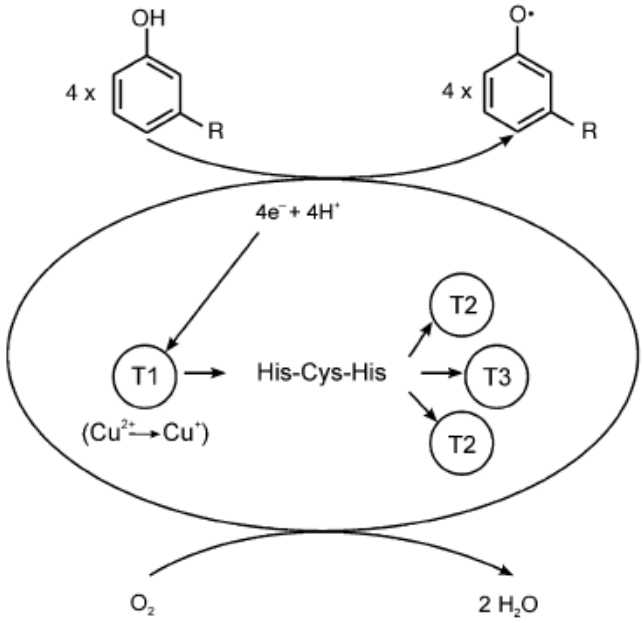

Fig. 2 Catalytic cycle of laccase

The oxidation of a reducing substrate typically involves the formation of a three (cation) radicals, which can further undergo the laccase-catalysed oxidation or non-enzymatic reactions, thus forming cross-bonds between monomers, polymers degradation or aromatic rings break.

1.1.1.3 Molecular biology of fungal laccases

The first laccase genes were isolated and sequenced about 15 years ago from the fungi *Neurospora crassa* (Germann et al., 1988), *Aspergillus nidulans* (Aramayo and Timberlake, 1990), *Coriolus hirsutus* (Kojima et al., 1990) and *Phlebia radiate* (Saloheimo and Niku-Paavola, 1991). Since then, the number of laccase genes sequenced has increased considerably, and searches in protein and gene sequence databases currently yield several hundreds laccase gene sequences.

A typical laccase gene codes for a protein of 500-600 amino acids. The coding regions of fungal laccase genes are usually intervened by 8-13 introns of about 50-90 basepairs in length. There are, however, also laccase genes that have only one intron (*N. crassa* laccase gene; Germann et al., 1988), as well as genes with even up to 19 introns (*P. ostreatus pox1*; Giardina et al., 1995). Most fungal laccases are secreted proteins, and typical eukaryotic signal peptide sequences of about 20 amino acids are found at the N-termini of the protein sequences. In addition to the secretion signal sequence, laccase genes from *N. crassa*, *Podospora anserina, Myceliophthora thermophila* and *Coprinun cinereus* contain regions that code for N-terminal cleavable propetides (Germann et al., 1988).

Many fungal genomes contain more than one laccase gene. *Trametes villosa*, for example, contains at least five laccase genes (Yaver et al., 1996); *Rhizoctonia solani* (Wahleithner et al., 1996), *P. sajor-caju* (Soden and Dobson, 2001) and *P. ostreatus* (Palmieri et al., 2003) contain at least four laccase genes.

Laccase proteins and thereby also laccase genes are identified by the presence of four highly conserved copper binding motifs, all involving the HXH sequence and containing altogether 10 conserved histidines and one conserved cysteine (Fernández-Larrea and Stahl, 1996; Kumar et al., 2003).

The expression levels of different laccase genes typically depend on culture conditions. Copper is a strong inducer of laccase gene transcription, being related to a defence mechanism against oxidative stress caused by free copper ions (Fernández-Larrea and Stahl, 1996; Palmieri et al., 2000). In addition to copper, other metal ions such as Mg^{2+}, Cd^{2+} or Hg^{2+} can stimulate laccase expression (Soden and Dobson, 2001). Certain aromatic compounds that are structurally related to lignin precursor, such as 2,5-xylidine or ferulic

acid, have also been shown to increase laccase gene transcription in *T. villosa*, *T. versicolor* and *P. sajor-caju* (Yaver et al., 1996; Collins and Dobson, 1997, Soden and Dobson, 2001). However, *T. villosa* and *P. sajor-caju* have also been shown to contain constitutively expressed laccase genes, and this may be related to different physiological roles of the various laccases in fungi (Yaver et al., 1996, Soden and Dobson, 2001).

The transcriptional induction of laccase genes is regulated by specific sequence blocks in the promoter regions of the genes, denoted as putative metal-responsive elements (MRE) (Giardina et al., 1995). Furthermore, putative heat-shock elements (HSE), xenobiotic response elements (XRE) and antioxidant response elements (ARE) have been identified in the promoter regions of laccase genes (Soden and Dobson, 2001; Giardina et al., 1995; Fernández-Larrea and Stahl, 1996), although the roles of these regulatory regions have not yet been experimentally demonstrated.

1.1.2 Role of laccase in nature

1.1.2.1 Laccase and lignin

Laccases are able to polymerise lignin monomers *in vitro* (Freudenberg et al., 1958) and their enzymatic activities are closely related to lignin deposition in developing xylem and to the early stages of lignification in living cells (Sterjiades et al., 1993).

Analysis of plant laccase sequences suggests that a number of fungal laccase might also be localized intracellularly. All data reported in literature about plant laccases can be interpreted as a strong indication of laccase acting as a multifunctional enzyme.

Given the oxidising characteristics (high redox potential) of laccase, and the great abundance of laccases in wood-rotting basidiomycete fungi, it has been suggested that the main role of fungal laccases is to contribute to lignin depolymerisation, i.e., to the break down into small components of the highly polydispersed polyphenolic lignin macromolecule (Hatakka, 1994). Laccase has also been shown to degrade non-phenolic lignin model compounds in systems incorporating naturally occurring or synthetic redox mediators (Call and Mucke, 1997). Redox mediators are oxidisable, low molecular weight, laccase substrate. These substrates yield radicals that are capable of diffusing away from the reactive site and acting as oxidants for other compounds. Importantly, some mediators can oxidise compounds such as lignin, which would not be oxidisable by laccase alone (Johannes and Majcherczyk, 2000). For most fungi, however, the precise role(s) of laccase in ligninolysis still remain to be fully understood (Thurston, 1994).

Lignin biodegradation by laccase-producing fungi probably occurs in nature through more complicated enzymatic mechanisms, which involve the synergistic effects of other

enzymes (such as peroxydase, glyoxal-, glucose-, aryl alcohol-oxidases) and non-enzymatic components that interact to establish equilibrium between enzymatic polymerisation and lignin depolymerisation.

1.1.2.2 Laccase in growth, development and resistance to stress and pathogens

Laccase activity has been reported to be associated with fungal mycelia growth (Das et al., 1997) and to be also involved in several biological events such as: i) development of rhizomorphs and sclerotia in certain basidiomycete and ascomycete taxa (Worrell et al., 1986; Rehman and Thurston, 1992): ii) conidia pigment production in *A. nidulans* (Clutterbuck, 1972): iii) *Cryptococcus neoformans* (Salas et al., 1996), *Cryphonectria parasitica* (Rigling et al., 1991) and *Botrytis cinerea* pathogenicity (Viterbo et al., 1994); iv) as alternative oxidase in the respiratory pathway in *P. anserina* (Frese et al., 1992) and in copper and iron homeostasis (Stoj et al., 2003).

Evidence of laccase-like enzymatic activities in the cuticle of larval and adult insects led the author to associate this phenoloxidase activity with the sclerotization process in insects (Dittmer et al, 2004).

Bacterial laccases play a role in sporulation and pigmentation of *Bacillus sphaericus* (Claus and Filip, 1997), *Marinomonas mediterranea* (Sanchez-Amat and Solano, 1997) and *B. subtilis* (Hullo et al., 2001), conferring resistance to stress factors such as UV radiation or hydrogen peroxide (Martins et al., 2002).

1.1.3 Applications of laccase

In addition to numerous studies on their strict biological functions, laccases are widely studied enzymes because of their use in several areas such as textile, paper, food and pulp industries. Given their versatility and broad substrate specificity, laccases are increasingly tested for their oxidative properties in a variety of practical applications: i) delignification of lignocellulosics (Elegir et al., 2005; Widsten and Kandelbauer, 2008); ii) purification of colored waste waters (Duran and Esposito, 2000); iii) textile dye decolourization (Shah and Nerud, 2002; Wesenberg et al., 2003); iv) beverage and food treatment (Minussi et al., 2002); v) the desulfurization and solubilization of coal for their use in enzyme-based biosensors (Marco and Barcelo, 1996); vi) the transformation and inactivation of toxic environmental pollutans (Gianfreda et al., 2006; Hwang et al., 2007); vii) hair dyeing process (Onuki et al., 2000; Pruche et al., 2000); viii) the generation of wood adhesives (Peshkova and Kaichang, 2003).

1.1.3.1 Laccase in Biopulping

About 25% of the wood pulp produced in the world is created using a mechanical pulping method, which has twice the yield of chemical pulping. Due to its importance in the pulp and paper industry, the removal of lignin from woody tissue is a process that has attracted a great deal of research. Laccase has been proposed for pulp and paper manufacture to enhance pulp bleaching, pulp refining, deinking, cellulose purification, deposit control, and papermaking (Wong and Mansfield, 1999). Biopulping results in nearly 30% saving of electricity, and biobleaching of pulp reduces chemical requirements of 50%. Moreover, laccases do not alter pulp brightness, improve auto-adhesion of fibres in medium density of mechanical pulp, and preserve tensile strength through calendaring (Buchert et al., 1998).

1.1.3.2 Laccase in Ethanol Production

To improve the production of fuel ethanol from renewable raw materials, a laccase from the white-rot fungus *T. versicolos* was expressed in *Saccharomyces cerevisiae* to increase its resistance to phenolic inhibitors present in lignocellulose hydrolysates (Larsson et al., 2001). The laccase-producing transformants were able to convert coniferyl aldehyde, enhancing yeast growth and consequent ethanol production, and this is definited an advantage for ethanol production from lignocellulose.

1.1.3.3 Laccase in the treatment of beverages

Several phenolic compounds (cumaric acids, flavans, and anthocyanins) are usually present in beverages such as wine, fruit juice, beer, and may, during their shelf life, cause undesirable and deleterious changes (discolouration, clouding, haze, and flavour changes). Positive effects of laccase action were observed on must and wine (Kiralp et al., 2003) as well as on fruit juice and beer (Elkaoutit et al., 2007). Servili et al. (2000) reported the laccase action on decolourization process by removing phenols from must of white grapes.

1.1.3.4 Laccase-based Biosensors

The use of laccases as analytic tools is well documented by several reports (Freire et al., 2003; Kulys et al., 2003). The ability of laccases to catalyze the electroreduction of oxygen via a direct mechanism, without the presence of an electrochemically active mediator, was used to develop a lot of biosensors based on laccases useful for: i) bioelectrochemical monitoring of phenolic compounds in flow injection analysis (Haghighi et al., 2003) and in environmental matrices (Freire et al., 2002); ii) detection of anionic toxic substances, which are known as enzyme inhibitors (Mousty et al., 2007); iii) catechol determination in compost bioremediation (Tang et al., 2008); iv) fabrication of biofuel cells using laccases immobilized into different specific matrices, such as carbon nanotubes-chitosan composite

(Liu et al., 2006) or alginate carbon beads (Khani et al., 2006) as the cathodic catalysts; v) developing a mediatorless and pseudoreagentless electro-enzyme-immunoassay (EEIA) (Kuznetsov et al., 2001).

1.1.3.5 Laccase in Bioremediation

Laccases have been reported to oxidize many recalcitrant substrates, such as chlorophenols (Fahr et al., 1999; Grey et al., 1998; Ricotta et al., 1996; Roy-Arcand and Archibald, 1991), lignin-related structures (Bourbonnais et al., 1995; Boyle et al., 1992), organophosphorous compounds (Amitai et al., 1998), non-phenolic lignin model compounds (Kawai et al., 1988; Majcherczyk et al., 1999), phenols (Bollag et al., 1988; Xu et al., 1996). Recent applications in bioremediation include an *ex planta* system of phytoremediation of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase (Wang et al., 2004).

An important laccase application in bioremediation is the treatment of industrial wastes, such as olive mill and textile wastewaters.

Olive oil production plays a leading role in the economy of most of Mediterranean countries. The vegetable water (olive-mill wastewater, OMW), produced during the olive oil production process, consists of the liquid fraction of the olive juice and the water used during the different phases of olive mill processing. OMW has significant polluting properties due to its high levels of chemical oxygen demand (COD), biochemical oxygen demand (BOD), and phenols. The structure of phenols contained in the OMW is similar to that of lignin, which makes them difficult to biodegrade. In a recent study, the treatment of OMW with several laccase-producing fungi led to the removal of up to 78% of the initial phenolic compounds in 12/15 day (Tsioulpas et al., 2002).

The frequently high volumetric rate of industrial effluent discharge, in combination with increasingly stringent legislation, makes the search for appropriate treatment technologies an important priority. By far the class of microorganisms most efficient in breaking down synthetic dyes are the white-rot fungi. In particular, basidiomycetes are microorganisms able to produce one or more extracellular lignin-modifying enzymes, which, due to their lack of substrate specificity, are also capable of degrading a wide range of xenobiotics, then also synthetic dyes (azoic, anthaquinones and phthalocyanines). Among extracellular enzymes produced from fungi, it is demonstrated that laccase plays a basic role in decolourization process of these pollutant and mutagenic compounds (Chung et al., 1992).

1.2 Tyrosinase

1.2.1 Properties of tyrosinase

1.2.1.1 Origin and distribution

Tyrosinases (monophenol, o-dipehnol:oxygen oxidoreductases, EC 1.14.18.1), often also called polyphenol oxidases, are copper containing metalloproteins and essential enzymes in melanin biosynthesis. Tyrosinases have been found widely distributed throughout the phylogenetic scale from bacteria to mammals and even with different characteristics in different organs of the same organism, such as roots (Sánchez-Ferrer et al., 1993a) and leaves (Sánchez-Ferrer et al., 1993b) of higher plants.

Tyrosinases and their corresponding genes have been characterized from various sources, including bacteria, fungi, plants and mammals. Fungal tyrosinases were firstly characterized from edible mushroom *Agaricus bisporus* (Wichers et al., 1996) because of enzymatic browning during development and postharvest storage, which particularly decreases the commercial value of the product. The most thoroughly characterized fungal tyrosinase both from structural and functional point of view are from *A. bisporus* (Wichers et al., 1996) and *N. crassa* (Lerch, 1983). Other microbial tyrosinase genes have been isolated from *Pseudomonas* (McMahon et al., 2007), *Bacillus* and *Myrothecium* (Echigo and Ohno, 1997), *Mucor* (Yamada et al., 1983), *Aspergillus*, *Chaetotomastia*, *Ascovaginospora* (Abdel-Raheem and Shearer, 2002), *Trametes* (Tomsovsky and Homolka, 2004), *Pycnoporus* (Halaouli et al., 2006), *Trichoderma* (Selinheimo et al., 2006) and *Streptomyces* (Della-Cioppa et al., 1998a, 1998b). Tyrosinase-related proteins have been characterized in human- (Know et al., 1987), mouse- (Know et al., 1988) and frog- (Takase et al., 1992). Descriptions of molecular properties of tomato- (Newman et al., 1993), potato- (Hunt et al., 1993), bean- (Gary et al., 1992), spinach- (Hind et al., 1995), apple- (Espín et al., 1995; Ni Eidhin et al., 2006), artichoke- (Espín et al., 1997d), avocado- (Espín et al., 1997c), pear- (Espín et al., 1997a), and strawberry-derived (Espín et al., 1997b) tyrosinases have been reported.

The sequence comparison of recently published tyrosinases reveals high heterogeneity concerning the length and overall identity. However, highly conserved regions can be found in the active site area among all tyrosinases (van Gelder et al., 1997; Halaouli et al., 2006; Marusek et al., 2006).

1.2.1.2 Modular structure and catalytic mechanism of tyrosinase

Tyrosinases belong to a large group of proteins, denoted as "type-3 copper proteins", which possess a diamagnetic spin-coupled copper pair in the active centre (Lerch et al., 1986). Tyrosinases differ from laccases, since they contain only two copper ions at one reaction site in each functional unit of the enzymes. These copper atoms (CuA and CuB) are coordinated by three highly conserved histidine residues each one and they act cooperatively in the catalytic reaction (Fig. 3). The copper ions are responsible for binding of both molecular oxygen and the reducing substrate, which is always either a phenol or a dihydric phenol (catechol).

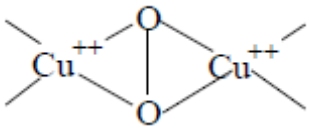

Fig. 3 Tyrosinase copper site organization

Tyrosinases catalyze two different reactions: *o*-hydroxylation of monophenol (creolase activity or "monophenolase") and the subsequent oxidation of the resulting *o*-diphenols into reactive *o*-quinones (catecholase activity or "diphenolase"). Molecular oxygen is used as an electron acceptor and it is reduced to water. Subsequently, the *o*-quinones undergo non-enzymatic reactions with various nucleophiles, producing intermediates, which associate spontaneously in dark brown pigments (Soler-Rivas et al., 2000).

Chemical and spectroscopic studies of tyrosinase have shown that its coupled binuclear active site can exist in three forms, called *met*, *oxy* and *deoxy*, according to the absence/presence of oxygen and the oxidation state of the copper ions. These enzymes have been extensively studied not only because of their biological interest, but also for their potential use in industrial applications. The oxygenated form (*oxy*tyrosinase, E_{oxy}) consists of two tetragonal Cu(II) atoms, each coordinated by two strong equatorial and one weaker axial N_{His} ligand. The exogenous oxygen molecule is bound as peroxide and bridges the two Cu centers (Fig. 4) (Solomon and Lowery, 1993).

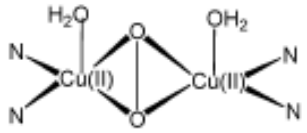

Fig. 4 Copper atoms coordination in *Oxy*tyrosinase

*Met*tyrosinase (E_{met}), like the *oxy* form, contains two tetragonal copper (II) ions antiferromagnetically coupled through an endogenous bridge, although hydroxide exogenous ligands, other than peroxide, are bound to the copper site (Fig. 5). This derivative can be converted by addition of peroxide to *oxy*tyrosinase, which in turn decays back to *met*tyrosinase when peroxide is lost (Himmelwright et al., 1980).

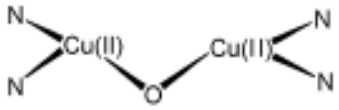

Fig. 5 Copper atoms coordination in *Met*tyrosinase

*Deoxy*tyrosinase (E_{deoxy}), by analogy with d*eoxy*hemocyanin, has a bicuprous structure [Cu^{I}-Cu^{I}] and can bind molecular oxygen to create the E_{ox}. Its presence was firmly established in 1938 by Kubowitz (Kubowitz, 1938), who demonstrated the binding of one carbon monoxide per two copper atoms after reduction of the enzyme with catechol (Fig. 6).

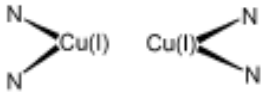

Fig. 6 Copper atoms coordination in *Deoxy*tyrosinase

These three copper states in the active site of tyrosinase led to the proposal of a structural model for the reaction mechanism involved in the *o*-hydroxylation of monophenols and oxidation of the resulting diphenols (D) (Fig. 6).

Monophenolic substrate initially coordinates to an axial position of one of the coppers of *oxy*tyrosinase, E_{oxy}. Rearrangement through a trigonal bipyramidal intermediate leads to the *o*-idroxylation of monophenol by formation of the E_{met} D complex. This E_{met} D form can either render free diphenol to fulfil the equilibrium E_{met} + D ↔ E_{met} D + $2H^{+}$ or undergo oxidation of the diphenolate intermediate bound to the active center, giving a free quinone and a reduced binuclear cuprous enzyme site (E_{deoxy}). *Oxy*tyrosinase is, then, regenerated after the binding of molecular oxygen to E_{deoxy}. If only diphenol is present, it binds to the E_{oxy} form to render E_{oxy} D, which oxidizes the diphenol to *o*-quinone and yields the antiferromagnetically coupled tetragonal Cu (II) form of the enzyme (E_{met}). The latter form transforms another *o*-diphenol molecule to *o*-quinone and is reduced to the bicuprous E_{deoxy} form (Burton, 1995).

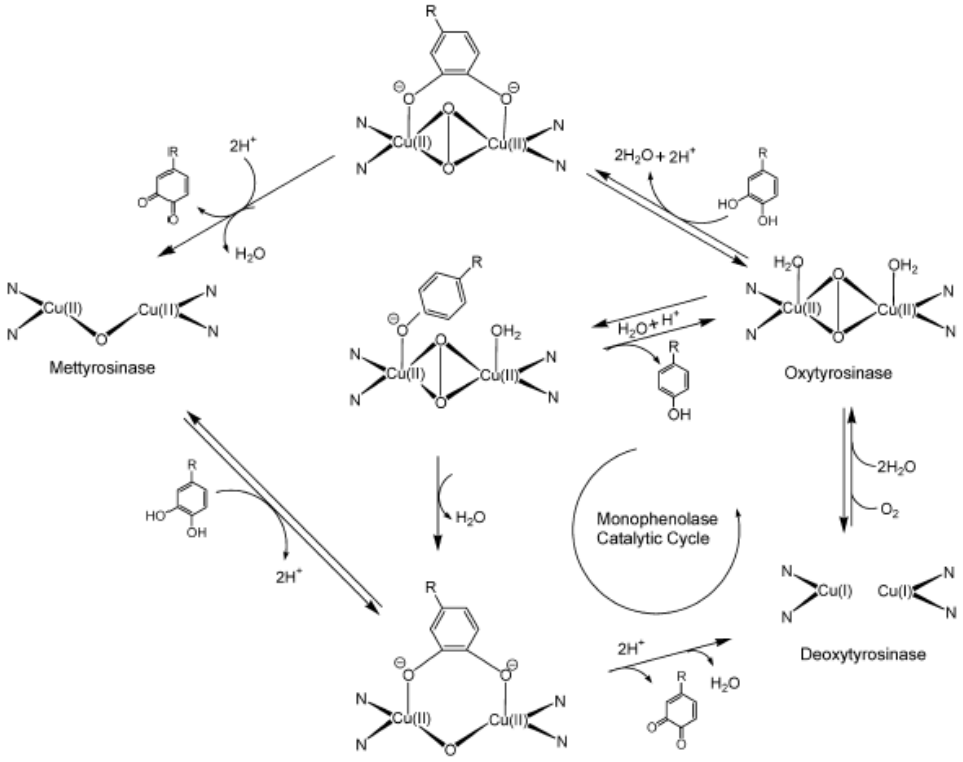

Fig. 6 Catalytic cycle for the oxidation of monophenol and diphenol substrates to *o*-quinones by tyrosinase in the presence of O_2.

1.2.1.3 Features of tyrosinases from different species

About twenty tyrosinase gene sequences are at the moment available, and their sequence homology is high within different taxa (higher eukaryotes, plants, fungi and bacteria) and conserved domains can be identified. However, differences in the size, processing, maturation and final location of tyrosinase are also large in the phylogenetic scale (Garcìa-Borròn and Solano, 2002).

Mammalian tyrosinases are synthetized with an N-terminal signal peptide that targets the nascent polypeptides to the endoplasmic reticulum for their folding, modification and sorting.

They are melanosomal membrane proteins with a carboxyl tail oriented to the cytoplasm and a single membrane-spanning helix located in the C-terminal portion of the proteins (Garcìa-Borròn and Solano, 2002). Fungal tyrosinases are considered soluble cytosolic enzymes (Wichers et al., 1996). In higher plants, the enzyme is mostly membrane bound in non-senescing tissues (Rodriquez and Flurkey, 1992).

1.2.1.4 Tyrosinase reaction system

Tyrosinase is known to exist in both latent and active forms, particularly in plants, fungi and invertebrates, possibly as a control mechanism *in vivo*. In the case of mushroom

tyrosinase, *ca* 99% of the total enzyme appears to be present in its latent form (Van Leeuwen and Wichers, 1996). Most of the plant tyrosinases seems to be latent in the mature form and in this inactive form the enzyme seems to be very stable (Cary et al., 1992; Fraignier et al., 1995; Robinson and Dry, 1992; Söderhäll, 1995). A number of reagents have been reported to be suitable activators of the latent enzyme, including chemical denaturing agents such as SDS (McIntyre and Vaughan, 1975; Moore and Flurkey, 1990), other detergents (Espín and Wichers, 1999) and urea, proteases such as trypsin (Sánchez-Ferrar et al., 1989), and various chemical agents e.g., alcohols, polyamines (King and Flurkey, 1987; Jimenez and García-Carmona, 1996a). The activation process has been recently shown to be reversible (Jimenez and García-Carmona, 1996b).

An additional important feature of tyrosinase reaction system is the phenomenon denoted as "suicide inactivation": the quinone produced by its enzymatic reaction can covalently bind to the active site of the enzyme, leading to its permanent inactivation (Burton, 2003).

1.2.2 Role in nature of tyrosinase

1.2.2.1 Tyrosinase and melanin synthesis

Melanin is one of the most widely distributed pigment and is found in bacteria, fungi, plants and animals. Melanins are heterogeneous polyphenol-like biopolymers with complex structure and colour varying from yellow to black (Prota, 1988). The colour of mammalian skin and hair is determined by a number of factors, being the degree and distribution of melanin pigmentation the most important. Melanin is formed in specialized pigment-producing cells known as melanocytes, within membranous organelles named melanosomes (Quevedo et al., 1982). There are basically two types of melanin in mammals, the eumelanins and the phaeomelanins, which are formed by a combination of enzymatic and chemical reactions. The initial phase of melanogenesis consists of the idroxylation of a monophenol (tyrosine) to an *o*-diphenol (L-dihydroxyphenilalanine or L-DOPA) and its oxidation to corresponding *o*-quinone (*o*-DOPAquinone), both catalyzed by tyrosinase. Subsequently, DOPAquinone is transformed, by several non-enzymatic reactions, in eumelanin and phaeomelanin, in the presence of copper or sulphur respectively (Sánchez-Ferrer et al., 1995). The interaction between the eumelanin and pheaomelanin compounds gives rise to a heterogeneous pool of mixed type melanins (Prota, 1988). Tyrosinase, being the key enzyme of melanin pathway, holds a fundamental role in the protection of melanin producers, and, in particular in mammals, it is responsible for skin, eye and hair pigmentation (Hearing and Tsukamoto, 1991; del Marmol and

Beermann, 1996). Tyrosinases are also suggested to be potential tool in melanoma (Morrison et al., 1985; Jordan et al., 2001) and Parkinson's disease treatment (Greggio et al., 2005). To date, information on the physiological role of the tyrosinases in microbes has been limited. However, it has been proposed that melanin has a role in the formation of reproductive organs, spore formation, virulence of phatogens, and tissue protection after damage (Lerch, 1983).

1.2.2.2 Role of tyrosinase in resistance of microorganisms and plants to stress and pathogens

Enzymatic browning of vegetables occurs when polyphenol substrates and tyrosinase come in touch, after their release due to plant cutting (Martinez and Whitaker, 1995). The role of tyrosinase in browning phenomena is well documented (Zhou et al., 2003; Veltman et al., 1999). Tyrosinases also play an important role in regulation of the oxidant-reduction potential, and the wound healing system in plants (Mayer, 1987; Walker and Ferrar, 1998; (Ray et al., 2006). Thipyapong et al. (2004) demonstrated that tyrosinase is clearly involved in the defence of plants against pathogens, it has also been reported that in invertebrates, tyrosinase interacts with defence reactions and sclerotization (Sugumaran, 2002). Instead, Jacobson (2000) demonstrated the role of fungal melanin in pathogenesis.

1.2.2.3 Clinical studies on tyrosinase

Various recent papers have focused on the involvement of tyrosinase in clinical studies.

Vitiligo, an autoimmune disease characterized by hair and skin hypopigmetation, is characterized by the presence of tyrosinase as an autoantigen, which is used as a marker for its diagnosis (Baharav et al., 1996).

Contradictory results are available regarding the role of mushroom tyrosinase in cancer, since it showed tumor-suppressing effects (Vogel et al., 1977), but it could also enhance the cell mutagenicity because it induces the production of phenolic and quinoid compounds (Papaparaskeva-Petrides et al., 1993).

The ability of tyrosinase to convert tyrosine into melanin allowed the production of new molecules, similar to tyrosine, which could be converted from inactive prodrugs into cytotoxic drug in melanoma cells (Jordan et al., 1999; 2001).

Recently, Shi et al. (2002) have reported that the extracts of *A. bisporus* prevented H_2O_2-induced oxidative damage to cellular DNA, thus allowing to correlate the genoprotective effect of *A. bisporus* with tyrosinase. In fact, the nature of the genoprotective activity of tyrosinase was found to be dependent upon the hydroxylation of tyrosine to L-Dopa and subsequent oxidation of L-Dopa to dopaquinone.

1.2.3 Application of tyrosinase

Tyrosinase have traditionally been utilized in plant-derived food production, e.g. tea, coffee, raisins and cocoa, where they promote the production of desired compounds (Seo et al., 2003). Tyrosinases retain many interesting applications in food and non-food processes, especially due to their capacity to catalyze formation of covalent bonds between peptides, proteins and carbohydrates (Aberg et al., 2004; Halaouli et al., 2005; Freddi et al., 2006). Tyrosinases proved to be also applicable in structure engineering of meat-derived food products (Mattinen et al., 2008).

1.2.3.1 Tyrosinase as a Biocatalyst

Use of tyrosinase in biocatalystic systems has been recently considered as an alternative to the chemical synthesis of *ortho*-hydroxylation of phenols, such as the production of L-Dopa for Parkinson's disease treatment (Fenoll et al., 2001).

Tailoring polymers in material science, for instance, grafting of silk proteins onto chitosan via tyrosinase reactions has also been reported (Freddi et al., 2006; Anhhileria et al., 2007).

The production of byphenyl products using tyrosinase is of particular interest because of their potential use for production of antioxidant and coloured products, with beneficial properties as food additives or pharmaceutical drugs (Janse van Rensburg et al., 2000). Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol) is a strong antioxidant which occurs naturally in olive oil and fruits, but which is not commercially available (Espín et al., 2000). The purified tyrosinase from *A. bisporus* (Espín et al., 2001) and *P. sanguineus* (Halaouli et al., 2005) have demonstrated effectiveness in the biosynthesis of hydroxytyrosol from p-tyrosol (2-(4-dihydroxyphenyl)ethanol), another monophenol found in agro-industrial by-products such as olive mill wastewaters (Lesage-Meessen et al., 2001).

Reactions using tyrosinase provide routes to a range of phenolic polymers. One potential application of such polymers is in their ability to bind proteins, thereby acting as anti-inflammatories (Jimenez and Garcia-Carmona, 1999), polypeptide polymers possessing high tensile strength (Zhu et al., 1997), synthetic polymer used in developing a method for linking biopolymers (Yamamoto et al., 1990).

1.2.3.2 Tyrosinase-based Biosensors

Tyrosinase-based biosensors represent a promising tool in the analysis of the total content of phenolics in order to assign the bitterness and pungency of virgin olive oil (Busch et al., 2006), to determine the amount of phenolic compounds (Böyükbayram et al., 2006), to

detect phenol compounds in other kind of beverages, as apple and citrus fruits juices, and to ascertain antioxidant properties of a beverage (Jewell and Ebeler, 2001).

1.2.3.3 Tyrosinase in Bioremediantion

Phenolic and aromatic compounds are present in the wastewaters of a large number of industries including coal conversion, petroleum refining, resins and plastics, wood preservation, dyes, chemicals and textiles (Karam and Nicell, 1997).

The enzymatic method for removal of phenols from industrial wastewater is a possible alternative to wastewater detoxification and recycling. Phenol-oxidizing enzymes, such as tyrosinases, are capable of catalyzing the oxidation of a wide range of phenolic pollutants.

Wada et al. (1993) suggested the use of mushroom tyrosinase in an immobilized form to remove phenolic compounds in wastewater.

Tyrosinase has been also indicated as a possible alternative to the more expensive peroxidase to accomplish the removal of phenols and aromatic amines from wastewaters (Ikehata and Nicell, 2000).

1.3 Recombinant expression of fungal oxidases

Fundamental studies to better understand the properties of the enzyme at the molecular and kinetic levels, or their use for biotechnological and environmental applications require large amount of readily available crude and purified enzyme.

The practical use of laccases and tyrosinases in biotechnological practices has led to search for new enzyme producers, the most suitable culture medium, the most appropriate, reproducible and inexpensive isolation procedures, and for mediators, which promote or facilitate enzyme action. Secreted laccases and cytosolic tyrosinases from wild-type fungal organisms might not be suitable for commercial purposes, mainly because unwanted preparation procedures (such as the presence of toxic inducers) are not economically advantageous. Moreover, laccase and tyrosinase genes are often expressed at very low levels in the native hosts. Therefore, it is thought that their heterologous expression in different hosts could be a valid approach to solve these problems.

Heterologous expression is better suited for large-scale production, because of the potential of expressing different enzymes in one selected optimised host. The choice of a particular host strain for the expression of a heterologous gene is one of the most important factors for the feasibility of an expression system. This choice is based on the complexity of the protein to be expressed, the protein production levels, and the properties of host cell. *Escherichia coli* and *Bacillus* spp. are the most commonly employed bacterial candidates.

Considerable information is available on their genetics and physiological characteristics and they often offer very high yields in heterologous protein production. *E. coli* is one of the most attractive host for heterologous protein expression because of its ability to grow rapidly and at high density on inexpensive substrates. Several expression systems designed for various applications and the host strain genetic background are highly important for recombinant expression, i.e. strains deficient in the most harmful natural proteases. However, a number of peculiar features of the foreign gene can be the reason of a lack of expression: i) the unique and subtle structural features of the gene sequence; ii) the stability and translational efficiency of mRNA; iii) the particular foreign protein folding; iv) the degradation of the protein by host cell proteases; v) the potential toxicity of the protein to the host; vi) the major differences in codon usage between the foreign gene and native *E. coli* genes.

However, bacteria lack post-translation modification systems and often produce eukaryotic proteins that are misfolded, insoluble, or inactive. Yeasts offer the ease of microbial growth and gene manipulation found in bacteria along with the eukaryotic environment and ability to perform many eukaryotic-specific post-translation modifications, such as proteolytic processing, disulfide bridge formation, and glycosylation. Yeasts are economical, usually give high yields, and are low demanding in terms of time and effort. Laccase and tyrosinase, like other oxidative enzymes, are difficult to express in non-fungal systems, however there are numerous data reporting successful heterologous expression of laccase and tyrosinase genes in several species. Fungal laccases have been expressed heterologously in *S. cerevisiae* **(**Kojima et al., 1990), *Trichoderma reesei* (Saloheimo and Niku-Paavola, 1991), *A. oryzae* (Sigoillot et al., 2004), *Pichia pastoris* (Jönsson et al., 1997; Soden et al., 2002; Liu et al., 2003), *A. sojae* (Hatamoto et al., 1999) and *A. niger* (Record et al., 2002; Larrondo et al., 2003), tobacco (LaFayette et al., 1999) and maize (Baiyle et al., 2004).

Starting from 1981, most of the recombinant proteins produced in yeasts have been expressed in *S. cerevisiae* strains (Hitzeman et al., 1981). The yeast *S. cerevisiae* is unique among eukaryotes in exhibiting fast growth both in the presence or absence of oxygen. *S. cerevisiae* has been used for thousand of years by mankind in brewing and baking and is regarded as GRAS (generally recognized as safe) organism. An overwhelming wealth of information on its genetics, molecular biology and physiology has been accumulated making this traditional species the best characterised eukaryotic system today. The sequencing of its entire genome has been completed (Goffeau et al., 1996) and thousands

of genes are characterised. *S. cerevisiae* is a suitable host for the expression of recombinant proteins from higher eukaryotes as it combines the ease, simplicity and cheapness of bacterial expression systems with the authenticity of the far more expensive and less convenient animal tissue culture systems. *S. cerevisiae* cell also provides a good environment for post-translational processing, such as proteolytic processing, folding, disulfide bridge formation, and glycosylation and secretion, resulting in a product that is often identical or extremely similar to the native protein. The expression of heterologous or homologous genes is obtained by fusion to a constitutive or inducible promoter element, generally derived from a highly expressed *S. cerevisiae* gene. A range of mutant strains further adds to the versatility of the yeast system, as the protease-deficient strains, able to improve the quality and yields of a large number of heterologous proteins (Romanos et al., 1992).

Several important factors that have an impact on expression must be considered, and among these, a key factor is the expression vector. A variety of vector systems for efficient expression of heterologous genes have been developed. Most of them are shuttle vectors with a bacterial part serving the purpose of cloning and amplification in *E. coli*. It is also possible to direct site-specific integration of a plasmid vector into the chromosome of the host strain. A range of different strong promoters is used in expression vectors; these promoter elements can either control a constitutive expression or can be induced by specific component added to the culture medium. The tight regulation of inducible promoter is advantageous in cases where the expressed protein might interfere with cell growth (Gellissen and Hollenberg, 1997). For secretion and for appropriate modification of many proteins from higher eukaryotes, it is necessary to fuse the protein sequence to a N-terminal leader that directs the nascent polypeptide into the secretory apparatus.

Heterologous expression of fungal tyrosinases has been obtained in *A. niger* (Halaouli et al., 2006), *E. coli* (Wichers et al., 2003) and *Pichia pastoris* (Westerholm-Parvinena et al., 2007).

Recent studies have reported *S. cerevisiae* as an excellent host for expression of heterologous genes, due its high transformation efficiency, DNA plasmid stability, growth rapidity. In particular, *S. cerevisiae* has already been successfully used to express laccase genes from *Melanocarpus albomyces* (Kiiskinen et al., 2004), *Myceliophthora thermophila* (Bulter et al., 2003), *Trametes* spp. (Klonowska et al., 2005), *T. versicolor* (Cassland and Jönsson, 1999) and *P. ostreatu*s (Piscitelli et al., 2005).

1.7.1 Laccases from *Pleurotus eryngii*

The white-rot fungus *P. eryngii* is a basidiomycete belonging to *Pleurotaceae* family. *P. eryngii* is a fungus variety widely spread, especially in Southern Italy in the summer-autumn. It is able to express multiple laccase genes encoding isoenzymes with interesting properties for both industrial applications and structure-function studies. Recently, several isoforms of laccases from the *P. eryngii* mycelium or fruitbody were purified, showing extremely attractive chemical-physical properties (Wang et al., 2006). In-depth studies about laccases purified from fungal mycelium show that these laccases have a simple protein structure with a reduced glycosylation level (1-7%) and a optimal stability at high pH values (8-12) (Munoz et al., 1997).

These data indicate that laccases of *P. eryngii* have a high potential in biotechnological applications, so studies aimed at the molecular characterization of genes encoding these enzymes are necessary to express them in heterologous system.

1.7.2 Tyrosinase from *Agaricus bisporus*

A. bisporus belongs to basiomycete class and it is a saprophyte and terricolous fungus. *A. bisporus* fungi are the most used for enzymes extraction and purification, directly from fungal fruitbodies, by chromatographic techniques, i.e. an acid phosphatase (AP) (Wannet et al., 2000), a threalose phosphorylase (Wannet et al., 1998), and, furthermore, *A. bisporus* is an optimal candidate for gene isolation and characterization studies.

Tyrosinase of *A. bisporus* is an heterotetramer composed by two heavy (H) and two light (L) chains, with a final molecular weight of 120 kDa (Strothkemp et al., 1976). There are two types of heavy chains, α and β, that produce respectively isoenzymes $H_2^{\alpha}L_2$ and $H_2^{\beta}L_2$ (Robb and Gutteridge, 1981).

AIM OF THE RESEARCH

The aim of the work was to characterize genes coding for oxidoreductases produced by fungi Basidiomycetes, in particular *ERY3* and *ERY4* genes coding for the Ery3 and Ery4 laccase of *P. eryingii* and the *PPO2* gene coding for the *A. bisporus* tyrosinase, and to select non-conventional yeast able to produce new oxidoreductase enzymes.

The main tasks of the present study were:

1. to express and characterize the *ERY3* laccase gene from *P. eryngii* in *S. cerevisiae* and to set up a new system for laccase expression and secretion consisting of whole cells of *S. cerevisiae* entrapped in calcium alginate gel beads;
2. to clone, express and characterize the *ERY4* laccase gene from *P. eryngii* and to produce different recombinant active laccase isoforms derived from it, by a mutation assay, starting from a non active Ery4 laccase in *S. cerevisiae*;
3. to construct engineered yeast by anchoring active recombinant laccase or tyrosinase isoforms on the cell wall in order to use it as a whole cell biocatalyst;
4. to clone, express and characterize the *PPO2* tyrosinase gene from *A. bisporus* in *S. cerevisiae*;
5. to investigate the application of oxidoreductase enzymes produced by non-conventional yeasts for the bioremediation of olive oil mill wastewaters.

Materials and methods

2. Materials and Methods

2.1 Media and solutions

All media and solutions used in this work are reported in the Appendix section. Any modification to the composition is described in the text.

2.2 Microorganism manipulation and isolation

2.2.1 Bacteria, yeast and fungal strains and growth conditions

The *Escherichia coli* strain employed in this study was DH5α (F-, ϕ80d*lac*ZΔM15, Δ*lac*ZYA-*arg*F).

The *S. cerevisiae* strains employed were CEN.PK2-1C (*MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8c SUC2*), W303-1A (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) and S288C (*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*).

The white-rot fungus *Pleurotus eryngii* (PS419) was obtained from International Bank of Edible Saprophytic Mushrooms (IBAF-CNR, Rome, Italy).

The *E. coli* strains were streaked from frozen stocks (stored at –70°C in 20% glycerol) onto the surface of LB (Luria Bertani) agar plate and incubated at 37°C for 18 h and were maintained in LB agar medium. Bacteria cells were inoculated in LB liquid medium and grown at 37°C with vigorous shaking at 250 rpm and were harvested at log phase. Selective media were supplemented with 50 μg/ml ampicillin.

Yeast cultures were grown using either a rich, YP medium, or a minimal medium, SM medium. Carbon sources for yeast cell growth were glucose (2%, w/v) or galactose (2%, w/v). Yeast strain maintenance was achieved by plating onto YP-glucose or SM-glucose medium supplemented with 2% (w/v) agar. All yeast strains were grown at 30°C and cultures were always harvested during the exponential phase of growth. Yeast induction was obtained by incubating cells, previously grown under repression conditions, for 72 h in SM medium supplemented with galactose and 1 mM $CuSO_4$ at 18°C.

The fungus was routinely grown on potato-dextrose agar plates at 25°C for 8-10 days. Inocula were prepared by scratching mycelia from plates and cultivating the fungus in 1 liter flasks containing 500 ml YPD (Sigma, USA).

2.2.2 Yeast and moulds isolation from OCW and OMW

The effluents used in this study derived from olive mills which use the continuous process for the extraction of olive oil. This extractive procedure is characterized by the use of rotating hammers to ground up the olives and the subsequent employment of a centrifuge to separate oil, wastewaters and pomace. The wastewater samples, produced during oil extraction, were collected, in sterile plastic containers, from local factories in five different locations (Casarano, Galatone, Martano, Scorrano and Torchiarolo) in Apulia (Southern Italy). The collected effluents were subsequently filtered through cheesecloth, centrifuged for 15 min at 4000xg, filtered through Whatman filter GF⁄A, and stored at -20°C until use. The main physic-chemical features of the five OMW samples ranged as follows: pH 4.7-4.9, Chemical Oxygen Demand (COD) 45.54 - 65.57 g/L, total phenol content 3.12 - 4.24 g/L and total solids 8.13 - 9.64 g/L.

Indigenous mycetes (yeast and moulds) were isolated from each Olive Centrifuge Water (OCW; directly collected from the vertical centrifuge) and from Olive Mill Wastes (OMW) samples (sampled from the storage ponds). Aliquots (100 µl) of appropriate dilutions in 1% saline solution were spread onto YPD agar, supplemented with 50 µg/ml ampicillin to inhibit bacterial growth. After incubation for 2–6 days at 28°C, 30 colonies of each sample were isolated according to shape, colour, surface feature and frequencies. The yeast cultures were preserved on YEPD plates at 4°C and sub-cultured every 6 months or kept at -80°C in liquid YEPD medium added with 50% sterile glycerol. All isolated strains were tested on solid media on plates containing 40 ml a sterile solution composed of 10%, 25%, 50%, 75% and 100% (v/v) OMW in water and 2% (w/v) agar, and then compared with the growth level on YEPD agar plates. Liquid cultures were conducted in triplicate, in 250 ml conical flasks containing 50 ml OMW medium (75% OMW, 25% sterile distilled water).

2.3 Nucleic acid extraction, competent cell preparation and transformation

2.3.1 Competent *E. coli* cells preparation

All steps in this procedure were done aseptically. Using a sterile platinum wire, *E. coli* cells were streaked from frozen stocks (stored at –80°C in 20% glycerol) onto the surface of LB agar plate and incubated at 37°C for 16 h. Fifty milliliter of LB liquid medium were inoculated with 0.5 ml saturated culture and grown at 37°C with vigorous shaking at 250

rpm. Bacterial cells were harvested at log phase (about 2.5 h after), kept on ice for at least 15 min and collected by 1.5 min centrifugation at 8000xg. Cells were re-suspended in 10 ml 0.1 M ice-cold sterile $CaCl_2$, incubated on ice for 1 h and then collected again by centrifugation (1.5 min at 8000xg). The pelleted cells were gently re-suspended in 5 ml $CaCl_2$ and stored for at least 4 h on ice before utilization.

2.3.2 *E. coli* transformation and colony screening

Two hundreds microliter of competent cells were mixed with about 50 ng DNA from ligation mixture in a sterile tube. The content was gently swirled and left on ice for 20 min. Heat shock treatment was done at 42°C for 1.5 min, followed by rapid chilling in ice bath for 10 min. The cells were thoroughly mixed with 0.8 ml LB (without antibiotics) and incubated at 37°C for 1 h with shaking at 220 rpm to allow recovery of the bacteria. After centrifugation for 2 min, 800 µl supernatant were removed and the bacterial pellet was re-suspended with the remaining LB medium. The bacterial suspension was gently spread over the surface of agarized LB medium, containing 50 µg/ml ampicillin and previously spreaded with 40 µl 2% w/v X-gal solution using a sterile bent glass rod, until the liquid was absorbed. The plates were inverted and incubated at 37°C overnight. The white bacterial colonies, carrying the insert, were selected and re-inoculated in another plate using sterile toothpicks.

2.3.3 Mini-preparation of plasmid DNA

The recombinant bacterial colonies were singly picked from the master plate and inoculated in 2 ml LB medium containing ampicillin (50 µg/ml). The culture was grown at 37°C for 16 h at 250 rpm. The bacterial culture was collected into a 1.5 ml eppendorf tube, centrifuged at 14000xg for 30 sec and the bacterial pellet recovered. The pellet was re-suspended in 350 µl STET lysis buffer and 15 µl lysozyme (20 mg/ml) and vortexed. Samples were then placed in boiling water bath for 40 sec, and immediately chilled on ice and then centrifuged at 14000xg for 20 min. The pellet of bacterial debris and genomic DNA were removed from the tube with a sterile toothpick. The supernatant was digested with 1.5 µl DNase-free-RNase A (10 mg/ml) at 37°C for 20 min. Plasmid DNA was extracted with 350 µl 1:1 (v/v) phenol/chloroform. The aqueous phase resulting by centrifugation at 14000xg for 10 min was extracted with 350 µl 24:1 (v/v) chloroform/isoamyl alcohol. After a centrifugation step at 14000xg for 10 min, the resulting aqueous phase was precipitated by the addition of 125 µl 5 M ammonium acetate

(pH 3.5) and 1 ml ice–cold absolute ethanol. Plasmid DNA was incubated at –20°C for 1 h and then centrifuged at maximum speed for 10 min. The pellet was washed with 70% ethanol and recovered by centrifugation at maximum speed for 10 min. After removing all the supernatant by gentle aspiration, the plasmid DNA was dried at room temperature, dissolved in 30-50 µl TE buffer (pH 8.0) and stored at -20 °C.
Recombinant plasmid DNA was extracted using the Plasmid Miniprep Kit (EuroClone, Italy) and submitted to sequence analysis, as reported in the next 2.4.4.4 Section.

2.3.4 DNA preparation from yeast

Total DNA was purified from a 2-10 ml culture of yeast grown in YPD to stationary phase at 30°C overnight with shaking at 180 rpm. The culture was spinned at 5 min in a tabletop centrifuge at 8000xg. The supernatant was poured off and the pellet re-suspended in 300 µl Breaking buffer and added with 0.3 g glass beads (Sigma) and 300 µl phenol:chloroform:isoamyl alcohol (25:24:1). Cells were disrupted by vortexing at highest speed for 3 min, added with 200 µl TE buffer, vortexed briefly and then centrifuged at 14000xg for 10 min. The aqueous phase was transferred to a clean microcentrifuge tube, added with 1 volume chloroform:isoamyl alcohol (24:1) and then centrifuged at 14000xg for 10 min. The aqueous layer was collected and transferred to a new tube and added with 1 ml 100% ice-cold ethanol. The samples was precipitated at –20°C for at least 1 h and subsequently centrifuged at 14000xg for 10 min at 4°C. The supernatant was removed and, after a wash with 70% ethanol, the pellet was dried, re-suspended in 50-100 µl sterile water and stored at –20°C.

2.3.5 Total RNA preparation from fungal mycelia

Isolation of total RNA from fungal mycelia was performed as previously described by Bleve et al. (2003). Briefly, mycelia were harvested by filtration, frozen and pulverised in liquid nitrogen and then resuspended in LETS buffer. Then 300 µl acid phenol (pH 4.3; Sigma-Aldrich, USA)-chloroform-isoamyl alcohol (25:24:1), 1 µl diethyl pyrocarbonate (DEPC; Sigma-Aldrich, USA), and about 60 mg of acid-washed glass beads were added. The preparations were treated by alternating 1-min cycles of vortexing with incubation on ice for about 5 min. Extracts were then centrifuged at 15000xg for 10 min at 4°C. The supernatant was extracted again with an equal volume of chloroform-isoamyl alcohol (24:1). These steps were repeated until a clear interface between aqueous and organic

layers was obtained after centrifugation. Total RNA was precipitated with 2 volumes ice-cold 100% ethanol and 0.1 volume 3 M potassium acetate and was left at -80°C for 1 h before the nucleic acids were pelleted at 15000xg for 15 min at 4°C. The pellet was washed with 70% ethanol and resuspended in 30 µl sterile DEPC-treated water. The sample was stored at -80°C until use.

Contaminating genomic DNA was removed from total RNA by incubation with RNase-free DNase I (Invitrogen, USA) in a reaction mixture containing RNase inhibitor (RNaseOUT; Invitrogen, USA), DNase assay buffer and DEPC-treated water. The reaction mixture was first incubated for 15 min at room temperature and then for 5 min at 65°C. The absence of any contaminating DNA was assessed by using the purified RNA as template in a PCR reaction.

2.3.6 Competent *S. cerevisiae* cell preparation, transformation and colony screening

S. cerevisiae cells were transformed by the improved lithium-acetate protocol (Gietz et al., 1995). The yeast strain was inoculated into 5 ml liquid medium (YPD or SM-D selection medium) and incubated overnight on a rotary shaker at 200 rpm and 30°C. The titer of the yeast culture was determined by spectrophotometer measuring the OD at 600 nm. Fifty milliliter of YPD or SM-D were inoculated with 2.5 x 10^8 cells (to give 5 x 10^6 cells/ml as initial concentration) and incubated on a rotary shaker at 30°C with 200 rpm. When the cell concentration was equivalent to 2 x 10^7 cells/ml, they were harvested by centrifugation at 3000xg for 5 min, washed in 25 ml sterile water and resuspended in 1 ml sterile water. One millilitre of carrier Salmon Sperm DNA (10 mg/ml) was boiled for 5 min and chilled in an ice/water bath while harvesting the cells. The cell suspension was then transferred to a 1.5 ml microcentrifuge tube, centrifuged for 30 sec and the supernatant discarded. Water was added to the pellet to a final volume of 1 ml and the mixture was vortexed vigorously to resuspend the cells. One hundred microliter of cell suspension (about 10^8 cells) were transferred into 1.5 ml microfuge tubes, centrifuged at 14000xg for 30 sec and the supernatant removed. To each transformation tube 360 µl Transformation Mix was added, the cells were resuspended by vortex mixing vigorously and incubated at 42°C for 1 h. Samples were then centrifuged at 14000xg for 30 sec and the yeast pellet was gently re-suspended by a micropipette into 1 ml sterile water. Appropriate dilutions of the cell suspension were plated onto selective SM-D media agar plates without the specific auxotrophic requirement that the plasmid enabled transformed yeast to synthesize. The

plates were incubated at 30°C for 3 to 4 days and then the number of transformants was counted.

2.4 Nucleic acid manipulation

2.4.1 Plasmid vectors

Bacteria. pGEM®-T Vector Systems (Promega, USA) and pYES2 Vector (Invitrogen, USA) for the PCR products cloning.

Yeast. pYES2 (Invitrogen, USA), p426 GDP (ATCC, USA) for heterologous expression of genes in yeast cells.

2.4.2 Reverse Transcription

The first strand cDNA was synthesized from total RNA by RT-PCR using the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA) with oligo dT and random hexamers primers.

Each reaction mixture (20 µl) contained 2 µl first-strand buffer (Invitrogen, USA), 0.3 mM deoxynucleoside triphosphates (dNTP), 5 ng the random hexamer (Invitrogen, USA) μl^{-1}, 20 U RNaseOUT, 10 mM dithiothreitol, 100 U SuperScript III reverse transcriptase (Invitrogen, USA), and 100-700 ng RNA. The reaction mixture was incubated first at 42°C for 50 min and then at 70°C for 15 min. An aliquot (2.5 µl) of the resulting cDNA was amplified by PCR.

2.4.3 DNA amplification by PCR and purification

PCR mix composition and PCR reaction conditions generally used are the following. 10-100 ng DNA or cDNA template were amplified by PCR using a 50 µl mixture which contained 1 unit Phusion DNA Polymerase (Finnzymes, Finland), 1× Phusion HF buffer (Finnzymes, Finland), 0.2 mM each dNTP and 0.25 µM each primer. The amplification program consisted of denaturation at 98°C for 30 s, 35 cycles of denaturation at 98°C for 10 s, specific primer pair annealing for 15-30 s, extension at 72°C for 15s/kb followed by a final extension at 72°C for 10 min. Long PCR amplification was performed in a 50 µl mixture which contained 1 unit DyNAzyme EXT DNA polimerase (Finnzymes, Finland), 1× Optimized DyNAzyme EXT buffer (Finnzymes, Finland), 0.2 mM each dNTP and 0.5 µM each primer (Tab.1). The PCR conditions employed were: 94°C 2 min (once) + 94°C

30 s, specific primer pair annealing 30 s, 72°C 2 min and 20 s (35 cycles) + 72°C 10 min (once).

Amplification reactions were performed using PCR Express System (Hybaid) and ONE personal PCR System (EuroClone, Italy). Control reactions were routinely carried out using also a negative (without DNA) as well as a positive (DNA from *S. cerevisiae* type strain CBS1171^T) control. The PCR products were analysed by electrophoresis in 1% agarose gels containing ethidium bromide (1 μg/μl). For direct yeast transformation, amplicons were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 2.5 volumes 100% ethanol and 1/10 volume 3M NaOAc, and resuspended in 20 μl sterile water.

2.4.4 DNA modifying reactions

2.4.4.1 Digestion with restriction enzymes

The PCR products and the cloning vectors were hydrolysed with appropriate restriction enzymes. Following the manufacturer's instructions, the amplification products were purified by the EuroGold Cycle-Pure Kit (EuroClone, Italy) and the digested vectors were purified using the EuroGold Gel Extraction Kit (EuroClone, Italy). Plasmid DNA or PCR products were digested with one unit of suitable restriction enzymes for 1 μg DNA, in the presence of specific restriction enzyme buffers. After incubation at 37°C for 1-2 h, preparation was analyzed by electrophoresis in 1% agarose gel in 1× TAE buffer, stained by ethidium bromide and visualized on an UV-source**.**

2.4.4.2 Dephosphorylation of the linearised plasmid

Antarctic phosphatase (New England Biolabs, USA) is the enzyme that catalyze the removal of 5'-phosphate groups from DNA and RNA. Since phosphatase-treated fragments lack the 5' phosphoryl termini required by ligases, they cannot self-ligate. The reaction was performed in 1× Antarctic Phosphate Reaction Buffer (New England Biolabs, USA) using 5 U enzyme and incubated at 37°C for 15 min. The phosphatase was inactivated at 65°C for 5 min.

2.4.4.3 Ligation of foreign DNA in a plasmid

Ligation reactions were generally performed in a final volume of 20 μl and using 50 μg vector DNA and the needed quantity of fragment resulting from an insert/vector ratio of 3:1 or 6:1. The reaction was performed in 1× Ligase buffer (New England Biolabs, USA) using T4 DNA ligase (New England Biolabs, USA) 20 U/μl and incubating at 4°C

overnight. Control reactions were also carried out: digested vector with ligase but without insert, digested vector with no ligase and supercoiled plasmid.

2.4.4.4 DNA sequencing

The PCR mix (final volume, 20 µl) contained 1× Ready mix (Applied Biosystems), 1 µl 3.2 µM specific primer, and 3ng/100 bp-length for fragments or 300 ng for plasmids < 7 kb-length and 500 ng for plasmids of 7-15 kb-length. Reactions were run for initial denaturation at 96°C for 2.5 min, followed by 25 cycles of 10 s at 96°C, 10 s at specific primer pair annealing, and 4 min at 60°C. After PCR reaction, the sample was purified by Centri-Sep Spin columns (Applied Biosystems) following the manufacturer's instructions and run on an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems, USA). The sequence data were analysed by employing the Chromas software (version 1.45).

2.5 Protein production, extraction, purification and analysis

2.5.1 Expression and induction of recombinant laccase in *S. cerevisiae*

The constructs of pYES2 plasmid carrying the laccase genes and the empty control pYES2 vector were used to transform the *S. cerevisiae* strain CEN.PK2-1C as described in Materials and Methods Section 2.3.6. To screen transformants for the secretion of recombinant laccase in the extracellular medium, a colorimetric assay on agar plate was first performed. Colonies were picked from SM-Ura plates supplemented with 2% glucose and transferred to SM-Ura plates supplemented with 2% galactose and with 5 mM ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] (Sigma, USA), and 0.5 mM $CuSO_4$. Plates were incubated for 3 days at 18-25°C and checked for the development of a green colour. In order to induce the expression of laccase genes, the laccase-secreting yeasts were inoculated into SM-Ura medium supplemented with 2% glucose and incubated with shaking at 30°C. When the turbidity of the culture reached an optical density at 600 nm = 0.8 (1 OD_{600} = 3 x 10^7 cells ml^{-1}), cells were harvested by centrifugation. Yeast cells were then washed once with water and re-suspended at 0.6 OD_{600} ml^{-1} concentration in SM-Ura medium, supplemented with 2% galactose and different concentration (0, 0.1, 0.25, 0.5 and 1 mM) of $CuSO_4$. Cultures producing laccase were grown at various temperatures (18, 25 and 30°C), and the samples were taken at different times for laccase activity determination. After performing the above experiments, the recombinant clones

exhibiting the highest laccase-secreting ability was selected for the subsequent experiments.

2.5.2 Laccase purification

One litre of cultured cells was centrifuged, the pellet discarded and the broth were pre-filtered with a micro-filtration system using a 0.45 μm membrane and then concentrated by the Stirred Ultrafiltration Cells equipped with a 10 kDa cut-off membrane filter (Millipore, USA). The concentrate (50 ml) was added with 7 volumes of Tris/HCl 50 mM pH 8, and filtered again as described above. The filtrate was then fractionated on a Bio-Rad Hight Q ion exchange column (1.6 x 10 cm; Bio-Rad, USA) equilibrated with Tris/HCl 50 mM pH 8. Laccases were eluted with a linear gradient from 0 to 0.4 M NaCl (30 ml) in Tris/HCl 50 mM pH 8 at a flow rate of 1 ml/min. Fractions containing enzymatic activity were pooled, further concentrated by filtration through the Amicon cell YM-10 kDa membrane. The filtrate obtained was loaded on Sephadex G-75 (1.6 x 60 cm; Amersham, USA) equilibrated with Tris/HCl 50 mM pH 8, added with 50 mM NaCl, eluting the enzyme at a flow rate of 0.5 ml/min. The fractions containing purified laccase were collected, pooled and then stored with 20% glycerol at -20°C.

Polyacrylamide gel electrophoresis and Western Blot were carried out as described in Materials and methods section 2.5.5. and 2.5.6 respectively.

2.5.3 Induction of tyrosinase expression

The recombinant pYES2 plasmid carrying the tyrosinase gene and the empty control pYES2 vector were used to transform the *S. cerevisiae* strain CEN.PK2-1C as described in Materials and Methods Section 2.3.6. In order to induce the expression of tyrosinase gene, the tyrosinase-expressing yeasts were picked from SM-Ura plates supplemented with 2% glucose and inoculated into SM-Ura medium supplemented with 2% glucose and incubated with shaking at 30°C. When the turbidity of the culture reached an optical density at 600 nm = 0.8 (1 OD_{600} = 3 x 10^7 cells ml^{-1}), cells were harvested by centrifugation. Yeast cells were then washed once with water and suspended at 0.6 OD_{600} ml^{-1} concentration in 1 liter SM-Ura medium, supplemented with 2% galactose and 1mM $CuSO_4$. Cultures producing tyrosinase were grown at 18°C for 1-2 days and then used for the subsequent experiments.

2.5.4 Tyrosinase extraction and purification

Recombinant yeasts expressing tyrosinase were harvested by sedimentation at 5000xg for 10 min and resuspended in lysis buffer pH 8 to a final volume of 90 ml for 7 g of cell pellet. Cells were lysed in a French Press (Thermo Electron Corporation, USA) and the homogenate was centrifuged at 4°C for 20 min at 30000xg in order to remove cell debris. If needed, the supernatant was filtered to take out particulate matter before column chromatography.

The filtered crude fresh extract was applied to a 5 ml Ni-NTA (Qiagen superflow agarose), gravity-fed column with 0.5 ml/min Flow/rate. Cell lysate was loaded onto the charged column equilibrated in lysis buffer, the column was washed with 40 column volumes of wash buffer. The tyrosinase protein was eluted from the column using 5 volumes of elution buffer. Individual column fractions were subjected to enzyme activity assays (Section 2.6.4) and the selected fractions were pooled and stored at -20°C.

2.5.5 Polyacrilamide gel electrophoresis assay

Gel Run was performed at 140 V and 25-30 mA in SDS Tris/Glicine buffer in a BioRad MiniProtean II gel electrophoresis equipment. Run system was disassembled and gels were immersed in at least 5 volumes of the Staining solution and placed on a slowly rotating platform for at least 1 h at room temperature. Then, the Staining solution was removed and the gels were firstly washed with Destaining solution and subsequently incubated in the same fresh buffer for 10-20 min on a slowly rocking platform. After destaining, the gels were stored in H_2O.

Recombinant tyrosinase samples, resuspended into Laemmli sample buffer 1x without β-mercapto-ethanol and without boiling, were loaded in onto 10% polyacrilamide native gel. Electrophoresis was carried out at 50 mA/gel at 4°C. After electrophoresis, the gels were washed in distilled water for at least 15 min and stained with 50 ml 10 mM L-Dopa solution at room temperature. To check the sensitivity of the staining method, different concentrations of purified enzyme and of commercial mushroom tyrosinase (Sigma, USA) were loaded and tested for the activity detection by staining.

Silver staining procedure was performed as previously described by Heukeshoven and Dernick (1985). Briefly, the gel was soaked in the Fixing solution for 30 minutes and then the Sensitizing solution was added and left shaking for at least 30 minutes. After removing the Sensitizing solution, the gel was washed with distilled water three times for 5 minutes each time; then, Silver solution was added and left shaking for further 20 minutes. After

removing the Silver solution, the gel was rinsed twice in distilled water for one minute each time; then, the Developing solution was added and left shaking for 2-10 minutes. The Developing solution was then removed and the gel was soaked in the Stop solution and left shaking for 10 minutes. Finally, the gel was washed three times for 5 minutes each time with distilled water.

2.5.6 Western blot analysis

Protein samples were separated using small one-dimensional gels, including protein molecular weight standard (SDS-PAGE Standard, broad range, Biorad), in one or more gel lanes. When electrophoresis was complete, gel sandwich was disassembled and stacking gel removed. The resolving gel was orientated by removing a corner. Gel was then soaked and equilibrated 20-30 min at room temperature in the Transfer buffer. A sheet of the transfer membrane (HybondTM-P PVDF Membrane, Amersham Biosciences) was cut to the same size as gel plus 1-2 mm on each edge. Membrane was pre-wetted in 100% methanol for 10 s and then washed in distilled water for 5 min and then it was equilibrated in the Transfer buffer for at least 10 min. Two sponges and two sheets of filter paper Whatman 3MM, cut to same size as gel, was pre-wetted with Transfer buffer. The transfer sandwich was assembled on the plastic transfer cassette placing in order, starting form the black or grey bottom half of the cassette, the sponge, a sheet of filter paper, the gel (removing any air bubbles between gel and filter paper by gently rolling a test tube or glass rod over the surface of gel and moistening it with Transfer buffer), the pre-wetted membrane directly on top side of gel (i.e. anode side, removing all air bubbles), another piece of filter paper (placed on anode side of membrane, removing all air bubbles) and the sponge on top of this filter paper. Assembly was completed locking top white half of the transfer cassette into place. The tank was filled with Transfer buffer and the transfer cassette-containing sandwich was placed into electro blotting apparatus in the correct orientation. The proteins were electrophoretically transferred from gel to membrane overnight at 30 V (constant voltage, at 130-140 mA) or 30 min-1 h at 100 V cooling at 4°C. At the end of this step, the power supply was turned off and the apparatus disassembled. The membrane was removed from blotting apparatus and cutting a corner or marking noted the orientation with a soft lead pencil.

The gel was stained for total protein with Coomassie staining solution to verify transfer efficiency, while PVDF membrane was reversibly stained with Ponceau S solution

incubating it for 5 min at room temperature and then destaining it for 2 min in water. Membrane was photographed and the molecular-weight-standard band locations marked with indelible ink. Membrane was completely destained by soaking an additional 10 min in water.

The membrane was placed on a tray with 10 ml of Blocking buffer and incubated with agitation on an orbital shaker or rocking platform at room temperature for 1 h. The membrane was then transferred into Blocking buffer added with the primary antibody [1:10000 dilution for the anti-laccase antibodies raised against the *Pycnoporus cinnabarinus* laccase (Record et al., 2002); 1:10000 dilution for the anti-tyrosinase antibodies raised against the *A. bisporus* tyrosinase] and incubated for 1-2 h at room temperature with constant agitation or overnight at 4°C. The membrane was then removed from the tray and washed four times for 10 min in 10 ml 1X TS. The filter was then incubated into Blocking buffer added with the secondary antibody [1:10000 dilution for the Anti-Rabbit IgG peroxidase conjugated (Sigma); 1:8000 dilution for the Anti-Mouse IgG peroxidase conjugated (Sigma, USA) and the membrane allowed to soak in the solution for 1 h at room temperature. The membrane was washed again as described above and then subjected to the protein detection procedure using the SuperSignal® West Pico System (Pierce). Briefly, the membrane was drained and placed face down on a sheet of clear plastic wrap. The wrap was folded back onto the membrane to form a liquid-tight enclosure. In a darkroom, the membrane was placed face down onto film (Kodak BioMax™ MR, Sigma) in a film cassette, and autoradiographed for a few seconds to overnight depending upon the sample. The film was immersed into a GBX Developer solution (Kodak Processing chemicals, Sigma) for 5 min, washed with water and fixed into a GBX Fixer solution (Kodak Processing chemicals, Sigma) for 5 min, and finally washed with water. The film was air dried and observed.

2.6 Enzymatic analysis

2.6.1 Laccase activity assay

Laccase activity was assayed at 25°C using, as substrates, (a) ABTS, (b) 2-methoxyphenol (guaiacol), (c) 2,6-dimethoxyphenol (DMP), (d) syringaldazine (SGZ) and (e) 3,3',5,5' tetramethylbenzidine (TMB) as follows. (a) The activity assay mixture contained 2 mM ABTS in 0.1 M sodium citrate buffer, adjusted to pH 3.0. Oxidation of ABTS was

followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}). (b) The assay mixture contained 10 mM guaiacol in 0.1 M sodium acetate buffer, adjusted to pH 6.5. Oxidation was followed by the absorbance increase at 460 nm (ε = 26,600 M^{-1} cm^{-1}). (c) The assay mixture contained 5 mM DMP in 0.1 M sodium acetate buffer adjusted to pH 4.5. Oxidation of DMP was followed by an absorbance increase at 477 nm (ε = 14,800 M^{-1} cm^{-1}). (d) The assay mixture contained 0.5 mM SGZ (dissolved in methanol) and 50 mM phosphate buffer, pH 6.5. Oxidation of SGZ was followed by absorbance increase at 525 nm (ε = 65,000 M^{-1} cm^{-1}). (e) The assay mixture contained 1 mM TMB and 100 mM citrate buffer, pH 3. Oxidation of TMB was followed by an absorbance increase at 655 nm (ε = 39,000 M^{-1} cm^{-1}). One unit was defined as the amount of enzyme which oxidized 1 μmol of substrate per minute. Enzyme activity was expressed as international units (IU). Enzyme activity measurements were performed on a Beckman DU-600 spectrophotometer (Beckman Instruments). Protein concentration was determined using the Protein Assay dye reagent (Bio-Rad, USA) with bovine serum albumin as a standard.

2.6.2 Laccase kinetic assays

Kinetic parameters were determined using a spectrophotometric method for all substrates. K_{cat} values were calculated using the total protein concentration in the enzyme solution. Control measurements without enzyme were carried out to correct for any chemical oxidation of the substrates at 25°C. Laccase (2.2 μM) was incubated with increasing concentrations of substrates in reaction buffer, and the reaction product formed was measured every 30 s for 10 min. Lineweaver-Burke analysis was used to analyze reaction kinetics parameter. All determinations were carried out in triplicate.

2.6.3 Temperature and pH dependent residual laccase activity

Enzymes activity at different pH and temperatures were determined using 2 mM ABTS as substrate. To test pH dependent activity, the enzymes were incubated for 60 h at 25°C in 0.1 M Sodium Citrate buffer pH 3, 0.1 M Sodium Acetate buffer pH 4.5 and pH 5.5, and in 0.1 M Phosphate buffer pH 6.5 and pH 8.0. The effect of temperature on stability of laccase isoforms was evaluated incubating the enzymes for 60 h at 10, 25, 40, 55 and 70°C in 0.1 M Phosphate buffer pH 6.5. All determinations were carried out in triplicate.

2.6.4 Tyrosinase activity assay

Tyrosinase activity was monitored spectrophotometrically using 50 mM Potassium phosphate buffer pH 6.5 and L-Tyrosine (ε_{280nm}=1,290 M^{-1} cm^{-1}), L-DOPA (ε_{475nm}=3,600 M^{-1} cm^{-1}), Pyrocatechol (ε_{420nm}=2,040 M^{-1} cm^{-1}), Pyrogallol (ε_{430nm}=2,600 M^{-1} cm^{-1}) as substrates. All reactions were carried out at room temperature. Enzyme activity was expressed in international units (IU). Protein concentration was determined using the Protein Assay dye reagent (Bio-Rad, USA) with bovine serum albumin as a standard.

2.6.5 Extracellular enzymes activity assay

The cellulolytic, xylanolytic, laccase, tyrosinase, lignin and manganese dependent peroxidase activities were separately assayed by plate tests, as described by Pointing (1999). The polyphenoloxidase activity in OMW was determined polarographically by measuring the oxygen uptake at 37°C with a Clark electrode connected to a Gilson K-IC Oxygraph (Medical Electronics, Middleton, Wisc., USA). The reaction mixture consisted of 0.01 M caffeic acid in 0.15 M Potassium phosphate buffer pH 6, and a suitable amount of OMW. The Ppo activity unit (U) is defined as the decrease of 1 mmol/L in O_2 concentration per minute. Protease activity was determined by the caseinolytic method (Mohawed et al., 1986) as reported in the next paragraph 2.6.6.

2.6.6 Protease activity assay

Protease activity was determined by caseinolytic method (Mohawed et al., 1986). One unit (U) of enzyme is defined as the amount of protease that produces free peptide fragments equivalent to one mg of bovine serum albumin (BSA) per unit time (min^{-1}), under the assay conditions (Patil and Shastri, 1981).

2.6.7 Antimicrobial activity assay

A spot test was employed to evaluate the antibacterial activity of OMW, using *B. megaterium* ATCC 25848 as indicator strain. Tryptic soya agar (Sigma, USA) plates were seeded with 0.3 ml bacterial suspension, containing ca 1.0 x 10^6 colony forming units (CFU/ml) and then allowed to dry at 25°C for 1 h. The OMW samples were previously filtered through 0.2 μm filter under sterile conditions and aliquots (100 μl each) were spotted on the surface of the inoculated plate. After incubation at 25°C for 24 h, the plates were analyzed for halos of inhibition around the OMW spots.

2.7 Isolation of yeast cell wall fractions and protein analysis

Yeast cells expressing the sequences carrying the different expression cassettes *FS-4NC3*, *FS-5LESS*, *PIR2-4NC3*, *PIR2-5LESS* were incubated with shaking at 24°C for two days. The cells grown in SM-Ura medium supplemented with 2% glucose and 1 mM $CuSO_4$ were harvested and washed three times in buffer A. The harvested biomass was resuspended in buffer A in a proportion of 5 ml *per gram* (wet weight) and was broken by five cycles at 1500 psi by French Press treatment. Breakage was confirmed by phase contrast microscopy and the walls were washed three times in buffer A. Removal of non-covalently bound proteins was achieved by boiling the walls in buffer A containing 2% SDS for 5 min. The cell wall-extracted proteins were recovered by centrifugation at 10000xg for 5 min. Cell walls were washed three times in buffer A and treated with 100 mU β-Glucanase, from *Aspergillus niger* (Sigma, USA), in 200 µl Na acetate 100 mM pH 5.5, 1 mM PMSF and supplemented with 20 µl/ml Protease Inhibitor Cocktail (Sigma, USA) and incubated at 37°C overnight. After treatment, the extracted and insoluble materials were separated by centrifugation at 10000xg for 5 min.

Cell wall-extracted proteins were separated by SDS-PAGE and the Western blot was performed, following the procedures reported in Materials and methods, Sections 2.5.5 and 2.5.6.

2.8 Indirect Immunofluorescence

The yeast cells, expressing mutant laccases on their cell walls, were analyzed by immunofluorescence microscopy according to the method of Redding et al. (1991). Yeast cells were fixed by adding, to cultures, formaldehyde to 8 % (vol/vol). After 30 min with gentle agitation (100 rpm), cells were pelleted (4000 rpm, 1 min), resuspended in formaldehyde to 4 % and gently shaken at room temperature (RT) for 1 h. Fixed cells were washed, re-suspended in SHA buffer and stored at 4°C for up to 2 wk. Fixed cells were adsorbed to polylysine-coated multiwell slides for 15 min, then washed with 1x PBS. To reduce background staining, the yeast cells coated on multiwell slides were incubated with PBS-B-N for 1 h at RT. Subsequently, wells were incubated for 2 h at RT with 10 µl primary antibody raised against *P. cinnabarinus* laccase (1:1000) diluted into PBS-B-N and then washed 10 times with 1x PBS. Sandwich amplification of the signal in cells expressing laccase was achieved by incubation, for 2 h at RT, with 10 µl Alexa Fluor 633-

conjugated secondary antibody (Molecular Probes, USA) (1:800) diluted into PBS-B-N, and then washed 10 times with 1x PBS. After the final washes, coverlips were applied with mounting solution and sealed. Cells were observed using a Zeiss LSM5 Pascal confocal laser-scanning microscope

2.9 Phenol concentration determination

Determination of phenol concentration was carried out in the filtered supernatant of OMW, using the Folin–Ciocalteu reagent and gallic acid as a standard. The Chemical Oxygen Demand (COD) was determined as reported by Hamdi et al. (1991). The soluble COD was measured after centrifugation of OMW samples for 10 min at 4000xg. Decolourization was assayed by measurement of the absorbance at 390 nm of filtered supernatant of OMW appropriately diluted in distilled water.

2.10 Yeast cell immobilization method

2.10.1 Immobilization of yeast whole cells producing laccase in Ca alginate gel beads

The following parameters were optimized for the preparation of beads: sodium alginate concentration, $CaCl_2$ concentration, initial inoculum quantity. Different concentrations of sodium alginate (1, 2, 3, 4%, w/v) and $CaCl_2$ (0.05, 0.075, 0.1, 0.125, 0.15 M) were tested. The initial biomass to be packed in the beads was ascertained by incorporating in the aqueous sodium alginate solution different amount of cells corresponding to 5, 10, 15, 20 or 25 OD_{600}. The yeast cells were grown in SM-Ura medium supplemented with 2% glucose with shaking at 30°C for 16 h. When the turbidity of the culture reached an optical density at 600 nm = 0.8, the requested volume was harvested. Cells were washed with water and then suspended in 5 ml Na-alginate (Sigma, USA) solution at the above indicated concentrations. The slurry was excluded drop-by-drop from a syringe through a blunt-ended needle (21 gauge) into 200 ml $CaCl_2$ at the tested concentration, with constant stirring at room temperature and under sterile conditions. The produced spherical beads (ca. 3 mm diameter) were cured in 0.1 M $CaCl_2$ solution for 4 h at 4°C. Beads were then washed with saline solution, added with SM-Ura medium (supplemented with 2% galactose, 0.5 mM $CuSO_4$, 0.05% $CaCl_2$) and subjected to submerged fermentation on a rotary shaker (180 rpm) at 18°C. Cell leakage from the Ca-alginate matrix was calculated by measuring the optical density of the culture medium at 600 nm. Optimized conditions

were selected for cell immobilization in repeated batch fermentation by carrying out each fermentation cycle up to 5 days. At the completion of each fermentation set, the beads were recovered from the exhausted medium, washed with sterile saline solution (0.9 % NaCl) and then aseptically moved to fresh medium to start the next fermentation cycle. Each experiment with free and immobilized yeast cells was carried out by performing at the same time three independent repetitions.

2.11 OMW treatment by immobilized *G. candidum* cells

After washing with saline solution, an amount of beads, corresponding to an inoculum equivalent to 5 x 10^8 cells, was added to 500 ml OMW medium (75% OMW, 25% sterile distilled water) and then subjected to submerged fermentation on a rotary shaker (180 rpm) at 25°C in the dark. Control experiments were similarly carried out by inoculating 500 ml OMW medium with an inoculum of 5 x 10^8 viable cells. Cell leakage from the Ca-alginate matrix and free cell cultures were periodically (0, 3, 6, 10 and 14 days) evaluated by viable counts on agar plates. This analysis was carried out by spreading tenfold serial dilutions of the OMW medium onto YEPD agar and incubating at 28°C overnight. At each time (0, 3, 6, 10 and 14 days), phenolic compound concentration using the Folin–Ciocalteu method, COD determination, decolourization test and antimicrobial activity assay were carried out on the filtered supernatant of OMW, inoculated with free and immobilized cells, appropriately diluted in distilled water. Each experiment, with free and immobilized yeast cells, was carried out by performing three independent repetitions.

2.12 Bioinformatic analysis

2.12.1 Bioinformatic analysis, primer construction and similarity search

Genomic and plasmid sequence were analysed by sequence analysis package Genamics Expression Version 1.1. Primer analyses were performed using the web program available on *Saccharomyces* Genome Database (http://seq.yeastgenome.org/cgi-bin/web-primer).
The search for sequence similarity with sequences in the GenBank database was performed by using the BLASTN algorithm.

2.12.2 Phylogenetic analysis

The analyses obtained with the CLUSTALW program (Thompson et al., 1994) were used to produce the alignment analysis of putative amino acid sequences coded by laccase genes from *Pleurotus* spp. obtained from SwissProt Database: POX1_Os (Q12729; *P. ostreatus*), POX2_Os (Q12739; *P. ostreatus*), LACa_Os (Q6STF0; *P. ostreatus*), LACb_Os (Q6RYA4; *P. ostreatus*), POXA1B_Os (Q9UVY4; *P. ostreatus*), POXA3_Os (Q96TR4; *P. ostreatus*), LCCK_Os (Q9UVY4; *P. ostreatus*), LAC1_Sc (Q7Z8S6; *P. sajor-caju*), LAC2_Sc (Q7Z8S5; *P. sajor-caju*), LAC3_Sc (Q7Z8S4; *P. sajor-caju*), LAC4_Sc (Q7Z8S3; *P. sajor-caju*), LAC5_Sc (Q7Z8S2; *P. sajor-caju*), LAC1_Sa (Q6A1A1; *P. sapidus*), LAC2_Sa (Q4VY49; *P. sapidus*), LAC2_Pu (Q2VT18; *P. pulmonarius*), LAC6_Pu (Q2VT19; *P. pulmonarius*), PEL3_Er (Q5MP11; *P. eryngii*), PEL4_Er (Q308C0; *P. eryngii*). *ERY3* cDNA sequence had been submitted to EMBL Database and the assigned EMBL accession number was AM773999. The protein sequence Lac_Ncra (P10574; *Neurospora crassa*) was used as outgroup in phylogenetic analysis. The phylogenetic trees were constructed using programs in the PHYLIP software package version 3.5 (Felsenstein, 1989). Dendrograms analysis was made with the assistance of the programs SEQBOOT, PROTDIST, NEIGHBOR (UPGMA and Neighbor-Joining methods) and CONSENSE. Parsimony trees were obtained with DNAPARS program using as input a multiple data set generated by SEQBOOT.

2.12.3 3D Modelling of recombinant laccases

Ery4. The 3D model of Ery4 was computed by comparison (Homology modelling) with the 3D structure of laccase from *Trametes versicolor* (PDB code: 1GYC – Piontek et al., PMID: 12163489), X-ray solved at 0.19 nm resolution. Sequence identity between ERY4/1GYC was approximately 61% . The template 1GYC fully covered target sequence with the exception of 15 residues at C-terminus. We found the template to build the C-terminus portion by the crystal structure of laccase from *Melanocarpus albomyces* (PDB code: 1GW0; Hakulinen et al., PMID: 12118243), know with a 0.24 nm resolution, sharing a sequence identity of 28% Ery4/1GW0 and 26% Ery4/1GYC (pairwise global alignment), with a RMSD (Root Mean Square Deviation) of 0.139 nm, after structural superimposition between 1GYC/1GW0. The C-terminus part was covered and modeled only considering the alignment with the template 1GW0. Alignment was done with ClustalW (Thompson et al., 1994), selecting Blosum matrix, default gap penalty for the opening and extension and

manually checked. To better mimic the folding of the functional protein, we placed the four copper ions and two water molecules deemed to contribute to the reaction of the substrate in the binding site. Modeling was performed with the program Modeller v9.4 (Sali et al., 1993). For a given alignment, ten 3D models were built and were evaluated and validated with the PROCHECK and PROSA2003 suites of programs (Laskowski et al., 1993; Sippl, 1993), choosing the model that showed the best stereo-chemical and energetics features according to the above mentioned tools.

Ery3. Since Ery3, a well functional laccase, and Ery4 share 57% residues in sequence, in order to make a structural comparison, we built a 3D model of Ery3, with the template 1GYC, 65% sequence identity, aligned by ClustalW, Blosum matrix, default gap penalty and manually checked. Ten obtained structures were validated with PROCHECK and PROSA2003.

Ery4-Chimera. Adopting the same modeling procedure described above, we proceed with the modeling of three different Ery4 chimeras, experimentally obtained with the substitution of the C-terminus of Ery4 with the C-terminus of Ery3 (Ery4-4C3) and the substitution of the NC-terminus of Ery4 with the NC-terminus of Ery3 (Ery4-4NC3), built with 1GYC as template, annotating a local sequence identity of 61% for both. The third Ery4-chimera protein was obtained from the substitution of the N-terminus of Ery4 with the N-terminus of Ery3 (Ery4-4N3) modeled with 1GYC/1GW0 as template, similarly to Ery4 modeling procedure, (local sequence identity of 61% Ery4-4N3/1GYC, and 30% Ery4-4N3/1GW0). Stereo-chemical and energetics validation were applied for each built model.

Single point mutations. Considering the 3D model of Ery4 as wild-type, we built three different models with a single residue mutation, changing the residue in position 532 from lysine to arginine (K532R), alanine (K532A) and glutamic acid (K532E), respectively. The models were built and validated in the same way as above.

C-Terminal deletion. In order to understand the structural changes due to the cut of C-terminus and try to explain the results experimentally tested, we realized the 3D model of 2Less, 5Less, 8Less, 11Less, 14Less, 18Less of the wild type Ery4. The input alignment for modeling procedure were developed for every single protein. The models were built and validated in the same way as above.

2.13 Statistic analysis

Statistical software SPSS 8.0 (SPSS Inc., Chicago, U.S.A.) was used for data analysis. Experimental points represent means ± s.d. of three replicates. Statistical analysis was carried out using ANOVA and by performing the *post hoc* tests (Bonferroni/Dunn). A P-value less than 0.05 was considered to be statistically significative.

2.13.1 Principal Component Analysis

Principal Component Analysis (PCA) is an unsupervised multivariate method that reduces the dimensionality of the original data matrix, retaining the maximum amount of variability (Forina et al., 1986), allowing the visualization of the different cases in a two-dimensional space, and identifying the directions in which most of the information is retained. It is therefore possible to explain differences among various samples by means of factors (principal components) obtained from the data set, and, at the same time, to determine which variable most contribute for such differences (Câmara et al., 2006).

Results

3. Results

3.1 Molecular cloning and heterologous expression of *ERY3* gene from *P. eryngii* in free and immobilized *S. cerevisiae* cells

3.1.1 pYES-*ERY3* construction and cloning

The first strand cDNA was synthesized from total RNA extracted from the *P. eryngii* PS419 mycelia as template by RT-PCR using the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA), using oligo dT as primers. The obtained cDNA was PCR-amplified using the primer pair ERY3_Eco.for/ERY3_Not.rev (Tab.1), designed after analysis of the nucleotide sequence of laccase genes belonging to *Pleurotus* spp. and available in the EMBL Database. The PCR amplicon (1.6-kbp in length) was digested with *Eco*RI and *Not*I, purified from agarose and ligated with a similarly digested pYES2 vector. *E. coli* strain DH5α was transformed with the ligation mixture and the recombinant clones were identified by plating onto LB medium plates containing ampicillin (50 μg ml^{-1}). The recombinant plasmid, denoted pY-*ERY3*, was isolated and submitted to sequence analysis. The sequence analysis showed that the *ERY3* gene is likely to be an allele of the not characterized *P. eryngii PEL3* gene (Q5MP11), as indicated by the high similarity level between the two sequences, i.e. 98% (not shown). The gene sequence have been confirmed by sequencing 5 independent clones, thus excluding the possibility that nucleotide differences were due to mistakes caused by the Taq polymerase-driven amplification. The isolated *ERY3* cDNA sequence consists of an open reading frame (ORF) of 1596 bp in length (EMBL Accession Nr. AM773999), which potentially codes for a polypeptide of 531 amino acids with an estimated molecular mass of 56.6 kDa. The predicted amino acid sequence of this ORF was aligned with previously reported fungal laccases, showing a high degree of similarity to known laccase sequences (Fig. 7 and Tab. 2). The amino acid sequence of Ery3 from *P. eryngii* PS419 exhibited 68-92% identity with laccases of other *Pleurotus* species and 99% identity with that of the not characterized Pel3 from *P. eryngii*. The first 23 amino acids block was likely to be the putative signal peptide, as indicated by the outcome of the bioinformatic analysis performed with the SPEPLip software (Fariselli et al., 2003). The Ery3 sequence contained the four copper binding regions used to identify the laccases (Kumar et al., 2003), denoted as L1 (residues 98-121), L2 (residues 138-158), L3 (residues 427-434) and L4 (residues 478-498). Two potential N-glycosylation sites

(Asn-Xaa-Ser/Thr) were found in Ery3 at positions 374 and 467, whereas Cys-119/Cys-516 and Cys-151/Cys-238 residues are potentially involved in disulphide bridge formation (Fig. 8).

3.1.2 Phylogenetic analysis

Laccase protein sequences of *Pleurotus* spp., available in the SWISS-PROT Database, were aligned with the Ery3 polypeptide and the obtained alignment was used to generate the clustering dendrograms. The consensus tree, obtained with the UPGMA method on the basis of the distances calculated with the PROTDIST software and the *N. crassa* laccase as the outgroup, is shown in Fig. 9. The phylogenetic tree showed that two primary branches departed from the bulk of all *Pleurotus* spp. selected laccases. On the whole, the 19 proteins clustered in three distinct groups (A, B and B1, Fig. 9), as indicated by the very significant values of bootstrap replicate placed at major nodes. The belonging to a group of any of the laccases was not related to the corresponding *Pleurotus* species. The Ery3 protein sequence (comprised in the group B) appeared to be clearly related to Pel3 of *P. eryngii* and to a cluster consisting of Pox1 and LacB of *P. ostreatus* and Lac1 of *P. sajor-caju*. Moreover, Ery3 indicated that it was unrelated to Pel4 of *P. eryngii*, which clustered with Pox1AB of *P. ostreatus* in the group B1 (Fig. 9).

3.1.3 Expression of *ERY3* gene in *S. cerevisiae*

To study the heterologous production of Ery3 protein in *S. cerevisiae*, the corresponding gene was episomally expressed in yeast. The pY-*ERY3* plasmid was used to transform the *S. cerevisiae* strain CEN.PK2-1C. A number of 50 yeast colonies, after selection of recombinants on SM-Ura plates, were screened by PCR analysis to confirm the presence of the heterologous gene sequence. Production of laccase was firstly assayed with a plate test, in which 2,2'-azinobis(3-ethylbenzo-6-thiazolinesulfonic acid (ABTS) was added to the agar medium, as laccase-specific substrate for a direct colorimetric reaction. Indeed, formation of a green halo, surrounding recombinant yeast colonies was detected after 2-3 days of incubation. The above halo was never observed for yeast line transformed with the empty vector (Fig. 10). Laccase production was studied in liquid cultures grown in synthetic medium added with 0.5 mM $CuSO_4$. The yeast recombinant strain was transformed with pY-*ERY3* and grown in glucose medium. Yeasts were subsequently transferred into a medium containing galactose, to induce the *GAL1* promoter activation and grown at 25°C. Laccase activity was found to be present in the culture medium,

indicating that Ery3 was produced and secreted in a biologically active form by the transformed yeast cells (Fig. 11). The native signal peptide of Ery3 protein demonstrated that it effectively directed secretion and proper proteolytic maturation of Ery3 in *S. cerevisiae*. The laccase produced by recombinant yeasts in the culture medium was studied by electrophoresis on an SDS-PAGE (Fig. 12a). No laccase bands were detected in yeast transformed with the control pYES2 vector, whereas a major band at about 60 kDa was observed for the supernatants of yeasts expressing the *ERY3* gene. The protein sample was probed in Western blot analysis with an antiserum to laccase and the assay gave a clear-cut reaction indicating that the 60 kDa band corresponded to the Ery3 recombinant laccase (Fig. 12b). In addition, a smear of larger proteins was detected, which indicate a possible over-glycosylation of the recombinant protein.

3.1.4 Effect of copper concentration and temperature on the production of active laccase

The effect of copper concentration on recombinant Ery3 laccase activity was investigated. When no copper was added to the medium, no laccase activity was detectable, whereas the increasing copper concentration enhanced laccase activity. The highest laccase activity (77 mU ml^{-1}) was reached after 3 days of induction using a copper concentration of 0.5 mM (Fig. 13A). Yeast lines transformed with pY-*ERY3* were also used to study the effect of temperature on Ery3 heterologous expression (Fig. 13B). Cultures were first grown at 30°C in SM-Ura medium supplemented with glucose and then transferred to the same medium supplemented with galactose and containing 0.5 mM $CuSO_4$ at 18, 25, and 30°C, respectively. The culture supernatants were assayed daily for 5 days. Laccase activity was enhanced by lower growing temperatures (25 and 18°C) and reached its peak after 3 days of recombinant yeast induction, thus resulting in the highest enzyme yield (88 mU ml^{-1}).

3.1.5 Optimization of yeast immobilization in calcium alginate

The initial cell biomass to be loaded in the beads was the first parameter considered in setting up the immobilized system. The laccase yield proportionally increased when the initial biomass in the beads was elevated from a total cell inoculum ranging from 5 to 25 OD_{600} (Fig. 14). However, at higher inoculum concentration (20 and 25 OD_{600}), the cell leakage into the fermentation medium clearly increased (Fig. 14). The optimization of further immobilization parameters allowed the improving of the bead properties, such as permeability and rigidity. The highest laccase yield was obtained with beads prepared

using 3% (w/v) sodium alginate (Fig. 15). At higher concentration of sodium alginate the pellet rigidity was improved, but the laccase yield decreased. The effect of $CaCl_2$ concentration on the rigidity of beads was then evaluated (Fig. 16). As in the case of sodium alginate concentration, lower concentration of $CaCl_2$ increased the cell leakage into the fermentation medium because of the reduced stiffness of the beads (not shown). When the $CaCl_2$ concentration was raised from 0.05 M to 0.1 M the laccase yield increased but, however, higher concentration of $CaCl_2$ (0.15 M) resulted in a decreased enzyme activity. The finding that almost identical laccase yields were obtained by applying the identical experimental parameters to three independent sets of experiments (Fig. 14, third column; Fig. 15, third column; Fig. 16, third column) supplied a strong evidence on the reliability of the obtained results. Considering minimum cell leakage and the maximum enzyme yield values, 0.1 M $CaCl_2$, 15 OD_{600} cell (total inoculum) and 3% Na-alginate concentration were chosen as the optimal parameters for the formation of beads with suitable rigidity and permeability.

3.1.6 Laccase production by immobilized cells

Cell immobilization gave the highest enzyme yield after 5 to 7 days of incubation. In fact, during this period the production peaked (139-125 mU ml^{-1}) and then enzyme yield decreased (Fig. 17). The calcium alginate entrapment technique led to an increased laccase production in comparison to free cell cultures. The enzyme yield obtained with a comparable cell inoculum (15 OD_{600} = 4.5 x10^8 cells ml^{-1}) in immobilized form (139 mU ml^{-1}; Fig. 17) increased 1.6-fold compared to the highest yield obtained with free cells (88 mU ml^{-1}; Fig. 13). The estimation of the minimum time for maximum enzyme production (5 days) was a fundamental parameter to be considered, since it indicated the cycle time for re-usability transfer in repeated batch fermentation. The possibility of re-using immobilized cells of *S. cerevisiae* for laccase production was investigated over a period of 35 days, which was equivalent to seven cycles of fermentation (Fig. 18). The immobilized cells showed for each cycle a daily laccase yield with a profile similar to that shown in Fig. 6, and indeed, they could be efficiently re-used for enzyme production seven times without any apparent loss of enzyme yield. The highest activity (146 mU ml^{-1}) was achieved at the second repeated cycle, nevertheless statistically similar laccase production (128-109 mU ml^{-1}) was detected until the seventh cycle (Fig. 18). Moreover, even after seven subsequent and efficient fermentation operations, the alginate beads showed good stability and they maintained 84% of the enzyme yield obtained in the first cycle.

3.1.7 Effect of cell immobilization on extracellular protease activity

In order to explain the increased laccase activity obtained using immobilized cells, we investigated the effect of cell immobilization on the extracellular protease(s) activity. The same initial total inoculum of recombinant yeasts (15 OD_{600}) was used to induce the expression of the *ERY3* gene at 18 and 30°C, either in free and immobilized cell. Biomass concentration (free cells), cell leakage (entrapped yeasts), laccase and extracellular protease activities were analysed at different time points (0 - 6 days). As expected, the yeast growth level in liquid medium and the cell leakage from alginate beads were enhanced when the temperature of incubation was 30°C (Fig. 19a). In accordance with the above reported data, the highest laccase yield was reached in free (77 mU ml^{-1}) and immobilized (141 mU ml^{-1}) cells, respectively after 3 and 5 days of incubation at 18°C, but no detectable laccase activity was found in the culture medium of both free and entrapped cells at 30°C (not shown). As shown in Figure 19b, proteolytic activity was assayed and the maximum specific activity of the extracellular proteases (6.9 mU ml^{-1}) was found in the medium of the free suspension culture incubated at 30°C, whereas a reduced activity (1.3 mU ml^{-1}) was found when the same cells were grown at 18°C. Indeed, the presence of extracellular proteases was dramatically reduced by cell immobilization. Extracellular proteolytic activity (2.1 mU ml^{-1}) was identified in the culture medium of alginate-entrapped yeasts incubated at 30°C, but no detectable protease activity was found in the culture medium deriving from the immobilized cells cultured at 18°C (Fig. 19b).

3.2 Role of the N- and C-termini of *Pleurotus eryngii* Ery4 laccase for the enzyme catalytic activity

3.2.1 Production and expression of the *P. eryngii ERY4* cDNA in *S. cerevisiae* and mutant *ERY4* genes

A study aimed to the characterization on undescribed genes from *P. eryngii*, was previously carried out employing an RT-PCR-based strategy (Bleve et al., 2008). By using total RNA extracted from the *P. eryngii* PS419 mycelia as template and the ERY4_Eco/ ERY4_Not primer pair, a 1602 bp fragment was amplified with the above primers by RT-PCR. The amplified DNA fragment, denoted *ERY4* was cloned in the pYES2 vector, under

the control of the strong galactose-inducible *GAL1* promoter, thus yielding the pY-*ERY4* vector. The sequence analysis showed that the *ERY4* gene is likely to be an allele of the not characterized *P. eryngii PEL4* gene (ABB30169, Rodríguez et al., 2008), as indicated by the 99% similarity level between the two sequences (not shown). The *ERY4* gene (EMBL Accession Nr. AM774000) potentially codes for a polypeptide of 533 amino acids with an estimated molecular mass of 58.1 kDa. The amino acid sequence of Ery4 from *P. eryngii* PS419 exhibited 62-94% identity with laccases of other *Pleurotus* species (Fig. 7) and 99% identity with that of the not characterized Pel4 from *P. eryngii* (not shown). The first 20 amino acids block was likely to be the putative signal peptide, as indicated by the outcome of the prediction performed with the SPEPLip software (Fariselli et al., 2003). The Ery4 sequence contained the four copper binding regions used to identify the laccases (Kumar et al., 2003), denoted as L1 (residues 84-108), L2 (residues 124-145), L3 (residues 414-422) and L4 (residues 465-486). Potential N-glycosylation sites (Asn-Xaa-Ser/Thr) were found in Ery4 at positions 221, 314, 490, 510, 513 and 362 (Brunak, http://www.cbs.dtu.dk/services/NetNGlyc/), whereas Cys-105/Cys-506 and Cys-137/Cys-224 residues are potentially involved in disulphide bridges formation (not shown).

The pY-*ERY4* plasmid was used to transform the *S. cerevisiae* strain CEN.PK2-1C. Production of laccase was firstly assayed with a plate test, in presence of ABTS as laccase-specific substrate for a direct colorimetric reaction. Indeed, the expected green halo surrounding recombinant yeast colonies, was never observed for yeast line transformed with the pY-*ERY4* (Fig. 10). Laccase production was also studied in liquid cultures grown in synthetic media added with 0.5 mM $CuSO_4$ final concentration. The yeast recombinant strain transformed with pY-*ERY4* was grown in glucose medium and subsequently transferred into a medium containing galactose, to induce the *GAL1* promoter activation and grown at 18°C. Although laccase activity was not found in the culture medium, the electrophoresis on an SDS-PAGE carried out on laccase produced by recombinant yeasts expressing the *ERY4* gene in the culture medium revealed a major band at about 60 kDa, whereas no laccase bands were detected in yeast transformed with pYES2 vector as a control (not shown). The protein sample was probed in Western blot analysis with an antiserum to laccase and the assay gave a clear-cut reaction, indicating that the 60 kDa band corresponded to the Ery4 recombinant laccase (Fig. 20). In addition, a smear of larger proteins was detected, which indicated a possible over-glycosylation of the recombinant protein.

In order to investigate the lack of biological activity of recombinant Ery4 protein, we decided to study the role of its N- and C-terminal regions in determining enzyme catalytic properties. *ERY4* gene was subjected to a mutational analysis following these approaches: i) C-terminal progressive deletion to study the role of specific amino acid residues at the C-terminus of Ery4 protein, ii) site-directed mutagenesis of the C-terminal region, iii) chimerical laccases derived from the substitution of both its terminal regions with the corresponding regions of *ERY3* gene (Fig. 21).

Three different categories of *ERY4* mutant genes were then produced: 1) mutants carrying a specific C-terminal deletion; 2) *ERY4* chimerical genes carrying specific portions deriving from the *ERY3* gene; 3) *ERY4* genes each carrying a different site-specific mutation at the C-terminus.

1) Mutants carrying C-terminal deletion were obtained by PCR amplification of *ERY4* gene using as forward primer the ERY4_ECO primer and as reverse primers the 2Lessrev, 5Lessrev, 8Lessrev, 11Lessrev, 14Lessrev, 18Lessrev primers, respectively (Tab.1).

2) The 4C3 chimerical gene was produced by PCR amplification, using the *ERY4* gene as template and the primer pair ERY4_Eco/ERY4CERY3. The ERY4CERY3 annealed with its 3'-terminal portion, from nt 1524 to nt 1545 of the *ERY4* sequence, whereas its 5'-terminal portion corresponded to the last 21 3'-terminal nucleotides of *ERY3* gene.

The chimerical 4N3 gene was obtained as follow. First, a 108 bp fragment was amplified by PCR using the *ERY3* gene as template and the primer pair ERY3_Eco.for/ERY3SSRev and, then, a 1553 bp fragment (missing the first 63nt of *ERY4* sequence) was amplified by PCR using the *ERY4* gene as template and the primer pair ERY4FUSFor/ERY4.Not (Tab.1). The two above mentioned amplicons shared a 30 nt block, respectively at their 3'- and 5'-terminal, which made it possible their PCR fusion through a single overlap extension. The PCR fusion was carried out by using the above gel-purified fragments as template and the primer pair ERY3_ECO/ERY4_NOT, which were added after the second cycle of the reaction. In order to produce the recombinant 4NC3 gene, the chimerical 4C3 and 4N3 genes were separately digested with the restriction enzyme *Kpn*I, which is present in the Ery4 sequence as single site at the position 516. The 5'-terminal portion of 4N3 and the 3'-terminal portion of 4C3 were purified by agarose gel electrophoresis and then ligated with T4 DNA ligase.

3) Point mutations were introduced in the *ERY4* sequence employing the Phusion Site-Directed Mutagenesis Kit (Finnzymes, Finland) according to manufacturer's instructions, using the *Not*I-digested pYES-*ERY4* DNA as template and the phosphorylated ERY4FMut

as forward primer. Mutagenic reverse primers (Tab. 1) were created by designing an opportune sequence mismatch: ERY4K/E.rev (to change Lys 532 to Glu), ERY4K/A.rev (to change Lys 532 to Ala), ERY4K/R.rev (to change Lys 532 to Arg) and ERY4P/A.rev (to change Pro 530 to Ala). The obtained genes were respectively denoted ERY4K/E, ERY4K/A, ERY4K/R and ERY4P/A.

All mutant versions of *ERY4* gene were digested with *EcoRI* and *NotI*, purified from agarose and then ligated with a similarly digested pYES2 vector (Invitrogen, U.S.A.). All *ERY4*-derived genes were separately transformed into *S. cerevisiae*, along with *ERY3* and the original *ERY4* genes. All the transformants were firstly assayed for laccase production with a plate test, in which ABTS was added to the agar medium as laccase-specific substrate for a direct colorimetric reaction. Indeed, formation of a green halo, surrounding recombinant yeast colonies, was detected after 2-3 days of incubation. The above halo was never observed for yeast line transformed with the gene carrying the K532R substitution, with the original *ERY4* gene and with the empty vector (not shown).

The *ERY3*, *ERY4* and the mutant derived genes were expressed in *S. cerevisiae*. The conditions for production of laccases in shake flask cultures were optimized in terms of $CuSO_4$ concentration, temperature and induction conditions, as follows: synthetic complete medium (SM-Ura) buffered to pH 4.8-5.0 and supplemented with 1 mM $CuSO_4$ at 200 rpm and 18°C. The specific activity of all produced laccase isoforms were analyzed in the culture medium by the enzymatic assay (Tab. 3). As expected, no activity was detected for the Ery4, but also for Ery4K532R mutant protein, although in both case laccase proteins were detectable by Western blot analysis into culture medium. The other mutant isoforms were biologically active after gene expression in *S. cerevisiae*. The specific activities (mU ml^{-1}) of the mutant proteins was considerably higher for 2Less (18-fold), 5Less (33-fold), 18Less (15-fold), 4C3 (16-fold), 4NC3 (21-fold) and K532E (13-fold) (Tab. 3). The obtained data showed that C-terminus progressive deletion, replacement of Ery4 C- and/or N-terminal portions with the homologous arms of Ery3 or a charge inversion K532E allowed production of enzymatically active Ery4-derived laccases.

All the produced isoforms were purified from the culture supernatant by anion exchange chromatography (Fig. 22A), followed by gel filtration chromatography (Fig. 22B). The adopted purification procedures (Tab. 4) showed that it was able to successfully purify at the homogeneity all the produced recombinant isoforms. Recombinant laccases were fractionated in order to purify the not glycosylated forms, which showed an apparent size of ~ 60 kDa (Fig. 22C). Fractions corresponding to a second pool of each laccase were

collected and further purified by gel filtration. An average level of 11.2 ± 2.47-fold purification respect to specific activity and a yield average of 4.3 ± 1.2 % of total protein recovered activity was achieved for all the purified laccase isoforms. Finally the average specific activity of purified proteins was 56 ± 13.4 U mg^{-1} (Tab. 4). As expected, Ery4 and Ery4K532R did not show any biological activity. The obtained results indicated that both N- and C-terminal sequences are fundamental for laccase activity. In particular, data showed that K532 and P530 residues have a crucial role in protein structure for regulation of enzymatic activity

3.2.2 The Ery4 modified isoforms showed different biochemical and physical features

The catalytic constants (K_{cat}) and the catalytic efficiency (K_{cat}/K_m) obtained for all the laccase isoforms (Tab. 5) showed that they have an optimal acidic pH for the oxidation of the substrate ABTS.

Among the three classes of mutants produced, the mutation that showed a considerable increase of K_m value on ABTS were 5Less (K_m= 28 μM) for the C-terminal progressive deletions, 4N3 (K_m= 51 μM) and 4NC3 (K_m= 53 μM) for the chimerical proteins and Ery4K532A (K_m= 58 μM) for the laccase derived by site-directed mutagenesis. However, the 2Less isoforms showed a 7.7-fold and a 4-fold increased K_m value (K_m= 216 μM) as compared with 5Less and with 4NC3, 4N3 or Ery4K532A, respectively. Consequently, the specificity constant increased about 22-fold (K_{cat}/K_m= 667 μM^{-1} min^{-1}) for the 5Less, about 7.7-fold (K_{cat}/K_m= 239 μM^{-1} min^{-1}) for 4NC3 and 8.8-fold (K_{cat}/K_m= 273 μM^{-1} min^{-1}) for 4N3 and about 3.3-fold (K_{cat}/K_m= 104 μM^{-1} min^{-1}) for Ery4K532A from 31 μM^{-1} min^{-1} of 2Less. Interestingly, considerable values of K_m were reached also by the two other isoforms carrying out single point mutations K532E (K_m= 84 μM) and P530A (K_m= 105 μM). These isoforms exhibited increased catalytic constant on ABTS ranging from 2.3 to 1.3-fold as compared with 2Less (Tab. 5).

The recombinant laccases were further characterized by studying their affinities for the following four different substrates: syringaldazine, 2,6-Dimethoxyphenol (DMP), tetramethylbenzidine (TMB), guaiacol (Tab. 6).

Among the phenolic substrates, high affinity and high catalytic constants were observed for syringaldazine and TMB, whereas oxidation of DMP and guaiacol was considerably slower and the respective K_m constant higher, demonstrating a lower affinity of all the mutated laccases for these two last substrates. The C-terminal deletions greatly influence

the catalytic parameters for the substrates syringaldazine and TMB. Although the K_m values for the 11Less and 18Less on syringaldazine (4.58 and 4.89 μM) were better than the K_m value for 5Less (6.6 μM), their catalityc constants were lower (K_{cat}/K_m= 109 μM^{-1} min^{-1} and K_{cat}/K_m= 138 μM^{-1} min^{-1}, respectively) than the corresponding value for the 5Less (K_{cat}/K_m= 222 μM^{-1} min^{-1}). Moreover, 5Less mutant showed the best performances also with TMB with a K_m=20 μM and a K_{cat}/K_m= 126 μM^{-1} min^{-1} for this substrate. Among the chimerical laccases the mutant that had the higher specificity constant on syringaldazine and on TMB was the 4NC3, which showed an increase of about six-fold (K_{cat}/K_m= 282.8 μM^{-1} min^{-1}) and of about 5-fold (K_{cat}/K_m= 121.5 μM^{-1} min^{-1}) as compared with 2Less (K_{cat}/K_m= 46.76 μM^{-1} min^{-1} and K_{cat}/K_m= 23 μM^{-1} min^{-1}). For the laccases produced by point mutations, the Ery4K532A mutant showed a high affinity for syringaldazine (K_m=3.47 μM) and an increased specificity constant of 1.8-fold (K_{cat}/K_m= 83 μM^{-1} min^{-1}) respect to 2Less, whereas a K_m value of 11 μM and a 2.7-fold increase in specificity constant was observed with TMB (K_{cat}/K_m= 61 μM^{-1} min^{-1}) for 4C3 mutant respect to 2Less (Tab. 6).

The 5Less and 4NC3 laccase isoforms showed the higher substrate affinities for DMP (K_m= 562 and K_m= 656 μM, respectively) and catalytic constants increased of 3.7-fold (K_{cat}/K_m= 23 μM^{-1} min^{-1}) and 2.3-fold (K_{cat}/K_m= 14 μM^{-1} min^{-1}) respect to 2Less (K_{cat}/K_m= 6.2 μM^{-1} min^{-1}). These two mutants were the only isoforms able to oxidize guaiacol, although oxidation activity was considerably slow and the respective Km constants very high (Tab. 6).

The activities of all mutant laccase isoforms and Ery3 were also examined at different pH values. All isoforms showed the best activities with ABTS at pH 3, whereas higher pH values sensitively affect laccase activities, which were absent at pH 8 (Tab. 7). All isoforms, except the three chimerical proteins and the Ery4K532A mutant, were quite stable after 60 h incubation at pH 6.5, revealing the highest stability values (97-100% residual activity). Residual activities of all the laccases after 60 h incubation at pH 3.0 were 25% or even less respect to the values obtained at pH 6.5. Among the C-terminal deletion mutants, stability of all the isoforms decreased with lowering pH values ranging from 70-80% to 15-25%, except the 8Less that showed a decreased stability at pH 5.5 and maintained the same activity at lower pH values (Fig. 23). Similarly to what was observed for Ery3 ($t_{1/2}$= 38 h at pH 3), the stability of these enzymes was almost unaffected by acidic pH ($t_{1/2}$= 34-42 h at pH 3; Tab. 9). Although all the chimerical proteins showed 60-70%

residual activities at pH 6.5 and all of them reduced their activities at lower pH values, 4NC3 isoform resulted the less stable protein. The point mutations that were able to maintain higher stability levels at low pH values were K523E and P530A, as they showed enzyme half-life values of 117 and 138 h at pH 5.5, two of the better $t_{1/2}$ values together with Ery3 ($t_{1/2}$= 126 h) and 14Less mutant ($t_{1/2}$= 122 h; Tab. 9).

In general, ABTS oxidation catalyzed by laccases was stimulated at elevated temperatures. Among C-terminal deletion-derived isoforms, 2Less, 5Less and 18Less demonstrated their best activities at 55, 70 and 55°C respectively, whereas highest ABTS oxidation rate for 8, 11 and 14Less was observed at 40°C (Tab. 8). However, while chimerical laccases, deriving from the single substitution of N- or C-terminal end, showed the highest activities at 70°C, 4NC3 mutant reached its maximum oxidation rate at 55°C. On the contrary, single point mutations in position 532 produced laccases with highest activities at 55°C, whereas P530A mutant had the best ABTS oxidation rate at 70°C. Thermal stability was investigated after 60 h incubation of enzymes in 0.1 M Phosphate buffer pH 6.5 with ABTS at different temperatures ranging from 10 to 70°C. At 10°C, all Ery4-deriving isoforms and Ery3 retained almost complete activities, whereas all they totally lose their catalytic activities after incubation at 70°C (Fig. 24). C-terminal deletion mutants retained their activities also after incubation at 25°C, but they reduced ABTS oxidation rate to 35-55% at 40°C and to 15-30% at 55°C. On the contrary, the 5Less isoform activity completely disappeared at 55°C, whereas 5 and 18Less proteins revealed a higher enzyme stability at 25 ($t_{1/2\ 5Less}$= 820 h, $t_{1/2\ 8Less}$= 1502 h) and 40°C ($t_{1/2\ 5Less}$= 73 h, $t_{1/2\ 8Less}$= 115 h) when compared to the Ery3 ($t_{1/2\ 25°C}$= 600 h, $t_{1/2\ 40°C}$= 48 h; Tab. 9). Chimerical proteins demonstrated to have the same behaviour after incubation at different temperatures, showing reduced activities to 80% at 25°C, to 65-85% at 40°C and about to 15-20% at 55°C. The half-life of these laccase isoforms were higher than the values revealed by Ery3 at 40°C (Tab. 9). However, the point mutation K532E produced a more stable laccase isoform at 40 and 55°C, respect to K532A and P530A, which reduced their activities to 35% at 40°C and completely lose their stability at 55°C (Fig. 24 and Tab. 8).

3.2.3 Statistical analysis of obtained data

In order to evaluate the different catalytic performances among the recombinant laccase, Principal Component Analysis (PCA) was applied to the data set of the Kcat/Km (min^{-1} μM^{-1}) value obtained by oxidation of five different substrates (ABTS, syringaldazine, DMP, TMB and guaiacol) for thirteen out the fifteen laccases produced, with the exception

of the Ery4 and K532R, both inactive (Fig. 25). The first principal component (PC1) accounted for 85.80% of the total variation, while PC2 explain 7.84% of the total variation. Along the first principal component (PC1), the isoforms were clearly grouped in two clusters: the first (negative values of PC1) include 4CN3, 4N3, and 5Less isoforms; the second cluster (positive values of PC1) grouped all the other ten laccase isoforms, which were not distinguishable according the selected variables.

The PCA scores plot indicated that the distribution of laccase isoforms on the plane was clearly dependent by their catalytic performances, thus indicating that this statistical assay allowed the identification of 5Less isoform as the best performing laccase with ABTS, DMP and guaiacol, whereas 4NC3 isoform was the most active in presence of syringaldazine. These two isoforms showed the same activity with TMB (Fig. 25).

The PCA was also applied to the data matrix constituted by the specific activity at five pH values (pH 3, pH 4.5, pH 5.5, pH 6.5 and pH 8) and five different temperatures (10, 25, 40, 55 and 70°C) for the above thirteen recombinant laccase isoforms using ABTS as substrate (Fig. 26). The first two principal components accounted for 95.6% of the total variation, with 91.15% and 4.45% explained by PC1 and PC2 respectively. Along the first component, the samples (laccase isoforms) were clearly grouped in three clusters. Samples 2Less, 5Less and 4NC3 were grouped in a single cluster on the left part of the plot, while the sample K532A on the other side of the plot grouped into a more scattered cluster. Finally the other samples (Ery3, 8Less, 11Less, 14Less, 18Less, 4C3, 4N3, K532E and P530A) were closely grouped in the central part of PC1 (Fig. 26).

3.2.4 3D modelling of Ery4, Ery3 and Ery4 mutant proteins.

The superimposition of the 3D models of Ery3 and Ery4, with a root mean square deviation (rmsd) equal to 0.41 Å, displayed two main differences between the two proteins: i) a loop in Ery3 that lacks in Ery4 (point out with a red ring); ii) the C-terminus in Ery4 is longer than that of Ery3 (arrows) (Fig. 27).

Several evidences revealed that the Lysine 532 residue is involved in the absence of enzymatic activity of Ery4 by *S. cerevisiae*. Indeed, the "nearest neighbor residue" analysis revealed that K532 residue is well stabilized by the negative charged D474, H131, T134 and C137. These bonds hedge the K532 residue in a sort of cage and block the Ery4 C-terminus, thus preventing any possible binding between substrates and laccase catalytic site (Fig. 28A).

The isoform carrying the K532 residue mutated in arginine residue (K532R) maintained the same interactions with other neighbour residues, with the excepted of the threonine in position 134 and it consequently didn't unlock the Ery4 C-terminus (Fig. 28B).
When the lysine 532 was substituted by a negative residue (glutamic acid, K5323E), it only established bonds with the histidine in position 131(Fig. 28C) and it was likely to allow the substrate interaction with enzymatic core site, as also indicated by enzymatic assay (Fig. 22D).
When in 532 position lysine residue was substituted by alanine (K532A), it could established an interaction bond only with H131 residue (Fig. 28D).
The introduction of the P530A substitution was likely to produce a more relaxed structure in the C-portion respect to wild type, since the proline residue (characterized by a limited polarity) was changed with an apolar aliphatic residue (Alanine). Although the nearest-neighbor residues were mainly maintained, a decrease of hydrophobic contacts around the mutated residue was observed. The main difference was the lost of interaction of the proline aromatic ring with the aspartic residue in position 478 and the new interaction with the glycine residue in position 133 (Fig. 28E-F).
The 3D models of Ery4-WT and the "lessrev" models, obtained with progressive deletion of C-terminus, highlights the occupancy of the C-terminus of Ery4-WT (Fig. 29A) and the increased accessibility to the Cu-cluster site due to the deletion of C-terminus residues (Fig. 29B-G).

3.3 Construction of whole cell laccase biocatalyst yeast

3.3.1 Construction of the cell surface expression plasmids and expression in *S. cerevisiae*

The genes encoding the laccase isoforms 5Less and 4NC3, which showed the best enzymatic performances on different substrates, were expressed in *S. cerevisiae* by displaying the recombinant enzyme on yeast cell surface. The mutant genes *4NC3* and *ERY4-5LESS* were respectively fused at their N-terminal end with the *S. cerevisiae* gene *PIR2* and *FLO1* (*FS* flocculin fragment).
To clone the *5LESS* and *4NC3* genes in N-terminal fusion with the *PIR2* and *FLO1* genes, the four genes were PCR-amplified respectively using the 4NC3Flag_Hind.for/5less_Xho.rev, 4NC3Flag_Hind.for/4NC3_Xho.rev,

PIR2_Eco.for/PIR2_Hind.rev and FS_Eco.for/FS_Hind.rev, as primer pairs, respectively (Tab.1).

After digestion with *Hind*III, the *PIR2/5LESS, FS/5LESS, PIR2/4NC3, FS/4NC3* couples were separately ligated with T4 DNA ligase and further digested with *Eco*RI and *Xho*I. After purification by agarose gel electrophoresis, they were separately cloned into the *Eco*RI and *Xho*I sites of p426-GDP vector (Invitrogen, U.S.A.), downstream the *GDP* promoter. The four obtained recombinant clones, designated p426-*PIR2—5LESS,* p426-*FS—5LESS*, p426-*PIR2—4NC3* and p426-*FS—4NC3*, were used to transform *S. cerevisiae* CEN.PK2-1C.

3.3.2 Characterization and cell wall localization of FS/PIR2 laccases

In order to screen yeast transformants for the production of recombinant laccase, a colorimetric assay on agar plate was performed. Cells harboring p426-*PIR2—4NC3*, p426-*PIR2—5LESS*, p426-*FS—5LESS*, p426-*FS—4NC3* or p426*,* as control, were picked from SM-Ura plates supplemented with 2% glucose, 5 mM ABTS and 0.5 mM $CuSO_4$. After incubation of 3 days at 18°C, the plate was checked for the development of a green halo colour. The results (Fig. 30) demonstrate that the cells harbouring the recombinant plasmids produced a green halo around the colony, while no colour formation was observed around the cells harbouring the control plasmid. This indicated that the former cells exhibited laccase activity due to the expression of the mutant isoforms of *PIR2* or *FS*/ laccase fusion genes.

The transformed yeast cells were subjected to enzymatic assays on liquid using several substrates for biochemical characterization. Laccase activity was assayed at 25°C using ABTS, guaiacol, DMP, syringaldazine and TMB as substrates. The result, shown in Tab. 10, demonstrated that the cells producing the mutant isoforms of laccase possess a detectable laccase activity, even if variable according to their substrate affinity and specificity constant on several substrates used. The fusion strategy of 5Less isoform to flocculin fragment FS produced yeast strains carrying detectable laccase activity on four to five tested substrates (no activity on syringaldazine), whereas, PIR2-5Less fusion strategy revealed the highest laccase activity on ABTS and DMP. The fusion strategy of 4NC3 to FS or PIR2 gene produced yeasts carrying detectable laccase activity only on DMP and TMB (Tab. 10).

The localization of the mutant laccases and their association with cell wall was determined by Western blotting analysis of cell wall proteins using antibodies raised against the

Pycnoporus cinnabarinus laccase. No bands were detected in yeast transformed with p426 control vector, whereas a major signal of about 175 kDa was observed for the FS/4NC3 and FS/5Less fusion proteins, and a signal of about 96 kDa was observed for the PIR2/4NC3 and PIR2/5Less fusion proteins (Fig. 31).

To further confirm the expression of recombinant laccases on yeast surface, an immunofluorescent labeling assay was carried out using the anti-laccase antibody as a primary antibody, and a fluorescent-conjugated secondary antibody. Cells harbouring p426-*FS—5LESS* (Fig. 32B), p426-*PIR2—5LESS* (Fig. 32C), p426-*FS*—4NC3 (Fig. 32D) and p426-*PIR2—4NC3* (Fig. 32E), clearly exhibited immunofluorescent signals at the cell surface. Cells harbouring a control plasmid did not exhibit immunofluorescent signals (Fig. 32A).

3.4 Heterologous expression, purification and biochemical characterization of *PPO2* tyrosinase from *A. bisporus* in *S. cerevisiae*

3.4.1 PPO2 tyrosinase production and characterization

The *PPO2* tyrosinase gene of *Agaricus bisporus* was amplified by PCR in order to add a six histidine tag to its C-term end, using as template the previously cloned *PPO2* gene, and PPO2_Eco.for/ PPO2.his_Not.rev as primer pairs. After digestion with *Eco*RI and *Not*I, the *PPO2* gene was cloned in pYES2 vector.

Yeast cells were lysed by using French Press and tyrosinase purification was carried out by affinity chromatography. All phases of purification on column were carried out using, initially, a Phosphate buffer containing 0.5 M NaCl. However, the tyrosinase purified using this saline concentration, lost its activity and the purification profile, viewed on SDS-PAGE, shown the presence of several protein pollutants co-eluted with tyrosinase (Fig. 33A). Subsequently, when a Phosphate buffer containing 0.15 M NaCl was used to lyse cells, to wash the column and to elute the proteins, most of the low-affinity binding proteins were eliminated (Fig. 33B). For the detection of tyrosinase activity on gels, purified tyrosinase samples were loaded in polyacrilamide native gel, and after electrophoresis, the gels were stained with L-Dopa solution. In order to check the sensitivity of the staining method, different concentrations of purified enzyme and of commercial mushroom tyrosinase (Sigma, USA) were loaded and tested for the activity staining. After 3-10 minutes, a brown band, corresponding to immobilized tyrosinase

within gel, appeared in the position taken up by the enzyme. In this way, it was possible to estimate readily the purification of an active tyrosinase. Using a lower saline concentration, it was possible to detect tyrosinase activity on L-Dopa as substrate and to view the enzyme in its active tetrameric (H_2L_2) or dimeric structure (HL) (Fig. 34).

Tyrosinase activity was biochemically characterized in liquid by assays in 50 mM Potassium Phosphate buffer pH 6.5, at 20°C using L-tyrosine, L-Dopa, pyrocathecol, pyrogallol as substrates, respect to the commercial purified *A. bisporus* tyrosinase enzyme (Tab. 11). Although the affinity values for all the tested substrates (K_m) were similar for Ppo2 protein and the commercial tyrosinase, catalityc constants were in generally higher for Ppo2 than for commercial tyrosinase. Indeed the K_{cat} values of Ppo2 showed an increase ranging from 9.3 fold (pyrogallol) to 2.8 fold (pyrocathecol) when compared to the values obtained by commercial tyrosinase. However, the activity of Ppo2 rapidly decreased during storage at room temperature and at -20°C (Tab. 11).

3.4.2 Tyrosinase display on yeast external surface

3.4.2.1 Plasmid construction and expression in *S. cerevisiae* cells

To clone the *PPO2* gene in N-terminal fusion with the *PIR2* and *FLO1* genes, the three genes were PCR-amplified using respectively the PPO2.Flag_Hind.for/PPO2_Xho.rev, PIR2_Bam.for/PIR2_Hind.rev and FS_Bam.for/Fs_Hind.rev, as primer pairs (Tab.1).

After digestion with *Hind*III, the *PIR2/PPO2* and *FS/PPO2* couples were separately ligated with T4 DNA ligase and further digested with *Bam*HI and *Xho*I. After purification by agarose gel electrophoresis, they were separately cloned into the *Bam*HI and *Xho*I sites of p426-GDP vector (Invitrogen, U.S.A.), downstream the *GDP* promoter. The two recombinant clones obtained, designated p426-*PIR2—PPO2* and p426-*FS—PPO2* were used to transform *S. cerevisiae* CEN.PK2-1C.

Tyrosinase activity was assayed in liquid and on plate using L-Dopa as substrate, as reported in the paragraph 2.6.4. Preliminary experiments revealed that it was not possible to detect any tyrosinase activity in plate neither in liquid in recombinant yeast strains expressing Ppo2 protein on their surface (data not shown).

3.5 Isolation and selection of yeasts and their immobilization for the treatment and detoxification of olive mill wastewater

3.5.1 Isolation and molecular identification of yeast and mould isolates

The effluents were collected in 5 different local olive mills, which adopted the continuous system for the extraction of olive oil. Samples were taken from the water obtained from the centrifuge of each olive mill (OCW) and from the corresponding storage pond (OMW), in order to obtain a complete microbiological characterization of the population dynamics occurring during the wastewaters storage. Samples denoted as OCW resulted from phase separation (aqueous and oily fractions) and they were directly collected from the vertical centrifuge. Samples designated as OMW were collected from wastewaters, after a two-months stabilization stage in small ponds positioned inside each of the olive mills. The total counts of moulds and yeasts present in each sample were carried out by incubating the agar plates at 25°C. The population isolated from the above samples was composed by mould and yeast isolates and the analysis of their morphologic characteristics (shape, size, pigmentation, etc.) made it possible to distinguish four main phenotypic groups (data not shown). In order to ascertain the natural mycete population associated to the five examined samples, 300 isolates, respectively 30 for each individual OCW and 30 for its deriving OMW, were taken in order to represent the existing colony phenotypes.

These isolates were identified according to their ITS sequence, obtained after amplification of the region between 18S rRNA and 28S rRNA, using the ITS1/ITS4 primers pair (Tab. 1) and total genomic DNA as template. Total genomic DNA from the strains was prepared according to the method used by Tristezza et al. (2009). The ITS1-5,8S-ITS2 region was amplified as described by Bleve et al. (2006) with the following modifications: initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of 30 s at 95°C, 30 s at 52°C and 1 min at 72°C, followed by a final extension at 72°C for 10 min. After agarose gel analysis, the amplicons were purified and then sequenced on an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems, USA). The search for sequence similarity with sequences in the GenBank database was performed by using the BLASTN algorithm.

The analysis of the five OCW samples showed that yeasts represented 85% of the total analyzed population, whereas the moulds, represented by *Penicillium* spp., corresponded to the remaining 15% (Tab. 12). It is interesting to note that the basidiomycete *Rhodotorula*

mucilaginosa was isolated only from the Casarano OCW sample, representing the 4% of the total isolated population. Moreover, the yeast population was represented by *Geotrichum candidum* (9.2 %), *Pichia holstii* (22 %), *P. fermentans* (14.2 %), *S. cerevisiae* (24.6 %), *Candida tropicalis* (8 %) and other *Candida* spp. (2.6 %). The screening of the stored OMW samples revealed the absence of moulds and it was observed that the yeast population was composed of *G. candidum* (48 %), *S. cerevisiae* (26.7 %), *P. holstii* (13.3 %) and *P. fermentans* (12 %) (Tab. 12).

3.5.2 Effects of OMW on microbial growth

In order to determine the maximum OMW concentration at which microbial growth was not inhibited, cultures of all 300 isolates were performed at 25°C, on solid media containing OMW, appropriately diluted in water. The ratio between the radial growth on OMW agar plate and the radial growth of the same isolate on YEPD agar plate was found to be affected by OMW concentration. Radial growth of all isolates was found to be generally unaffected by OMW concentrations lower than 50% (data not shown). On the plates containing 75% OMW in water, which was the value chosen as a threshold, the ratio was higher than 80% only for 12 isolates (Tab. 13). The above strains and the nucleotide sequence of their ITS region were respectively deposited in the ISPA culture collection (http://www.ispa.cnr.it/ Collection) and in the EMBL Nucleotide Sequence Database (Tab. 13). According to these preliminary results, all the following trials were performed with the selected 12 yeast strains in sterile OMW medium, without any addition of nutrients or pre-treatment.

3.5.3 Detoxification of OMW by selected yeasts

The reduction of total phenol content operated by the twelve selected yeasts was investigated. The same initial total inoculum of the above microorganisms (1×10^7 CFU ml^{-1}), as determined by counting in a Bürker chamber) was used to inoculate separately 50 ml of sterile OMW medium. The cultures were incubated at 25°C for 20 day and the biomass concentration and total phenol amount were analysed at different time points (0, 4, 7, 11, 14, 17, 20 days). The results obtained are shown in Fig. 35, where the data concerning only the yeast isolates which were able to reduce phenol concentration are reported. Indeed, the isolates 10455 (*C. membranifaciens*), 10464 (*P. holstii*), 10464 (*S. cerevisiae*) and 10465 (*S. cerevisiae*) were able to grow in the OMW medium but they were not capable to remove phenolic compounds (data not shown). The other eight yeast

isolates also successfully used OMW for their growth and they presented a typical profile of biomass accumulation, showing that the highest biomass concentration was reached after 7-14 days of incubation and then the cell concentration slightly decreased. Phenolic compound concentration, measured during microbial growth for 20 days, was rapidly and significantly decreased by the *G. candidum* strains, which showed a maximum phenolic removal ranging from 25% to 31% of the initial content (Fig. 35; Tab. 14). Three other isolates showed an aptitude to a lower degradation, being able to reduce the initial phenol content by 25% (isolate 10456, *C. tropicalis*), 26% (isolate 10462, *P. fermentans*) and 17% (isolate 10463, *P. holstii*). The treatment with all the selected isolates did not induce significant colour reduction, thus showing that they were not correlated with the removal of phenolic compounds (Tab. 14).

The biological properties of the eight yeast isolates able to remove phenolic compounds were further investigated in order to evaluate their potential use in OMW detoxification. In fact, it was seen that their growth in OMW medium promoted a decrease in COD (Tab. 14). Reduction of the COD was scored at day 20 of incubation and it ranged from 23% (*G. candidum* isolate 10460) to 15% (*P. holstii*, isolate 10463). The five *G. candidum* isolates under investigation showed the best performance in reducing COD during incubation in OMW with COD reduction values ranging from 23% to 20% (Tab. 14). The effect of the eight selected yeast isolates on the reduction of OMW antibacterial activity, was investigated by a plate assay using *B. megaterium* ATCC 25848 as a sensitive strain (Tab. 14). Untreated OMW medium showed a significant antimicrobial activity against the indicator microorganism. On the contrary, the yeast-free OMW medium, obtained by culturing for 20 days the eight yeast isolates described above, showed no or reduced antimicrobial activity (Tab. 14). In particular, cell-free supernatants from four out of the five *G. candidum* isolates under study (10457, 10458, 10460 and 10461) showed strong reduction of antimicrobial activity. These data also indicated that, in addition to OMW detoxification, none of the above four *G. candidum* isolates produced any antibacterial compounds.

3.5.4 Extracellular enzyme activities

The presence of extracellular ligninolytic activities was determined for the eight phenol-degrading yeast isolates by dedicated plate tests. Appropriate dilutions of yeast cultures were plated on solid media containing the different substrates for the detection of cellulase, xylanase, lignin and Mn dependent peroxidase, laccase and tyrosinase. Enzyme production

assays (Tab. 15) revealed that the five *G. candidum* isolates detained cellulase (Ce), xylanase (Xy), lignine/manganese-dependent peroxidase (LMp), laccase (La) and tyrosinase (Ty) activities, with the exception of the isolate 10459 (no Xy and LMp activities) and 10460 (no Xy activity). The *C. tropicalis* isolate showed that it was not able to carry out cellulase activity, whereas none of the investigated extracellular enzyme activities were shown by the *P. fermentans* and *P. holstii* selected strains (Tab. 15).

3.5.5 Immobilization of *G. candidum* whole cells in Ca-alginate gel beads and OMW treatment

The *G. candidum* 10461 isolate was chosen for whole-cell immobilization in alginate beads. The initial cell biomass to be loaded in the beads, the concentration of sodium alginate and $CaCl_2$ to improve the bead properties of permeability and rigidity, were set according to Bleve et al. (2008). Briefly, the yeast cells were grown in YPD medium with shaking at 25°C for 16 h. When the turbidity of the culture reached an optical density of 0.8 at 600 nm, the requested volume was harvested. Cells were washed with water and then suspended in aliquot 3% Na-alginate (Sigma, USA) solution to obtain a final concentration of 3 OD/ml. The slurry was excluded drop-by-drop with a syringe through a blunt-ended needle (21 gauge) into a solution of 0.1 M $CaCl_2$, with constant stirring at room temperature and under sterile conditions. The spherical beads (ca. 3 mm diameter) produced were cured in 0.1 M $CaCl_2$ solution for 4 h at 4°C. The cell growth and viability together with detoxification effects were studied during incubation of immobilized and free cells in OMW medium. The concentration of *G. candidum* as free cells in the medium, the COD values, the concentration of phenolic compounds, the medium decolourization and the antimicrobial activity were measured at predefined interval times for either immobilized and free *G. candidum* biomasses (Fig. 36). Indeed, the COD removal and phenolics degradation by calcium alginate entrapped cells were higher than those of free cells. The statistical analyses of results shown in Fig. 36A, indicated that the COD significantly decreased during the first 10 days of treatment with the immobilized cells, whereas the highest level of COD removal was promoted by free cells at the sixth day incubation. In fact, after 14 days of incubation, the treatment of OMWs by alginate beads produced COD values significantly lower (22.18 ± 0.68 g L^{-1}) than those found after treatment with free cells (35.39 ± 0.93 g L^{-1}). Indeed, beads immobilization allowed *G. candidum* to produce a COD decrease of 52.3% respect to the initial value, versus the 23.8% produced by free cell culture. Interestingly, a negligible cell leakage was detected

during the incubation of the alginate-immobilized cells ($1.1 \pm 0.11 \times 10^4$ CFU ml^{-1}; Fig. 36A). An increased reduction rate of phenolic compound concentration and OMW colour intensity was also observed in the OMW samples treated with the immobilized cells (Fig. 36B). In fact, phenolics were rapidly and significantly decreased by the immobilized *G. candidum*, from 4.07 ± 0.58 g L^{-1} to 2.07 ± 0.13 g L^{-1} (49.1% reduction), whereas OMW colour was diminished about 26% of the initial value (Fig. 36B). As expected, no significant colour reduction and a moderate removal of phenolic compounds (final concentration 3.07 ± 0.33 corresponding to 24.5% phenolic reduction) were detected in the control experiment, where OMWs were treated with *G. candidum* free cells (Fig. 36B). Untreated OMW medium did not show valuable differences in COD, phenolic content and colour after 14 days of incubation at 25°C (data not shown). The activity of secreted phenol oxidases, rapported to the number of yeast cells detected during the time course, was recorded by measuring the oxygen consumption during OMW treatment with either immobilized and free *G. candidum* cells. The oxygen consumption, associated to oxidase activity, was recorded in the OMW treated with the immobilized cells (Fig. 37A). During the time course of the experiment, the enzymatic activity peaked from the 2^{nd} to the 4^{th} day of incubation and, then, it decreased. No detectable cell leakage was recorded, thus indicating that no variation occurred in the inoculum concentration (Fig. 37A). In the control experiment, the concentration *G. candidum* free cells exponentially increased during the first three days of incubation and then it reached a plateau, showing that it increased by 28 times the initial cell concentration. The enzymatic activity of free-cells-treated OMW, shown as oxygen consumption related to cell concentration, was also monitored along the time course of the experiment and it showed to be very low and constant during the time course experiment (Fig. 37A).

In order to explain the increased ability to reduce COD and phenol compound content of OMWs using immobilized cells, we investigated the effect of cell immobilization on the extracellular protease(s) activity. As shown in Figure 36B, proteolytic activity was assayed and the maximum specific activity of the extracellular proteases (130.8 ± 7.2 U L^{-1}) was found in the OMWs inoculated with the free suspension culture 14 days post inoculation. No significant activity was found neither in OMWs containing the alginate-entrapped *G. geotrichum* cells nor in untreated OMWs. Taken together, the above data showed that the presence of extracellular proteases was dramatically reduced by cell immobilization. The plate assay using *B. megaterium* ATCC 25848 as a sensitive strain revealed that immobilized cells were also able to reduce antimicrobial activity after the first 3 days of

treatment and that they did not produce any antibacterial compounds during OMW detoxification (not shown).

Discussion

4. Discussion

4.1 Molecular cloning and heterologous expression of *Ery3* gene from *P. eryngii* in free and immobilized *S. cerevisiae* cells

In this first part of thesis, we describe the isolation and, for the first time, the heterologous expression in an immobilized system of a new laccase isoform from the white-rot fungus *P. eryngii*. The Ery3 protein is clearly distinguishable from the only three already characterized *P. eryngii* laccases (Munoz et al., 1997; Wang and Ng, 2006), since its N-terminal portion is absolutely different from the corresponding regions of previously described laccases of *P. eryngii*. This finding indicates that the enzyme described in the present study represents a novel laccase isoform produced by *P. eryngii*. Two laccases isoforms, similar to Ery3 and denoted Pox1 (Giardina et al., 1995) and PoxC (Palmieri et al., 1993), have been isolated from *P. ostreatus* and biochemically characterized. However, no heterologous expression have been documented for Pox1. Moreover, when PoxC was expressed in *S. cerevisiae* (Piscitelli et al., 2005), no detectable activity was measured, because of the very low amount of the produced recombinant protein. The Ery3 protein sequence contains the four laccase signature sequences (Kumar et al., 2003), which include the multiple histidine and single cysteine copper-binding residues analogous to those of laccase from *Coprinopsis cinerea* (Ducros et al., 1998). The bioinformatic analysis, performed on Ery3 and other *Pleurotus* spp. laccase sequences (Fig. 9), showed that the gene phylogeny does not strictly follow the species phylogeny, as already shown by a previous detailed phylogenetic analysis of basidiomycetes laccases (Hoegger et al., 2006).

S. cerevisiae was chosen as an host to attempt the heterologous production of Ery3 laccase. The recombinant secreted proteins were analyzed by Western blot analysis, revealing the presence of proteins with a molecular weight higher than the expected size (Fig. 12b). The obtained high molecular weight protein smear is likely due to laccase over-glycosylation, as suggested by previous investigations (Romanos et al., 1992; Kiiskinen and Saloheimo, 2004). The production of active Ery3 laccase is directly proportional to copper ion amounts in the growth medium, thus confirming that the correlation between copper availability and laccase activity was due to the enzymes requirement for copper (Guo et al., 2005). The yeast growth temperature has been identified as one of the most important parameters to optimize the production of heterologous laccase in yeast systems, as shown by laccase

gene expression experiments in *S. cerevisiae* (Cassland and Jonsson, 1999) and *P. methanolica* (Guo et al., 2005). Indeed, according to our results, yields of active heterologous Ery3 laccase are strongly influenced by the temperature. In this study, the Ery3 laccase yields proofed to be higher with respect to those obtained by previously documented expression of laccase genes in *S. cerevisiae* (Klonowska et al., 2002; Necochea et al., 2005). However, it resulted poor if compared to laccase yields obtained by employing other recombinant systems such as: (i) yeasts, as *P. pastoris* (Soden et al., 2002) and *P. methanolica* (Guo et al., 2005); (ii) filamentous fungi, as *A. oryzae* (Hoshida et al., 2005), *A. niger* (Record et al., 2002) and *T. reesei* (Kiiskinen et al., 2004); (iii) basidiomycetes, as *P. cinnabarinus* (Alves et al., 2004) and *C. cinerea* (Kilaru et al., 2006). Nevertheless, we decided to employ the *S. cerevisiae* expression system as a model to set up an immobilised system of viable cells for the production of a recombinant laccase, since this yeast demonstrated to efficiently secrete eukaryotic recombinant proteins, under low secretion level of endogenous proteins (Kiiskinen and Saloheimo, 2004). Previous studies have reported the use of laccase-producing fungi (Font et al., 2006) and of the enzyme itself (Abadulla et al., 2000) in immobilized form, to treat toxic industrial wastewater and to decolorize textile dyes, respectively. However, to date no reports on production of recombinant laccase in an immobilized system are available in the literature. The use of immobilized systems for recombinant protein production offers many advantages over conventional free cells fermentations, including: (i) prolonged activity and stability of the biocatalyst; (ii) elimination of non-productive cell growth phases; (iii) feasibility of continuous processing; (iv) easier product recovery; (v) regeneration and re-use of the biocatalyst (Kourkutas et al., 2004). Moreover, application of cell immobilization in recombinant protein production already demonstrated to be a strategy in overcoming the drawbacks related to plasmid instability and in delaying overgrowth of plasmid-free cells (Kumar et al., 1991; Barbotin, 1994). Optimization of the immobilization conditions is highly requested to reduce the constraints produced inside the gels by the immobilization process, which could affect the yeast physiological behaviour (Mattiasson et al., 1984). The optimal concentration of alginate in biocatalyst beads reveals to be a quite critical parameter. In fact, high alginate concentrations induce decrease of laccase yield, because of diffusion limitations reducing the nutrients transfer into the gel (Ellaiah et al., 2004), whereas beads prepared using lower concentrations of sodium alginate appear fragile (Konsoula and Liakopoulou-Kyriakides, 2006). The mechanical strength of alginate beads

also shows to be directly dependent on $CaCl_2$ concentration into gelling solution (Martinsen et al., 1992). The data reported in this study indicated that the repeated batch processing can represent a suitable tool to enhance the productivity of a microbial culture, because of the extension of the culture production phase (Fenice et al., 2000). Our results indicate that after cell immobilization, laccase yield is increased likely by the fact that the immobilization process leads to changes in microenvironmental conditions. These modifications could enhance either (i) the biological stability of *S. cerevisiae* or (ii) the yeast tolerance to stress conditions or (iii) the cell resistance to toxic by-products due to the cell metabolism or (iv) the proteolytic stability of the secreted protein. As far as the first two hypotheses are concerned, there is a significant body of evidences indicating that the physiology of immobilized yeast is different from that of free cells (Verbelen et al., 2006). Melzoch and coworkers (1994) showed that Ca-alginate entrapped *S. cerevisiae* cells are able to survive for a time as long as 5 years, thus demonstrating that the process of immobilization significantly enhances and maintains stability of viable yeast cells. Indeed, yeast cells immobilized in Ca-alginate beads actively produce Ery3 laccase even after 40 days of storage at 4°C, exhibiting a behavior similar to that described for an immobilized *K. lactis* system used for glucoamylase production (de Alteriis et al., 2004). Immobilized yeast cells showed an increased ethanol tolerance due to cell encapsulation, which induces a modification of fatty acid concentration in cell membranes (Norton and D'Amore, 1994). In the same paper, the authors demonstrated that the osmotic stress caused by the immobilization techniques was able to induce the intracellular production of regulative molecules such as polyols, which lead to higher tolerance to toxic compounds. Moreover, comparative studies on the metabolic activity of free and immobilized cells showed that entrapped yeasts revealed an activation of their energetic metabolism and an increase in both storage (i.e. glycogen and trehalose) and structural sugars (glucan and mannan) (Galazzo and Bailey, 1990; Jirku et al., 2000). The increase in storage polysaccharides does not primarily function as a reserve, but it acts as a highly efficient protecting agent to keep structural integrity of the cytoplasm under environmental stress conditions (Wiemken, 1990).

Several investigations have shown that extracellular proteolysis may represent a considerable obstacle to the production of secreted heterologous proteins in *S. cerevisiae*. The reported results indicate that, under the described experimental conditions, yeast immobilization in alginate beads lowers extracellular protease(s) activity (Siegel and

Brierley, 1990; Turner et al., 1991; Chung and Park, 1998). As expected, the protease(s) assays, performed in this study on the culture medium of free and immobilized cells, indicated that at higher growth temperature (30°C) a higher protease activity was obtained in both systems. On the other hand, when the assays were carried out after cell growth at lower temperature (18°C), no or very low proteolytic activity was detectable (Fig. 19b). It seems unlikely that the protease activity is due to the autolysis of yeast cells, which is characterized by the release of intracellular proteins and by the rapid reduction of cell viability (Hernawan and Fleet, 1995). In fact, we did not detect any release of intracellular proteins into the extracellular environment (Fig 12a), nor any apparent decrease of cell viability up to 72 h (data not shown). The inhibition of extracellular protease activity observed in the immobilized cell system can be explained by hypothesising a severe reduction of extracellular protease(s) secretion induced by Ca-alginate entrapping, which has been already demonstrated to occur in *A. niger* immobilized cells (Liu et al., 1998). However, the extracellular degradation activity might be also associated with whole cells (Kang et al., 2000), thus suggesting that a cell-bound protease(s) present on the *S. cerevisiae* cell surface is possibly responsible for the Ery3 protein degradation in the free cell system.

4.2 Role of the N- and C-termini of *Pleurotus eryngii* Ery4 laccase for the enzyme catalytic activity

A gene and the corresponding cDNA encoding new laccase isoenzyme in *P. eryngii*, never described before and denoted *ERY4*, was cloned, sequenced and its amino acid sequences deduced. The laccase coded by *ERY4* showed a high identity with other fungal laccases, especially with those reported in *Pleurotus* species and the maximum identity (95.3%) was found with *P. ostreatus* POXA1b laccase (Giardina et al., 1999). The differences found between Ery4 sequence and the dendrogram obtained from the alignment of several phenol oxidases (Fig. 4), suggest that Ery4 belongs to a phylogenetic group different from that including Ery3 (Fig. 9, named Pel4_Er). However, both sequences belong to the cluster that included laccases from different species belonging to the *Pleurotus* genus. Recently, a more extensive phylogenetic analysis of different multicopper oxidases has provided the basis to reserve the name of laccases only for the enzymes produced by fungi (Hoegger et al., 2006). Although biochemical characterization of native enzymes has not been possible,

according to Hoegger and coworkers (2006), the *ERY4* gene can be considered as a laccase coding gene.

The *ERY4* gene was cloned and expressed in *S. cerevisiae*. The recombinant enzyme, purified to homogeneity and detected by immunoblot analysis, did not show laccase activity in its native form. A similar evidence has been obtained by Rodriguez and coworkers (2008), who expressed in *Aspergillus niger* an allele of *ERY4* (denoted by the authors as *PEL4*). These authors produced the expected polypeptide depleted of its biological properties and they explained this evidence as being due to the instability of the recombinant enzyme.

In order to explain the lack of enzymatic activity of the recombinant Ery4, it could be hypothesized that this evidence might be due to an incorrect or absent C-terminal processing of this enzyme. Although the role of C-terminal processing of laccase has not been fully ascertained, several authors have suggested that it should be involved in the enzyme activation (Hakulinen et al., 2002; Autore et al., 2009; Andberg at al., 2009)

A comparison of *P. eryngii* Ery4 laccase protein sequence with that of laccases with known 3D structure was performed by means of the Clustal W program. The presence of a more protruding C-terminal tail of sixteen amino acids (residues 517–533 in the sequence) was thus shown in Ery4, compared to the other analysed laccases from basidiomycetes. On the other hand, a C-terminal tail (of 13–14 amino acids), usually cleaved by proteolysis in the active form, was found in deduced amino-acidic sequences of laccases from the ascomycetes *Podospora anserina* (Fernandez Larrea and Stahl., 1996), *Neurospora crassa* (Germann et al., 1988;), *Melanocarpus albomyces* (Kiiskinen and Saloheimo, 2004b) and *Myceliophthora thermophila* (Bulter et al., 2003; Zumárraga et al., 2008a). If this C-terminal extension acts as a cap obstructing the T2/T3 channel, its cleavage is required to then allow the access of the oxygen molecule and following exit of water through the above channel. A similar plug has been recently described in C-terminus of *M. thermophila* laccase, whose four last residues do not allow oxygen and water to reach the trinuclear T2/T3 copper cluster (Zumarraga et al., 2008b). The closing of the access to laccase catalytic site affects the functionality of Ascomycete laccases although it is not clear the interaction modulating the conformational modifications that permit the passage of oxygen to the T2/T3 site.

To date, one does not know if this feature is also associated to laccases from basidiomycetes. However, the C-terminal domain can affect the function of fungal laccases

from Basidiomycetes, as reported for *T. versicolor* where the redox potential of the TvL1GYC isoform changes when its C-terminus is truncated of eleven amino acids (Gelo-Pujic et al., 1999)

The PoxA1b, a laccase isoenzyme isolated from *P. ostreatus*, having a 95% similarity with Ery4 protein, was demonstrated to undergo a C-terminal processing. (Giardina et al., 1999). In fact, the Edman's sequence analysis showed that the purified PoxA1b C-terminal was be heterogeneous. Three overlapping C-terminal sequences were identified easily, as follows: -Leu-Pro-Ala-Pro-Leu-Lys (relative abundance, 40±45%); -Leu-Pro-Ala-Pro-Leu (40± 45%); and -Leu-Pro-Ala-Pro (10±20%). The three C-terminal sequences appeared to be generated from the same polypeptide chain. It is important to note that none of the obtained terminal sequences ends with Ala, it being the C-terminal residue deduced from the cDNA sequence. The authors explained the heterogeneity observed by the existence of C-terminal processing of the protein, occurring either *in vitro* and/or during the purification procedure (Giardina et al., 1999). The high similarity among PoxA1b and Ery4 primary structures, which share an identical sequence in 24 out of the last 25 amino acid residues of their C-termini, led us to hypothesize that the lack of enzymatic activity of Ery4 laccase expressed in *S. cerevisiae* was due to its incorrect or absent C-terminal processing. In fact, if C-terminus can really affect laccase activity, it could be responsible for the different affinity between native and recombinant Ery4 towards ABTS.

The 3D model of the recombinant Ery4 laccase revealed that the C-terminal tail was strongly stabilized by Lysine 532 residue electrostatic interactions and hydrogen bonds with neighbor residues in a cage (Fig. 28A). This structure could be responsible for the closure of dioxygen or of any other molecule (substrate) entrance, affecting the function of the Ery4 laccase. This unique feature has been demonstrated only in the laccase from *M. albomyces*, but Andberg and coworkers (2009) suggested that C-terminal blocking could be a common feature of Ascomycete. This hypothesis is in accordance with the data obtained by Hakulinen and coworkers (2002), who demonstrated that the protruding C-terminal of *M. albomyces* laccase from extension plays as a plug obstructing the trinuclear (T2/T3) channel. The above data further indicated that conformational changes induced by C-terminal proteolytic cleavage can be required also in laccase of Basidiomycetes in order to obtain the mature form of the enzyme (Hakulinen et al., 2002).

To check if the presence of a more protruding C-terminal tail was related to a role of this protein domain in affecting the catalytic or stability properties of Ery4 laccase, the *ERY4*

gene was used as template to obtain: i) C- terminal truncated mutants (each respectively missing 2, 5, 8, 11, 14, 18 residues); ii) mutants carrying site-specific mutations at their 3'-terminus; iii) mutants specific portions derived from the *ERY3* gene

All the mutant Ery4-derived isoforms carrying progressive deletions of their C-terminal sequence were correctly secreted. This evidence indicates that the above region is not essential for secretion of the functional recombinant enzyme, as also recently shown for PoxA1b of *P. ostreatus* (Autore et al., 2009). The Ery4 does not share the above property with the *M. thermophila* laccase, whose C-terminus is essential for early post-translational processing step (Bulter et al., 2003).

It is significant to note that the truncation of the two last amino acids was able to restore the enzymatic activity. This finding allows to hypothesize that the deletion of the above two terminal residues is requested to remove the plug that stop the access of oxygen and water to the trinuclear T2/T3 copper cluster, in this differing from *P. ostreatus* Poxa1b and *M. thermophila* laccases, where the terminal plug is constituted by the last four residues. The deletion of the last five C-terminal residues (isoform 5Less) resulted in the best performing recombinant laccase, since it showed the highest second order rate constant (k_{cat}/K_m) ratios towards all the substrate tested. The k_{cat}/K_m ratio is the direct measure of the efficiency of the enzyme in transforming a substrate and it indicates the catalytic efficiency of the enzyme itself. The partial deletion of Ery4 C-terminal tail could influence binding/interaction of the laccase with the reducing substrate and the different sizes of the analysed substrates could be responsible for the different observed effects. Moreover, truncation of a more extended C-terminal region does not have any positive effect on catalytic properties towards all the analysed substrates.

Site directed mutagenesis of selected amino acid residues in the very C-terminal portion of Ery4 shed light on the role of the lysine in position 532 in the activation of catalytic activity. In fact, because of its positive charge the above residue is stabilized by a network of electrostatic interactions established with the surrounding residues, which is likely to block the plug obstructing the trinuclear (T2/T3) channel. This evidence is confirmed by several lines of evidence: i) the substitution of the above lysine with a similarly charged arginine residue did not allow to obtain an isoform denoted by catalytic activity; ii) its exchange with either a negatively charged glutamic acid or a neutral alanine, both able to establish a single interaction with the histidine in position 131, allowed to obtain two biologically active laccase isoforms.

It is noteworthy that the substitution of proline with an alanine in position 530 of Ery4 amino acid sequence restore laccase catalytic properties. The position 494 is located in a loop at the C-terminus that, in agreement with modelling analysis, constitute a kind of stopper blocking the channel through which the substrate access the enzyme catalytic site. The above loop hat has already been ascertained to have an effect on the function of fungal laccases (Hakulinen et al., 2002). Therefore, it can be hypothesized that the substitution of proline in position 530 could destabilize the final part of the C-terminal loop, thus allowing oxygen and water molecules to reach T2/T3 copper cluster.

Compared to the shuffling of sequences mutated by rationale deletions or site-directed mutagenesis, recombination of related sequences allows large distances in sequence space to be travelled without disturbing the function and⁄or structure. This method has recently been applied to laccase either by in vivo homologous recombination for *Trametes* sp. 30 (Cusano et al., 2009) or by chimerical laccase construction for Lcc1 and Lcc4 of *Lentinula edodes* (Nagakawa et al., 2010).

However, for the first time in our knowledge, we have produced three chimerical laccase isoform by substituting the N- and/or the C-terminal non catalytic portion of Ery4 with the equivalent regions of Ery3. We were able to produce three chimerical laccase which exhibit enzymatic activity and, in particular, the 4NC3 isoform that showed interesting K_{cat}/K_m ratios towards all the substrate tested. No expression problems were recorded for any of the three chimerical genes, in this differing from the data described by Nakagawa and coworkers (2010) about the non-production of Lcc1/4 chimerical laccase.

Is noteworthy that the expression of 4N3 gene, that carry the substitution of Ery4 N-terminal portion with the equivalent region of Ery3, produced a biologically active enzyme even if the C-terminal region of the chimerical laccase was identical to that of the non active Ery4. This evidence shed light on the more complex structural interaction between laccase domain and on a possible role of the N-terminal region in laccase structure and catalytic activity.

4.3 Construction of whole cell laccase biocatalyst yeast

Surface-display of proteins in yeast can be realized by fusion of the selected gene with tag gene encoding surface proteins, i.e. α-agglutinin (cell-wall mannoproteins); Flo1 (lectin-like cell-wall protein); Pir family, (proteins covalently bound to cell wall β-1,3-glucan) (Schreuder et al., 1996). The α -agglutinin fusion strategy was used to express, on the yeast

cell wall, several proteins, such as a β-glucosidase from *Aspergillus oryzae* (Kaya et al., 2008), a glucoamylase from *Rhizopus oryzae* and α-amylase from *Bacillus stearothermophilus*, codisplayed on yeast cell wall (Murai et al., 1999), a xylanase I from *Trichoderma reesei* and a β-glucosidase from *A. oryzae*, co-displayed on yeast cell wall (Katahira et al., 2004). The Flo1 fusion strategy was used to express an organophosphorus hydrolase from *Flavobacterium* species (Fukuda et al., 2010), a lipase (Matsumoto et al., 2002) and a glucoamylase (Sato et al., 2002) from *R. oryzae,* whereas examples of the use of Pir proteins family concerned the surface display of three different glycosyltransferases from S*chizosaccharomyces pombe* (Abe et al., 2003), a human α -1,3-fucosyltransferase (Abe et al., 2004), a xylanase A from *Bacillus* sp. BP7 (Andrés et al., 2005), a lipase A from *B. subtilis* (Mormeneo et al., 2008).

In the yeast-based cell-surface display system, the target protein N-terminal end is commonly fused with the C-terminal end of yeast cell wall protein, especially for the target proteins that have their active site spatially near to the C-terminus (Matsumoto et al., 2002).

In this work, the Pir2 and flocculin fragment of Flo1p (FS) (Matsumoto et al., 2002), were chosen as cell wall anchors to express the *4NC3* and *5LESS* mutant laccase genes on the *S. cerevisae* cell surface. The fusion strategy of mutant laccases to carboxy-terminal end of Pir2 and FS cell wall proteins was likely to be suitable to display a laccase enzyme on the cell wall. In fact, Andrés and coworkers (2005) and Matsumoto and coworkers (2002) demonstrated, respectively, that the target protein fused at the C-term end of an homologous of Pir2 protein (Pir4) or FS domain is most likely to be correctly folded and to retain activity, besides being correctly targeted to the cell wall. The resulting fusion proteins was thus correctly targeted to the *S. cerevisiae* cell wall and they conferred laccase activity to the cells (Tab. 10).

The localization of recombinant laccases on yeast cell walls was confirmed by immunofluorescent labeling assay. Immunofluorescent signals were detected at the cell surface of all strains harbouring recombinant fusion proteins, although not all the cells had the same label intensity (Fig. 32), maybe probably because of differences in the expression levels of fusion genes among the cells. The present preliminary results represented, for the first time, data concerning the construction of a functional whole cell biocatalysts carrying laccase exposed on cell wall.

Although laccase proteins were associated with the cell walls of strains harbouring recombinant fusion proteins, kinetic parameters demonstrated that the recombinant laccases displayed on yeast cell wall had a less activity if compared to laccase activity obtained by yeast cells secreting recombinant mutant laccases (Tab. 6 and Tab. 10). This evidence could be due to the fact that the Pir2 protein with its other homologous proteins (Pir1, Pir3 and Pir4) and Flo1p are endogenous yeast proteins normally localized at the surface of the cells. When the recombinant proteins were expressed on yeast cell walls, they are likely to compete in finding a localization on cell wall with the endogenous Pir and Flo1p proteins.

As reported by Abe and coworkers (2003), the yeast cells, producing a target protein fused with a Pir protein and having the *PIR* gene family disrupted, exhibited a significant increase in enzymatic activity on the cell wall.

Another possible explanation of low laccase activity on cell wall could be attributed to the length of Flo1 protein portion used to display a target protein on cell wall. Sato and coworkers (2002) demonstrated that surface-displayed enzyme was more accessible and more reactive to polymer substrate when a longer portion of Flo1p was used. Therefore, it could be possible that the recombinant laccases fused to the flocculin domain (FS) of Flo1 protein were exposed to the extracellular environment, but partially blocked in the space to react with the substrate.

4.4 Heterologous expression, purification and biochemical characterization of *PPO2* tyrosinase from *A. bisporus* in *S. cerevisiae*

A recombinant *A. bisporus* tyrosinase gene (*PPO2*), tagged at its 3-terminus with a block encoding six histidine, was expressed in *S. cerevisiae*. The recombinant protein was purified and it appeared to be biologically active.

The purification of the recombinant Ppo2 was carried out by affinity chromatography using a Nickel gravity-fed column. This chromatographic step, that allowed the elimination of several protein contaminants, was exploited by using a buffer containing NaCl, a substance to which the fungal tyrosinase are very sensitive. The *A. bisporus* tyrosinase has been described as a protein with a tetrameric or dimeric structure (Strothkamp et al., 1976). In the chromatographic procedures, when 0.5 M NaCl was used in lysis, wash and elution buffer, the purified protein had no biological activity because of the salt high concentration. In fact, the electrophoresis assay showed that the subunits of the purified

tyrosinase were dissociated and did not possess any enzymatic activity by their own (Strothkamp et al., 1976). The use of lower NaCl concentration (0.15 M) in the chromatographic procedures allowed to purify the recombinant tyrosinase in an active form and, as expected, its active tetrameric and dimeric structure were detected by electrophoresis analysis (Fig. 34).

The activity of purified tyrosinase was determined using 5 different substrates and it was compared with the performances of the commercial *A. bisporus* tyrosinase. Although both enzymes exhibited similar K_m values for all tested substrates, the recombinant tyrosinase was more reactive than the commercial one. This result may be related to the purity level of recombinant tyrosinase which was purified at the homogeneity from the yeast *S. cerevisiae*, whereas the commercial tyrosinase preparation was contaminated by other proteins (data not shown).

The N-terminus of Ppo2 tyrosinase was fused with the C-terminus of Pir2 and Flo1 proteins in order to express the enzyme on the *S. cerevisae* cell wall. Although the genetic constructs were correct in their sequences, it was not possible to detect any tyrosinase activity in plate nor in liquid in recombinant yeast strains expressing Ppo2 protein on their surface. The absence of tyrosinase activity may probably be ascribed to an unsuccessful exposure on the yeast cell surface, due to an incorrect assembly on its active tetrameric or dimeric structure. According to literature data, heterologous expression of *A. oryzae melO*-encoding tyrosinase cDNA in *S. cerevisiae* resulted in the production of an intracellular inactive proenzyme (Fujita et al., 1995) and, without acidifying treatment, no tyrosinase activity was observed. No data are available concerning the stability and the catalytic properties of purified recombinant tyrosinase isoforms expressed in *S. cerevisiae*. In fact, Nakamura and coworkers (2000) functionally expressed several *A. oryzae melO* mutant genes in *S. cerevisiae* but data on purification of these enzymes were not produced. Induction and expression of the *A. bisporus AbPPO2* encoding tyrosinase cDNA in *E. coli* cells also resulted in the production of an inactive pro-tyrosinase of 64 kDa (Wichers et al., 2003). Only a tyrosinase-encoding gene from the white-rot fungus *Picnoporus sanguineus* was expressed in *A. niger* and secreted in the extracellular medium, under the control of glucoamylase pre-pro-sequence of *A. niger*. The maturation process was effective in *A. niger* and the recombinant enzyme was fully active (Halaouli et al., 2006). The use of this fungus could be investigated as a suitable system for heterologous expression of a stable and correctly processed, may be secreted, *A. bisporus* Ppo2 tyrosinase.

To our knowledge, this is the first time that an *A. bisporus* tyrosinase was functionally expressed in the heterologous host *S. cerevisiae*.

4.5 Isolation and selection of yeasts and their immobilization for the treatment and detoxification of olive mill wastewater

Several recent investigations have studied microorganisms associated to the OMW, i.e. bacteria (Jones et al., 2000; Rincón et al., 2006), yeasts and moulds (Giannoutsou et al., 2004; Ben Sassi et al., 2006; 2008; Morillo et al., 2008), in order to evaluate the ability of these microbes in OMW detoxification. Among the mentioned microbiota, yeasts are the more adapted to growth in OMW, as they can resist the high concentrations of phenols (Shivarova et al., 1999; Yan et al., 2005) and low pH values of mill wastes, allowing them to be the dominant microorganisms in this environment (Ben Sassi et al., 2006). The present study started with the isolation of yeast and mould strains in different OCWs and OMWs collected from five different Apulian olive mills.

Strains belonging to *Penicillium*, *Geotrichum*, *Saccharomyces*, *Pichia*, *Rhodotorula* and *Candida* were the mycetes detected by molecular identification in this study. These genera have previously been isolated from different OMW including TPOMW, demonstrating that they are likely to be part of the natural microbiota of these residues (Assas et al., 2000; Ettayebi et al., 2003; Millan et al., 2000; Ben Sassi et al., 2008; Giannoutsou et al., 2004; Morillo et al., 2008). In particular, in the OCW samples *S. cerevisiae* were the most representative species compared with *G. candidum*, whereas this trend was inverted in stored OMWs, where *G. candidum* corresponded to 48% of the population and the presence of *S. cerevisiae* was reduced to 26.7%.

It is known that OMW contains all the elements essential for microbial growth (e.g. carbon and nitrogen sources, minerals), but also several growth inhibitors, such as organic acids and phenolic compounds. Its composition depends on the maturity and variety of the olive fruit but mainly on the oil extraction technology used (Fiestas Ros De Ursinos and Borja-Padilla, 1992). Out of the 300 isolates identified, twelve isolates belonging to *Candida membranifaciens*, *C. tropicalis*, *G. candidum*, *P. fermentans*, *P. holstii* and *S. cerevisiae* species, demonstrated that they could use OMW as a unique nutrient source for their growth. According to the observations made by Morillo and coworkers (2008), the use of the indigenous microbiota could be a promising bioremediation approach, since indigenous

micro-organisms have a range of different biodegrading activities. The data produced during our investigations confirmed previous reports indicating that phenol removal from OMW by yeasts seems to be a strain-dependent process (Papanikolaou et al., 2008), since in similar conditions some yeast strains can and others cannot grow in OMW, even when the amount of phenolic compounds is low. We have showed that eight wild yeast isolates were able to reduce the phenol content of OMW (Fig. 35) but they were not able to promote OMW decolourization. Yeast strains belonging to the *Pichia* genera have previously demonstrated to significantly reduced the concentration of total phenols (Ben Sassi et al., 2008). The two *P. fermentans* (11462) and *P. holstii* (11463) strains described here decreased phenol content without reducing colour levels of treated OMW, thus showing a behaviour similar to that described by Ben Sassi and coworkers (2008). Furthermore, the capacity of *C. tropicalis* (isolate 10456) to reduce the OMW initial total phenol content by 29% was not surprising, since this yeast already demonstrated phenol degradation activity (Ettayebi et al., 2003; Yan et al., 2005). The filamentous yeast *G. candidum* has been recently reported to hydrolyze phenolic compounds and to reduce colour in mill wastewaters supplemented with glucose and/or nitrogen sources (Assas et al., 2002; Asses et al., 2009). The five *G. candidum* strains here described, were capable of actively growing in OMW as the sole carbon source, giving rise to a partial phenol removal (at values ranging from 25 to 31%), being also unable to induce decolourization. The behaviour of *G. candidum* strains here described was partially consistent with previously reported data (Assas et al., 2000; Ayed et al., 2005), where no biodegradation and rapid growth inhibition were obtained when *G. candidum* strains were incubated with stored oxidized OMW as the sole carbon source. OMW contains some phytotoxic and antimicrobial compounds, as described by several authors (Peredes et al., 1986; Perez et al., 1992; Ramos-Cormenzana et al., 1996; Kissi et al., 2001). After effluent fermentation with all the selected yeast strains and, in particular with the *G. candidum* isolates, we observed a reduced toxicity of the OMW medium, which was proportional to the reduction total phenols and COD in the treated samples (de la Rubia et al., 2008).

In this study, the *G. candidum* isolates demonstrated to reduce COD (from 20 to 23%) and the phenols (from 25 to 31%) in treated OMW. However, these reductions resulted lower than to those described by OMW treatment with other microorganisms such as other *G. candidum* strains (Assas et al., 2002; Fadil et al., 2003; Asses et al., 2009) or other mycetes belonging to *Candida* (Ettayebi et al., 2003; Ben Sassi et al., 2008; Gonçalves et al., 2009),

Aspergillus (Fadil et al., 2003; Fausto Cereti et al., 2004) and *Yarrowia* (Lanciotti et al., 2005; Goncalves et al., 2009) genera. Nevertheless, we decided to employ one of the selected *G. candidum* strains as a model to set up an immobilized system of viable cells for mill waste bioremediation. In fact, several studies have indicated this organism as a good candidate for OMW management because of its ability to grow on the wastewaters and to decrease their organic charge (Assas et al., 2002; Fadil et al., 2003; Asses et al., 2009). Previous investigations have reported the use of basidiomycetes, such as *Phanerochatae chrysosporium* (Mebirouk et al., 2006) and *Lentinula edodes* (D'Annibale et al., 1998) to treat olive mill wastewater. The ascomycetes *C. tropicalis*, under metabolic induction by hexadecane, has also been tested in immobilized form for phenol biodegradation in nutrient-added OMW (Ettayebi et al., 2003). However, to date no reports on employment of yeasts in an immobilized system for direct OMW detoxification are available in the literature. The use of immobilized systems for recombinant protein production offers many advantages over conventional free cells fermentations, including: (i) prolonged activity and stability of the biocatalyst; (ii) elimination of non-productive cell growth phases; (iii) feasibility of continuous processing; (iv) regeneration and re-use of the biocatalyst (Kourkutas et al., 2004). The COD and phenolics reduction obtained with *G. candidum* immobilized cells respectively showed a 2.2- and 2-fold higher if compared to the removal obtained with free cells. Our results also indicate that the incubation with alginate-entrapped cells strongly enhanced the presence of oxidative enzymes activity in treated OMW, which has been associated to microbial detoxification of olive mill effluents (Tsioulpas et al., 2002).

The above lines of evidence can be explained likely by the fact that the immobilization process leads to changes in microenvironmental conditions. These modifications could enhance either (i) the biological stability of *G. candidum* or (ii) the yeast tolerance to stress conditions or (iii) the cell resistance to toxic by-products due to the cell metabolism or (iv) the proteolytic stability of the secreted protein. As far as, the first two hypotheses are concerned, there is a significant body of evidences indicating that the physiology of immobilized yeast is different from that of free cells (Verbelen et al., 2006). Melzoch and coworkers (1994) showed that Ca-alginate entrapped *S. cerevisiae* cells are able to survive for a time as long as 5 years, thus demonstrating that the process of immobilization significantly enhances and maintains stability of viable yeast cells. Comparative studies on the metabolic activity of free and immobilized cells showed that entrapped yeasts revealed

an activation of their energetic metabolism and an increase in both storage (i.e. glycogen and trehalose) and structural sugars (glucan and mannan) (Galazzo and Bailey, 1990; Jirku et al., 2000), that enhance the cell resistance to toxic secondary products. Several investigations have shown that extracellular proteolysis may represent a considerable obstacle to the production of secreted proteins in yeasts. The reported results indicate that, under the described experimental conditions, *G. candidum* immobilization in alginate beads lowers extracellular protease(s) activity (Siegel and Brierley, 1990; Turner et al., 1991; Chung and Park, 1998; Bleve et al., 2008). The inhibition of extracellular protease activity observed in the immobilized cell system can be explained by hypothesising a severe reduction of extracellular protease(s) secretion induced by Ca-alginate entrapping, which has been already demonstrated to occur in *A. niger* immobilized cells (Liu et al., 1998). However, the extracellular degradation activity might be also associated with whole cells (Kang et al., 2000), thus suggesting that a cell-bound protease(s) could be present on the *G. candidum* cell surface, which could be responsible for protein degradation in the free cell system.

Conclusions

Main objective of this thesis was the characterization of genes encoding basidiomycetes oxideoreductases, ie. laccases and tyrosinases, to increase knowledge about the relationship between structure and function in this class of enzymes of great biotechnological importance.

A further goal of the present investigation was to obtain improved oxideoreductases for industrial applications either by application of recombinant techniques or by selecting microorganisms producing the above biocatalysts.

The *ERY3* and *ERY4* laccase genes of *Pleurotus eryingii* and the *PPO2* gene coding for the *Agaricus bisporus* tyrosinase were cloned and expressed in *Saccharomyces cerevisiae*.

In particular, immobilized *S. cerevisiae* cells were here for the first time employed for recombinant protein production and in particular to express a fungal laccase *ERY3* gene. The stability shown by the developed biocatalyst system suggests its possible application in semi-continuous processes for recombinant laccase production, thus indicating that the use of whole-cell immobilization can be a suitable alternative method for large scale laccase production.

The expression of *P. eryngii ERY4* in yeast led to the production of a biologically inactive protein. The C-terminal tail of the Ery4 was shown to affect both catalytic performance and stability properties of the enzyme. Molecular dynamics simulations and biochemical data allowed us to demonstrate that C-terminal tail acts as a plug that blocks the access of oxygen and water to the trinuclear T2/T3 copper cluster, whereas the C-terminal tail could influence binding/interaction with reducing substrate. The progressive truncation of C-terminal amino acids in Ery4 laccase has resulted in enzymatically active laccase isoforms with specific properties towards different substrates. The role of the lysine in position 532 in the activation of catalytic activity was elucidated. In fact, it is stabilized by electrostatic interactions with the surrounding residues and blocks the plug obstructing the trinuclear (T2/T3) channel.

For the first time, evidences on the complex structural interaction between laccase domain and on a possible role of the N-terminal region in laccase structure and catalytic activity were produced. The mutagenesis experiment has led to new and more efficient variants of this enzyme paving the way to new strategies to tailor laccase catalysts for different applications and to increase the knowledge of the structure-function relationship of this class of enzymes.

The fusion strategy of mutant laccases to carboxy-terminal end of Pir2 and FS cell wall proteins was demonstrated to be suitable to display a laccase enzyme on the cell wall. To our knowledge, this is the first example of a functional laccase immobilized on yeast surface.

The Ppo2 tyrosinase of *A. bisporus* was functionally expressed for the first time in the heterologous host *S. cerevisiae*. The recombinant protein was purified and it showed to be biologically active. The future employment of other heterologous expression systems, such as *Aspergillus niger* could be exploited for a more efficient production of the above biocatalyst.

A molecular and biotechnological characterization of natural yeast strains from OMW samples in Italy and the population dynamics occurring during the natural stabilization process of olive mill wastewaters was produced. The results described here indicate that Ca-alginate immobilized *G. candidum* might be applicable to an OMW treatment system for the removal of phenols, COD and antimicrobial compounds. In fact, COD and phenolic reduction was twice as high in OMW treated with immobilized cells as in wastewaters treated with free cells, since cell immobilization enhanced the proteolytic stability of microbial degradative enzymes.

Figures

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P.os_poxA       --MAVAFVALVSLALALVRVEAS---IGPRGTLNIANKVIQPDGFYRSTVLAG-------
P.er_Ery4       --MAVAFIALVSLTLALVRVEAS---IGPRGTLNIANEVIKPDGFSRSAVLAG-------
P.os_lccK       MFPGARILATLTLALHLLHGTHA--AIGPTGNMYIVNEDVSPDGFARSAVVARSVPATDP
P.os_lacA       MFPGARILATLTLALHLLHGTHA--AIGPTGNMYIVNEDVSPDGFARSAVVARSVPATDP
P.os_pox2       MFPGARILATLTLALHLLHGAHA--AIGPAGNMYIVNEDVSPDGFARSAVVARSVPATDP
P.sa_lac4       MFPGARILATLALALHLLHGALA--AIGPVGDMYIVNEDVSPDGFSRSAVVARSVPITGP
P.pu_lac2       MFPGARILATLTLALHLLHGALA--AIGPVGDMYIVNEDVSPDGFSRSAVVARSVPITGP
P.sp_lac1       MFPGARILATLTLALHLLRGTHA--AIGPIDDMYIVNEDVSPDGFTRSAVVARSVPATGP
P.sa_lac1       MFPGARILATLTLALHLLHGAHA--AIGPVGDMYIINEDVSPDGFTRSAVVARSAPSTES
P.os_lacB       MFPGARILATLTLALHLLHGTHA--AIGPTGDMYIVNEDVSPDGFTRSAVVARSDPTTNG
P.er_Ery3       MFPGARILATLTLALHLLHGTHA--AIGPIADMYIVNEDVSPDGFARSAVVARSVPATDP
P.sa_lac2       MRALSHFIASLTLVLWFRPGANAVETIGPVGSMRIVNKVVSPDGFSRSAVVAG------K
P.sa_lac5       MAPLVRALA--CLAILAVTNVYG-ATVGNNADIYIANKVIAPDGFERSGVLAG-------
P.os_pox3       -MVLSTKLAALVASLPFVLAATK------KLDFHIRNDVVSPDGFERRAITVN-------
P.sp_lac2       -MVLSTKLAALVASLPFVLAATK------KLDFHIRNDVVSPDGFERRAITVN-------
P.sa_lac3       -MVLSTKLVALVASLPFVFAVTK------KLDFHIRNDVVSPDGFERRAITVN-------
P.pu_lac6       -MVLSTKLVALVASLPFVFAVTK------KLDFHIRNDVVSPDGFERRAITVN-------
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                                                                  T2 T3
P.os_poxA       ----GSYPGPLIKGKTGDRFQINVVNKLADTSMPVDTSIHWHGLFVKGHNWADGPAMVTQ
P.er_Ery4       ----GSYPGPLIKGETGDRFQINVVNKLADTSMPVDTSIHWHGIFVRGHNWADGPAMVTQ
P.os_lccK       TPATVSIPGVLVQGNKGDNFQLNVVNQLSDTTMLKTTSIHWHGFFQAGSSWADGPAFVTQ
P.os_lacA       TPATVSIPGVLVQGNKGDNFQLNVVNQLSDTTMLKTTSIHWHGFFQAGSSWADGPAFVTQ
P.os_pox2       TPATASIPGVLVQGNKGDNFQLNVVNQLSDTTMLKTTSIHWHGFFQAGSSWADGPAFVTQ
P.sa_lac4       TPATASIPGVLVQGNKGDNFQLNVVNQLSDTTMLKTTSIHWHGFFQAGSSWADGPAFVTQ
P.pu_lac2       TPATASIPGVLVQGNKGDNFQLNVVNQLSDTTMLKTTSIHWHGFFQAGSSWADGPAFVTQ
P.sp_lac1       APATVSIPGVLVQGNKGDNFQLNVVNQLSDTTMLKTTSIHWHGFFQFGTSWADGPAFVTQ
P.sa_lac1       TSETGSTPGVLVQGNKGDNFQLNVLNQLSDTTMLKTTSIHWHGIFQTNSTWADGPAFVNQ
P.os_lacB       TSET--LTGVLVQGNKGDNFQLNVLNQLSDTTMLKTTSIHWHGFFQSGSTWADGPAFVNQ
P.er_Ery3       TPASVSVPGVLVQGNKGDNFQLNVRNQLSDSTMLKTTSIHWHGFFQSGSTWADGPAFVNQ
P.sa_lac2       TAAGATNPGVLVHGWKGNNFKLNVINELTDTTMLKTTSIHWHGFFQAGTSWADGPAFVTQ
P.sa_lac5       TAAGVEVIGPLVRGWKGNRFRLNVINQLSDSSMLKTTSIHWHGFFQEGSSWADGPAFVTQ
P.os_pox3       ----GIFPGTPVILQKNDKVQINTINELTDPGMRRSTSIHWHGLFQHKTSGMDGPSFVNQ
P.sp_lac2       ----GIFPGTPVILQKNDKVQINTINELTDPGMRRSTSIHWHGLFQHKTSGMDGPSFVNQ
P.sa_lac3       ----GIFPGTPVILQKNDKVQIKTINELTDPGMRRSTSIHWHGLFQHKTSGMDGPSFVNQ
P.pu_lac6       ----GIFPGTPVILQKNDKVQIKTINELTDPGMRRSTSIHWHGLFQHKTSGMDGPSFVNQ
                       *  :   ..:..:::. *:*:*. *   ********.*    .  ***:.*.*
                                                                    L1
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                                  T3 T3
P.os_poxA       CPIVPGHSFLYDFEVPDQAGTFWYHSHLGTQYCDGLRGPLVVYSKNDPHKRLYDVDDEST
P.er_Ery4       CPIVPGHSFLYDFEIPDQAGTFWYHSHLGTQYCDGLRGPFVVYSKNDPHKRLYDVDDEST
P.os_lccK       CPVASGDSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPSDPHLSLYDIDNADT
P.os_lacA       CPVASGDSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDSSDPHLSLYDIDNADT
P.os_pox2       CPVASGDSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPSDPHLSLYDIDNADT
P.sa_lac4       CPVAPGDSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPTDPHLSLYDIDNADT
P.pu_lac2       CPVASGDSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPTDPHLSLYDIDNADT
P.sp_lac1       CPIASGDSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPSDPHLSLYDIDNADT
P.sa_lac1       CPIASGNSFLYDFNVADQAGTFWYHSHLSTQYCDGLRGPLVIYDPSDPHLSLYDIDNADT
P.os_lacB       CPIASGNSFLYDFNVPDQAGTFWYHSHLSTQYCDGLRGPFIVYDPSDPHLSLYDVDNADT
P.er_Ery3       CPIASGNSFLYDFNVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPSDPHLSLYDVDNADT
P.sa_lac2       CPIAAGNSFLYDFEVPDQAGTFWYHSHLSTQYCDGLRGAFVVYDLFDPPRLRYDIDNADT
P.sa_lac5       CPIASGDSFLYDFRVPDQAGTFWYHAHLSTQYCDGLRGAFVVYDLLDPHRLRYLVDNEDT
P.os_pox3       CPIPPNSTFLYDFDTAGQTGNYWYHSHLSTQYCDGLRGSFIVYDPNDPLKHLYDVDDEST
P.sp_lac2       CPIPPNSTFLYDFDTAGQTGNYWYHSHLSTQYCDGLRGTFIVYDPNDPLKHLYDVDDEST
P.sa_lac3       CPIPPNTTFLYDFDTAGQTGNYWYHSHLSTQYCDGLRGSFIVYDPNDPLKHLYDVDDEST
P.pu_lac6       CPIPPNTTFLYDFDTAGQTGNYWYHSHLSTQYCDGLRGSFIVYDPNDPLKHLYDVDDEST
                **: .. :***:*  ..*:*.:***:** .********.::*.  **    * :*: .*
                                                    L2
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P.os_poxA       VLTVGDWYHAPSL-------SLTGVPHPDSTLFNGLGRSLNGPASPLYVMNVVKGKRYRI
P.er_Ery4       VLTVGDWYHAPSL-------SLSGVPHPDSTLFNGLGRSLNGPASPLYVMNVVKGKRYRI
P.os_lccK       VITLEDWYHIVAP-------QNAAIPTPDSTLINGKGRYAGGPTSPLSIINVESNKRYRF
P.os_lacA       VITLEDWYHIVAP-------QNAAIPTPDSTLINGKGRYAGGPTSPLSVINVESNKRYRF
P.os_pox2       VITLEDWYHIVAP-------QNAAIPTPDSTLINGKGRYAGGPTSPLAIINVESNKRYRF
P.sa_lac4       VITLEDWYHIVAP-------QNAAIPTPDSTLINGKGRYAGGPTSPLAVINVESTKRYRF
P.pu_lac2       VITLEDWYHIVAP-------QNAAKSHPDSTLINGKGRYAGGPTSPLAVINVESTKRYRF
P.sp_lac1       VITLEDWYHVVAP-------QNAVLPTPDSTLINGKSRYAGGPTSPLAIINVESTKRYRF
P.sa_lac1       VITLEDWYHVVAP-------QNAVLPTADSTLINGKGRFAGGDTTALAVINVESNKRYRF
P.os_lacB       IITLEDWYHVVAP-------QNAVLPTADSTLINGKGRFAGGPTSALAVINVESNKRYRF
P.er_Ery3       VITLEDWYHVAAP-------QGAVLPTPDSTLINGKGRFAGGPTSPLAIINVESNKRYRF
P.sa_lac2       VITLEDWYHVVAP-------QNGPIPTPDSTLINGLGRYAGGPTSPLAVVRVLWGLRYRF
P.sa_lac5       VITLADWYHTVAP-------QAGLVPVPDSTLINGRGRYAGGPTVPLSLIRVLWGVKYRF
P.os_pox3       IITLADWYHDLAPHAQNQFFQTGSVPIPDTGLINGVGRFKGGPLVPYAVINVEQGKRYRF
P.sp_lac2       IITLADWYHDLAPHAQNQFFQTGSVPIPDTGLINGVGRFKGGPLVPYAVINVEQGKRYRF
P.sa_lac3       IITLADWYHDLAPHAQNQFFQTGSVPIPDTGLINGVGRFKGGPLVPYAVINVEQGKRYRF
P.pu_lac6       IITLADWYHDLAPHAQNQFFQTGSVPIPDTGLINGVGRFKGGPLVPYAVINVEQGKRYRF
                ::*: **** :       .   . .*: *:** .*  .*   .  ::.*    :**:


P.os_poxA       RLINTSCDSNYQFSIDGHTFTVIEADGENTQPLQVDQVQIFAGQRYSLVLNANQAVGNYW
P.er_Ery4       RLINTSCDSNYQFSIDGHAFTVIEADGENTQPLQVDQVQIFAGQRYSLVLNANQAVGNYW
P.os_lccK       RLVSMSCDPNFTFSIDGHSLLVIEADAVNIVPITVDSIQIFAGQRYSFVLTADQTVGNYW
P.os_lacA       RLVSMSCDPNFTFSIDGHSLLVIEADAVNIVPITVDSIQIFAGQRYSFVLTADQTVGNYW
P.os_pox2       RLVSMSCDPNFTFSIDGHSLLVIEADAVNIVPITVDSIQIFAGQRYSFVLTANQAVDNYW
P.sa_lac4       RLVSMSCDPNFTFSIDGHSLLVIEADAVNIVPITVDSIQIFAGQRYSFVLTADQTPDNYW
P.pu_lac2       RLVSMSCDPNFTFSIDGHSLLVIEADAVNIVPITVDSIQIFAGQRYSFVLTADQTPDNYW
P.sp_lac1       RLVSMSCDPNFMFSIDGHSLQVIEADAVNIVPITVDSIQIFAGQRYSFVLTADQTVGNYW
P.sa_lac1       RLVSMSCDPNFTFSIDGHSLQVIEADAVNIVPIVVDSIQIFAGQRYSFVLNANQTVDNYW
P.os_lacB       RLISMSCDPNFTFSIDGHSLQVIEADAVNIVPIVVDSIQIFAGQRYSFVLNANQTVDNYW
P.er_Ery3       RLISMSCDPNFTFSIDGHSLQVIEADAVNIVPLVVDSIQIFAGQRYSFVLNANQAVDNYW
P.sa_lac2       RLVSLSCDPNWTFSIDGHKLLVIEADAVNVEPVEVDSIQIFAGQRYSFVLRANQQIANYW
P.sa_lac5       RLVSLSCDPNYTFSIDGHKMTIIEVDGVNTKPLEVDSIQIFAGQRYSFVLHANQRIANYW
P.os_pox3       RLIQISCRPFFTFSIDNHTFDAIEFDGIEHDPTPAQNIDIYAAQRASIIVHANQTIDNYW
P.sp_lac2       RLIQISCRPFFTFSIDNHTFDAIEFDGIEHDPTPAQNIDIYAAQRASIIVNANQTIDNYW
P.sa_lac3       RLIQISCRPFFTFSIDNHTFDAIEFDGIEHDPTPAQNIDIYAAQRASIIVNANQTIDNYW
P.pu_lac6       RLIQISCRPFFTFSIDNHTFDAIEFDGIEHDPTPAQNIDIYAAQRASIIVNANQTIDNYW
                **:. ** . : ****.* :  ** *. :  *  .:.::*:*.** *::: *:*   ***


P.os_poxA       IRANPN----SGDPGFENQMNSAILRYKGARSIDPTTPEQNATNPLHEYNLRPLIKKPAP
P.er_Ery4       IRANPN----SGDPGFANQMNSAILRYKGARNVDPTTPERNATNPLREYNLRPLIKEPAP
P.os_lccK       IRANPN----LGSTGFDGGINSAILRYAGATEDDPTTTSSTS-TPLLETNLVPLENPGAP
P.os_lacA       IRANPN----LGSTGFDGGINSAILRYAGATEDDPTTTSSTS-TPLLETNLVPLENPGAP
P.os_pox2       IRANPN----LGSTGFVGGINSAILRYAGATEDDPTTTSSTS-TPLLETNLVPLENPGAP
P.sa_lac4       IRANPN----LGSTGFAGGINSAILRYVGAADADPTTTSSTS-TPLLETNLVPLENPGAP
P.pu_lac2       IRANPN----LGSTGFAGGINSAILRYVGAADADPTTTSSTS-TPLLETNLVPLENPGAP
P.sp_lac1       IRANPN----LGSTGFAGGINSAILRYAGATEDDPTTTSSTS-TPLLETNLVPLENPGAP
P.sa_lac1       IRANPN----LGSTGFDGGINSAILRYAGATEDDPTTTSATS-TPLEETNLVPLENPGAP
P.os_lacB       IRADPN----LGSTGFDGDINSAILRYAGATEDDPTTTSSTS-TPLEETNLVPPENPGAP
P.er_Ery3       IRANPN----LGSTGFEGGINSAILRYAGATENDPATTSSTS-TPLLETNLVPLENPGAP
P.sa_lac2       IRAEPN----LGTVGFAGGINSAILRYILAPNADPTTTAPASPVQLAETDLVPLSNPGAP
P.sa_lac5       IRANPN----LGTRGFEGGLNSAILRYYGAPNRDPTTTQETSVNPLVETNLVPLRDAGAP
P.os_pox3       IRAPLTGGNPAGNPNLDISLIRAILRYKGAPAVEPTTVATTGGHKLNDAEMHPIAQE-GP
P.sp_lac2       IRAPLTGGNPTGNPNLDVSLIRAILRYKGAPAVEPTTVATTDGHKLNDADMHPIAQE-GP
P.sa_lac3       IRAPLTGGNPAGNPNLDVSLIRAILRYKGAPAVEPTSVATTEGHKLNDADMHPIAQE-GP
P.pu_lac6       IRAPLTGGNPAGNPNLDVSLIRAILRYKGAPAVEPTSVATTGGHKLNDADMHPIAQE-GP
                ***  .     *  .:   :  *****  *   :*::        * : :: *  .  .*
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P.os_poxA       GKPFPGGADHNINLNFAFDPATALFTANNHTFVPPTVPVLLQILSGTRDAHDLAPAGSIY
P.er_Ery4       GKPFPGGADHNINLNFAFDPATVLFTANNYTFVPPTVPVLLQILSGTRDAHDLAPAGSIY
P.os_lccK       GPAVPGGADININLAMAFDFTNFELTINGVPFIPPTAPVLLQILSGASSAASLLPSGSIY
P.os_lacA       GPTVPGGADININLAMAFDFTTFELTINGVPFIPPTAPVLLQILSGASSAASLLPSGSIY
P.os_pox2       GPPVPGGADININLAMAFDFTTFELTINGVPFLPPTAPVLLQILSGASTAASLLPSGSIY
P.sa_lac4       GPAVPGGADININLAMAFDFTTFELTINGVPFHPPTAPVLLQILSGASSAASLLPSGSIY
P.pu_lac2       GPAVPGGADININLAMAFDFTTFELTINGVPFHPPTAPVLLQILSGASSAASLLPSGSIY
P.sp_lac1       GPAVPGGADININPAMAFDFTNFELTINGAPFHAPTAPVLLQILSGATTAASLLPSGSIY
P.sa_lac1       GPAVPGGADININLAMAFDFTNFELTINGSPFQAPTAPVLLQILSGATDAASLLPSGSIY
P.os_lacB       GPAVPGGADININLAMAFDVTNFELTINGSPFKAPTAPVLLQILSGATTAASLLPSGSIY
P.er_Ery3       GPAVPGGADININLAMGFDFTNFEMTINGSPFKAPTAPVLLQILSGATPAASLLPSGSIY
P.sa_lac2       GPPVPGGADVNINLAMNFDFTNFELIINGQAFHPPTAPVLLQILSGATTAASLLPAGSIY
P.sa_lac5       GVPGVGNADVNINLAMAFDFPSLQFTINGAPWVSPTSPVLLQIISGASSASELLPPGSIY
P.os_pox3       GNLGTGPPDMAITLNIAQPNP-PFFDINGISYLSPSVPVLLQMLSGARKPQDFLPSEQVI
P.sp_lac2       GNLGTGPPDMAITLNIAQPNP-PFFDINGISYLSPSVPVLLQMLSGARKPQDFLPSEQVI
P.sa_lac3       GNLGTGPPDMAITLNIAQPNP-PFFDINGISYLSPSVPVLLQMLSGARKPQDFLPSEQVI
P.pu_lac6       GNLGTGPPDMAITLNIAQPNP-PFFDINGISYLSPSVPVLLQMLSGARKPQDFLPSEQVI
                *    * .*  *.  :    .   :  *. .: .*: *****::**:  . .: *. .:

                                T1  T2 T3
P.os_poxA       DIKLGDVVEITMPALVFAGPHPIHLHGHTFAVVRSAGSSTYNYENPVRRDVVSIGDDPTD
P.er_Ery4       DIKLGDVVEVTMPALVFAGPHPMHLHGHSFAVVRSAGSSTYNYENPVRRDVVSIGDDPTD
P.os_lccK       ALEPNKVVEISMPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTGAND
P.os_lacA       ALEPNKVVEISMPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTGAND
P.os_pox2       ELEANKVVEISMPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTGAND
P.sa_lac4       ELAPNKVVEISMPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTGAND
P.pu_lac2       ELAPNKVVEISMPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTGAND
P.sp_lac1       ELEANKVVEISIPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTGAND
P.sa_lac1       APEANKVVEISIPALAVGGPHPLHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTDAND
P.os_lacB       SLEASKVVEISIPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGTDAND
P.er_Ery3       ALEANKVVEISIPALAVGGPHPFHLHGHTFDVIRSAGSTTYNFDTPARRDVVNTGIDAND
P.sa_lac2       ELPPNKVIELSIPALAIGGPHPFHLHGHTFDVIRSAGMTTYNFDTPARRDVVNTGVGAED
P.sa_lac5       TLPRNKVVELSIPALAIGGPHPIHLHGHAFDVVRSAGSSTYNYNNPVRRDVVSTGTGS-D
P.os_pox3       ILPANKLIEVSIPG---AGAHPFHLHGHTFDIVRTSNSDVVNLVNPPRRDVLPI---NGG
P.sp_lac2       ILPANKLIEVSIPG---AGAHPFHLHGHTFDIVRTSNSDVVNLVNPPRRDVLPI---NGG
P.sa_lac3       ILPANKLIEVSIPG---AGAHPFHLHGHTFDIVRVSNSDVVNLVNPPRRDVLPI---NGG
P.pu_lac6       ILPANKLIEVSIPG---AGAHPFHLHGHTFDIVRVSNSDVVNLVNPPRRDVLPI---NGG
                    ..::*:::*.   .*.**:*****:* ::* :.  . *  .* ****:      .
                                 L3

                          T3T1T3  T1     T1
P.os_poxA       NVTIRFVADNAGPWFLHCHIDWHLDLGFAVVFAEGVNQTAAANPVP---EAWNNLCPIYN
P.er_Er44       NVTIRFVADNAGPWFLHCHIDWHLDLGFAVVFAEGVNQTAVANPVP---EAWNDLCPIYN
P.os_lccK       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIS--APP----AAWDDLCPIYD
P.os_lacA       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIS--APP----AAWDDLCPIYD
P.os_pox2       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIS--APP----AAWDDLCPIYN
P.sa_lac4       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIS--APP----AAWDALCPIYD
P.pu_lac2       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIS--APP----AAWDALCPIYD
P.sp_lac1       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIS--APP----AAWDDLCPIYD
P.sa_lac1       NVTIRFTTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIA--APP----AAWDDLCPIYD
P.os_lacB       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVTSIT--APP----AAWDDLCPIYD
P.er_Ery3       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAVVFAEDVASIK--APPAAW-AAWDDLCPIYD
P.sa_lac2       NVTIRFVTDNPGPWFLHCHIDWHLEIGLAIVFAEDAPIVNTENPP----SSWDDLCPIYD
P.sa_lac5       NVTIRFKTDNPGPWFLHCHIDWHLEIGLAVVFAEDTASTSKQRPP----KSWSDLCPIYD
P.os_pox3       NTTFRFFSGNSGAWFLHCHIDWHLEAGLAVVFAERPAEVNEGEQAQIVTQDWRTLCPAYD
P.sp_lac2       NTTFRFFSGNSGAWFLHCHIDWHLEAGLAVVFAERPAEVNDGEQAQIVTQDWRTLCPAYD
P.sa_lac3       NTTFRFFSGNSGAWFLHCHIDWHLEAGLAVVFAERPAEVNEGEQAQIVTQDWRTLCPAYD
P.pu_lac6       NTTFRFFSGNSGAWFLHCHIDWHLEAGLAVVFAERPAEVNEGEQAQIVTQDWRTLCPAYD
                *.*:** :.*.*.**********: *:*:**** *  *** *:
                                L4
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P.os_poxA       SSNPSKLLMGTNAIGRLPAPLKA
P.er_Ery4       SSNPSKLLMGTNAIGRLHAPLKA
P.os_lccK       A------LSDNDKGGIVPS----
P.os_lacA       A------LSDNDKGGIVPS----
P.os_pox2       A------LSDNDKGGIVPS----
P.sa_lac4       A------LSDADKGGIIP-----
P.pu_lac2       A------LSDTDKGGIIP-----
P.sp_lac1       A------LSDSDKGGIV------
P.sa_lac1       A------LSDTDKGGIA------
P.os_lacB       A------LSDSDKGGIA------
P.er_Ery3       A------LSDSDRGGIA------
P.sa_lac2       A------LTEEEKGGLLPT----
P.sa_lac5       S------LTEEQKGGIV------
P.os_pox3       G------LAPEFQ----------
P.sp_lac2       G------LAPEFQ----------
P.sa_lac3       G------LAPEFQ----------
P.pu_lac6       G------LAPEFQ----------
                .      *
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Fig. 7. Alignment of laccase sequences of *Pleurotus* species available in the GenBank Database. Sequence regions, L1–L4, used to identify the laccases, are indicated (Kumar et al., 2003). Conserved residues ligating with the copper centers (Solomon et al., 1996). type 1 (T1), type 2 (T2) and type 3 (T3) are indicated in grey. A description of protein sequences used for multi-alignment is reported in Table 2.

Fig. 8 The translation product of the *P. eryngii ERY3* cDNA (EMBL accession number AM773999). The end of the signal peptide and beginning of the mature protein is indicated (⇩). His and Cys residues predicted to be involved in the binding of copper are indicated in white with a black background. Asterisks (*) indicate Cys residues predicted to be involved in the formation of disulphide bridges. Potential N-glycosylation sites (N-X-S/T) are indicated (#)

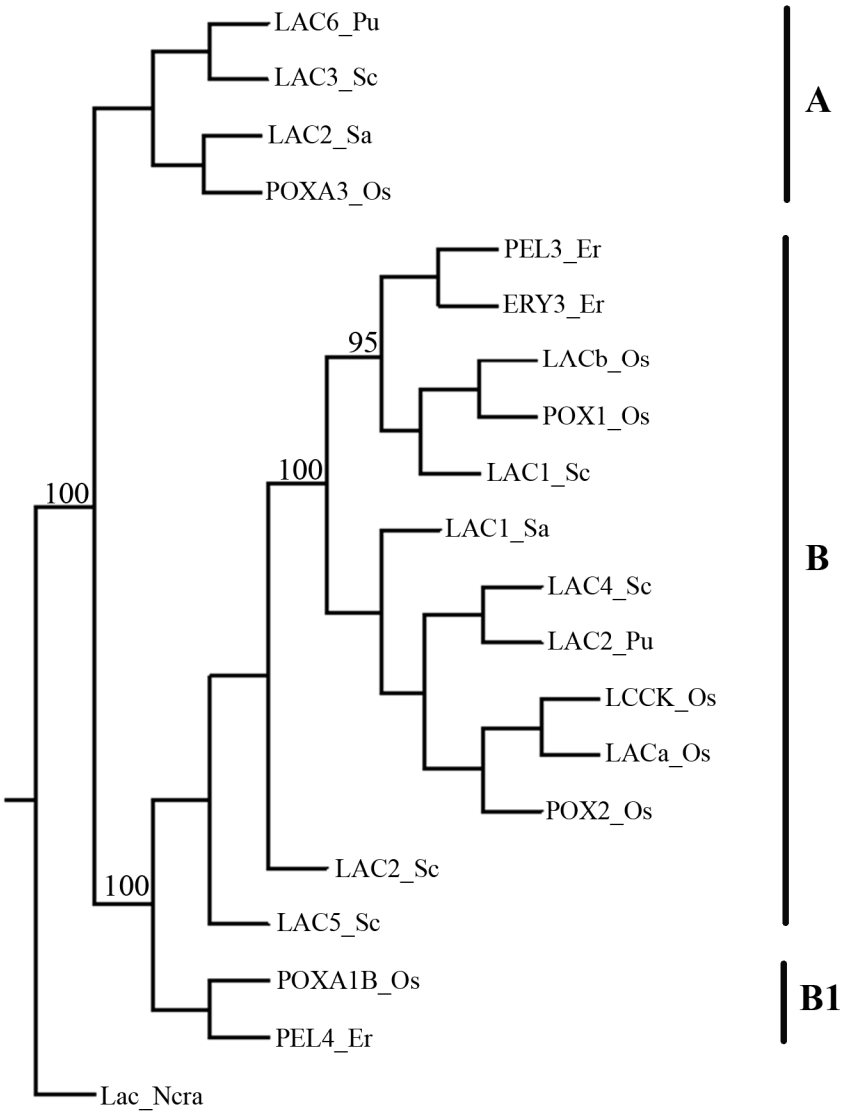

Fig. 9 Clustering dendrogram of the Ery3 protein from *P. eryngii* and other laccases from *Pleurotus* spp. *N. crassa* laccase precursor was used as outgroup sequence. Bootstrap scores for a total of 100 replicates are noted on the relevant horizontal branches. Sequence grouping (A, B and B1) is reported.

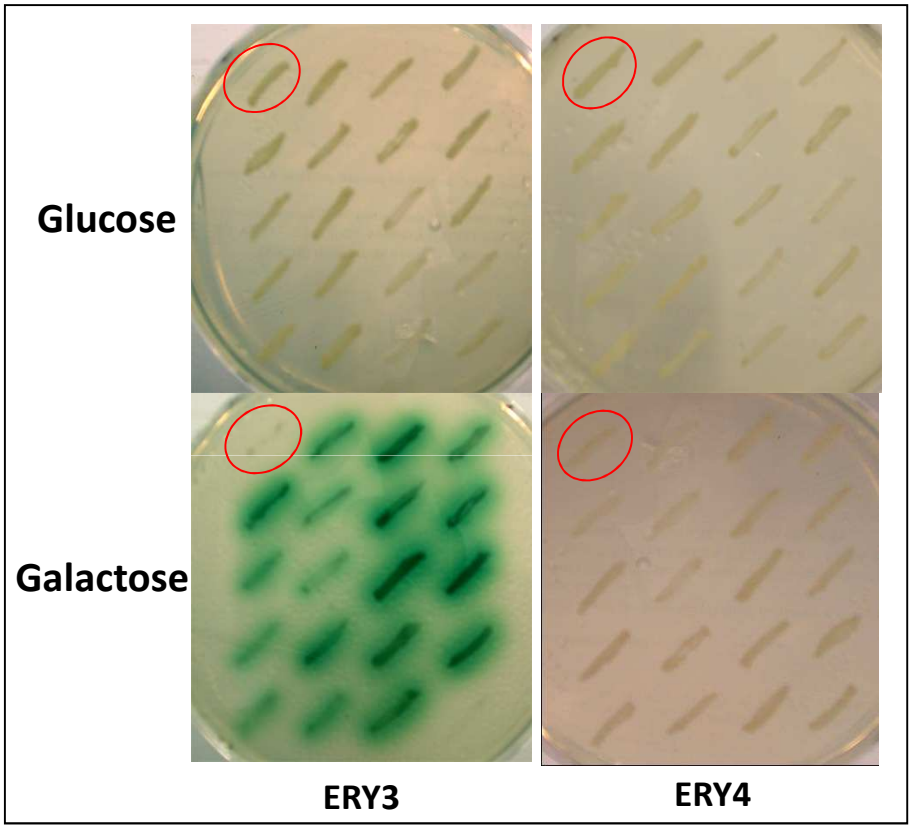

Fig. 10. Laccase enzymatic assay on plate. Recombinant CEN.PK2-1C clones, separately transformed with the pY-*ERY3* and pY-*ERY4* vectors, were grown on minimal medium with glucose added as carbon source. The same clones were spotted onto galactose medium in the presence of ABTS as laccase-specific substrate for a direct colorimetric reaction after inducing the expression of *ERY3* and *ERY4* genes. The pYES2 vector was used as control (red ring).

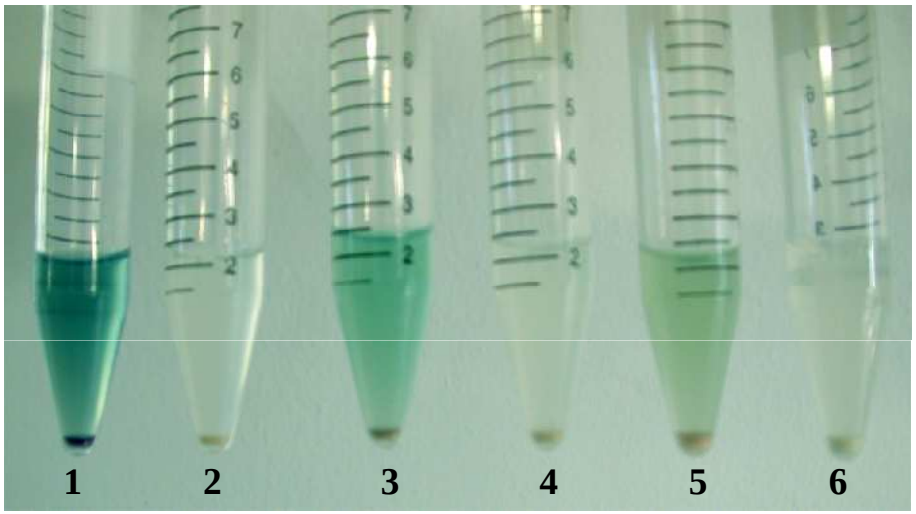

Fig.11. Laccase enzymatic assay in liquid medium. Three different recombinant CEN.PK2-1C clones, transformed with the *pY-ERY3* vectors, were grown on minimal medium with galactose added as carbon source in presence of ABTS, as laccase-specific substrate for a direct colorimetric reaction, after inducing the expression of *ERY3* gene (odd numbers). The pYES2 vector was used as control (even numbers).

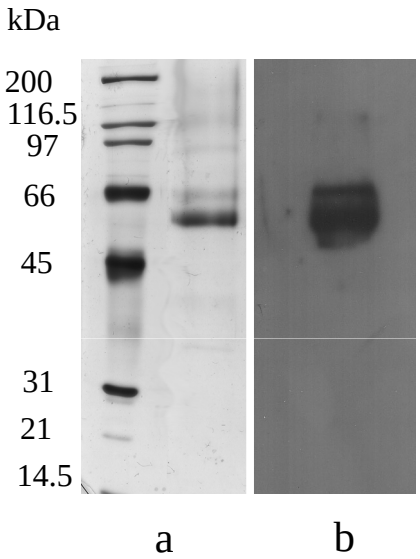

Fig. 12. SDS-PAGE gel (**a**) and Western blot (**b**) analyses of proteins secreted by *S. cerevisiae* cells transformed with pY-*ERY3*. Molecular weight marker in kDa is indicated on the left of the figure.

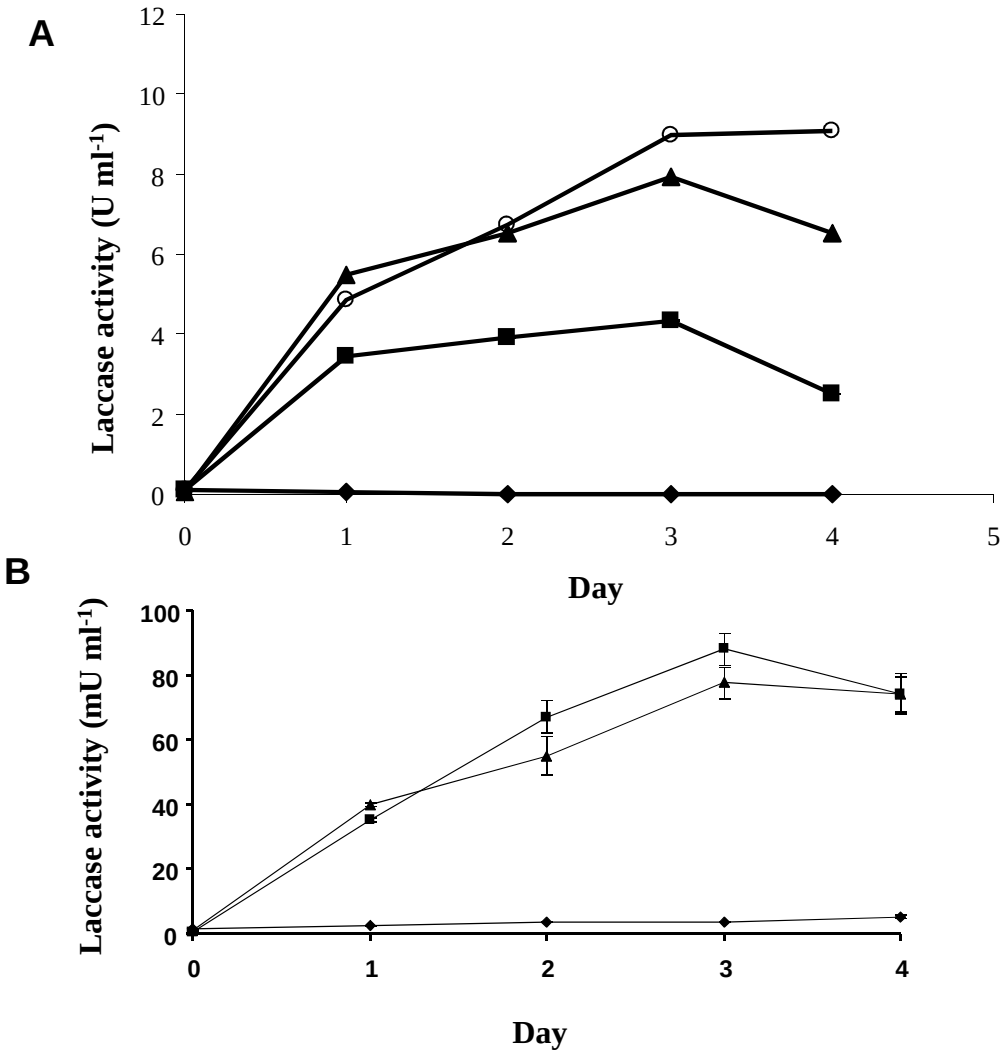

Fig. 13. A. Laccase activity level of transformant pY-*ERY3* incubated at 30°C in BMMY medium at different copper concentrations. The $CuSO_4$ concentrations evaluated were 0 mM (♦), 0.1 mM (▪), 0.25 mM (▲), and 0.5 mM (○). **B.** The effect of temperature on the production of laccase from yeast cells expressing the *P. eryngii ERY3* gene. Yeast were grown in SM-Ura medium [supplemented with 2% (w/v) galactose, 0.5 mM $CuSO_4$]. The temperatures evaluated were 18°C (■), 25°C (▲), and 30°C (♦).

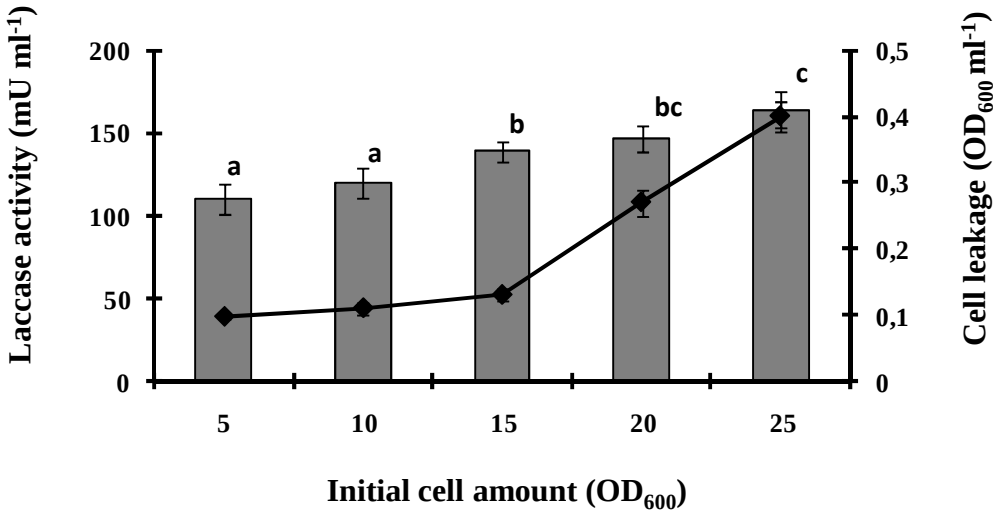

Fig. 14. Effect of initial cell amount on the production of laccase and on cell leakage of yeast cells expressing the *P. eryngii ERY3* gene, immobilised in gel beads prepared with 3% Na-alginate and 0.1 M $CaCl_2$. Beads were incubated at 18°C in SM-Ura medium [supplemented with 2% (w/v) galactose, 0.05% (w/v) $CaCl_2$, 0.5 mM $CuSO_4$]. The amounts of cells immobilized on Ca-alginate beads are expressed as the total cell inoculum corresponding to 5, 10, 15, 20 and 25 OD_{600} (1 OD_{600} = 3 x 10^7 cells ml^{-1}). Cell leakage is also reported (—♦—). Letters on the top of each bar indicate the results of the Bonferroni/Dunn test ($P<0.05$); values with shared letters in the same graph are not significantly different.

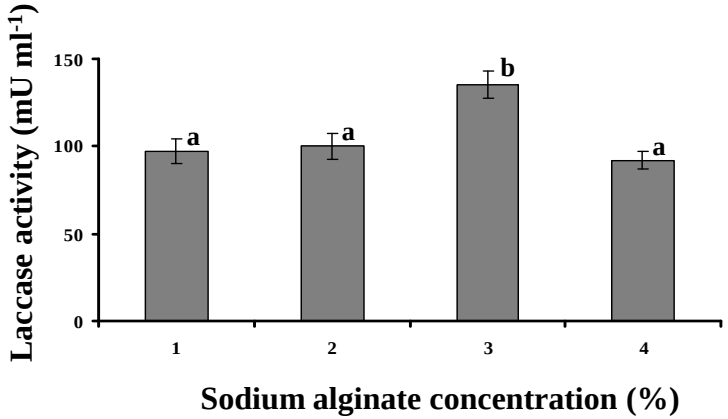

Fig. 15. Effect of sodium alginate concentration on the production of laccase from yeast cells expressing the *P. eryngii ERY3* gene, immobilised in Ca-alginate gel beads. The initial cell amount immobilized in Ca-alginate corresponds to 15 OD_{600} (1 OD_{600} = 3 x 10^7 cells ml^{-1}). Beads were incubated as described in Fig. 3. Letters on the top of each bar indicate the results of the Bonferroni/Dunn test ($P<0.05$); values with shared letters in the same graph are not significantly different.

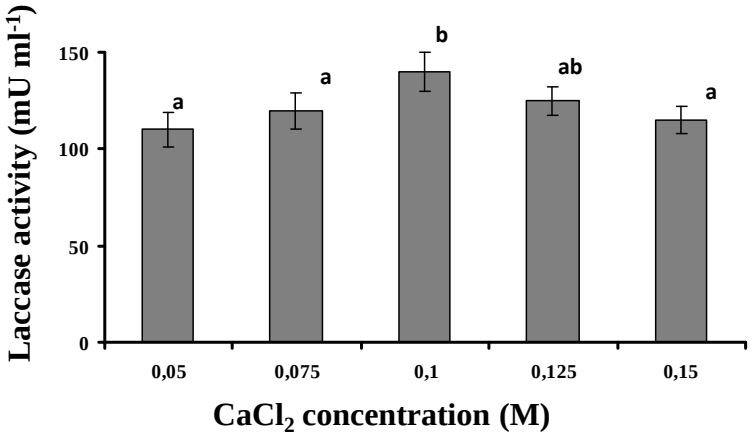

Fig. 16. Effect of $CaCl_2$ concentration on the production of laccase from yeast cells expressing the *P. eryngii ERY3* gene, immobilised in Ca-alginate gel beads. Beads were prepared and incubated as described in Fig. 4. Letters on the top of each bar indicate the results of the Bonferroni/Dunn test ($P<0.05$); values with shared letters in the same graph are not significantly different.

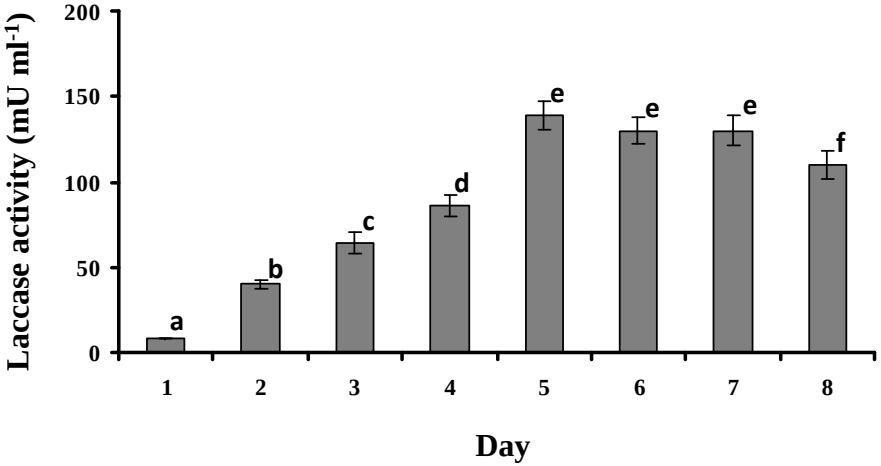

Fig. 17. Time course of laccase production from yeast cells expressing the *P. eryngii ERY3* gene, immobilised in Ca-alginate gel beads. Beads were prepared and incubated as described in Fig. 4. Letters on the top of each bar indicate the results of the Bonferroni/Dunn test (P<0.05); values with shared letters in the same graph are not significantly different.

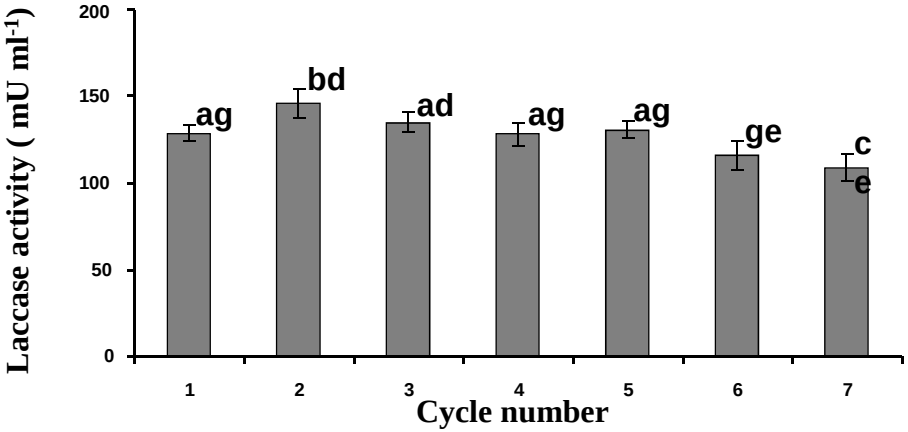

Fig. 18. Cycles of batch fermentations of yeast cells expressing the *P. eryngii ERY3* gene, immobilised in Ca-alginate gel beads. Beads were prepared and incubated as described in Fig. 4. Each cycle was carried out for 5 days in a rotary shaker. At cycle completion, the exhausted medium was removed and the laccase activity assayed. The beads were then collected, washed with sterile saline solution (0.9 % NaCl) and re-incubated for the enzyme production. Letters on the top of each bar indicate the results of the Bonferroni/Dunn test (P<0.05); values with shared letters in the same graph are not significantly different.

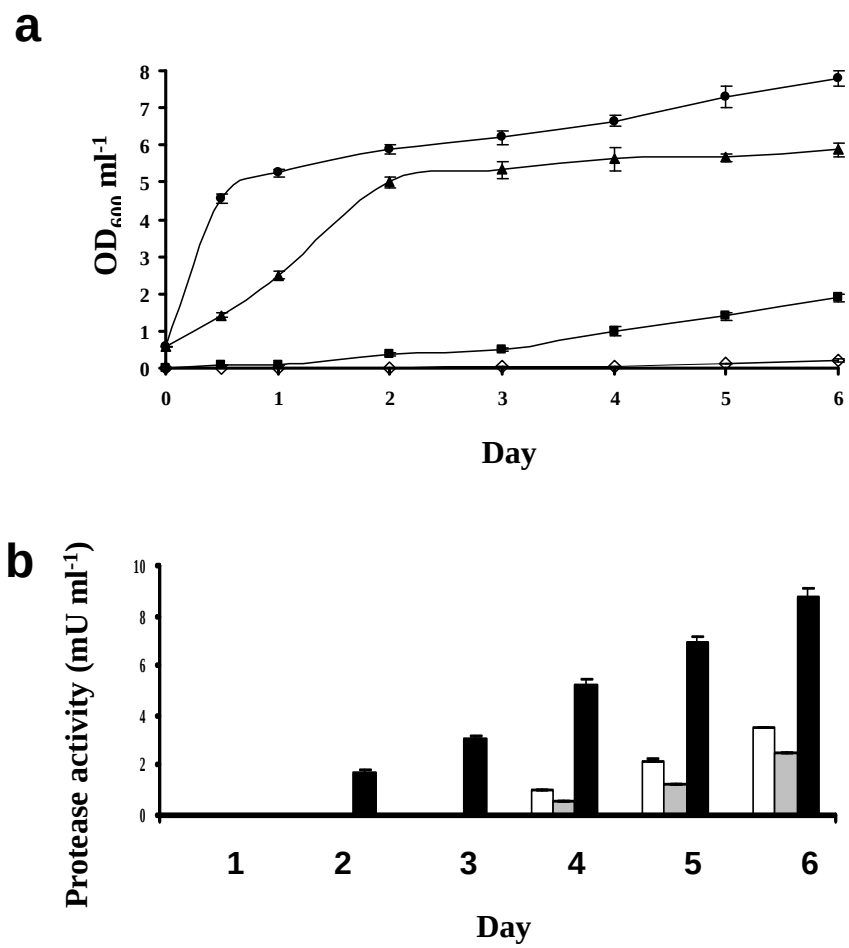

Fig. 19. (**a**) Time courses of cell growth in freely suspended cultures grown at 30 (—●—) or 18°C (—▲—) and cell leakage from alginate beads incubated at 30 (—■—) or 18°C (—◊—); (**b**) protease activity detected in the culture medium of free cell grown at 30 (black column) or 18°C (grey column) and of immobilized cells incubated at 30 (white column) or 18°C (dashed column).

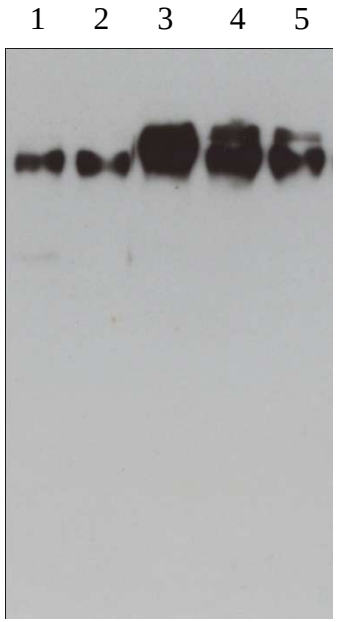

Fig. 20. Western blot analysis of proteins secreted by *S. cerevisiae* cells transformed with pY-*ERY3* (Lane 1), pY-*ERY4* (Lane 2), pY-*5LESS* (Lane 3), pY-*4NC3* (Lane 4) and pY-*K532E* (Lane 5), performing using anti-laccase antibodies raised against the *Pycnoporus cinnabarinus* laccase.

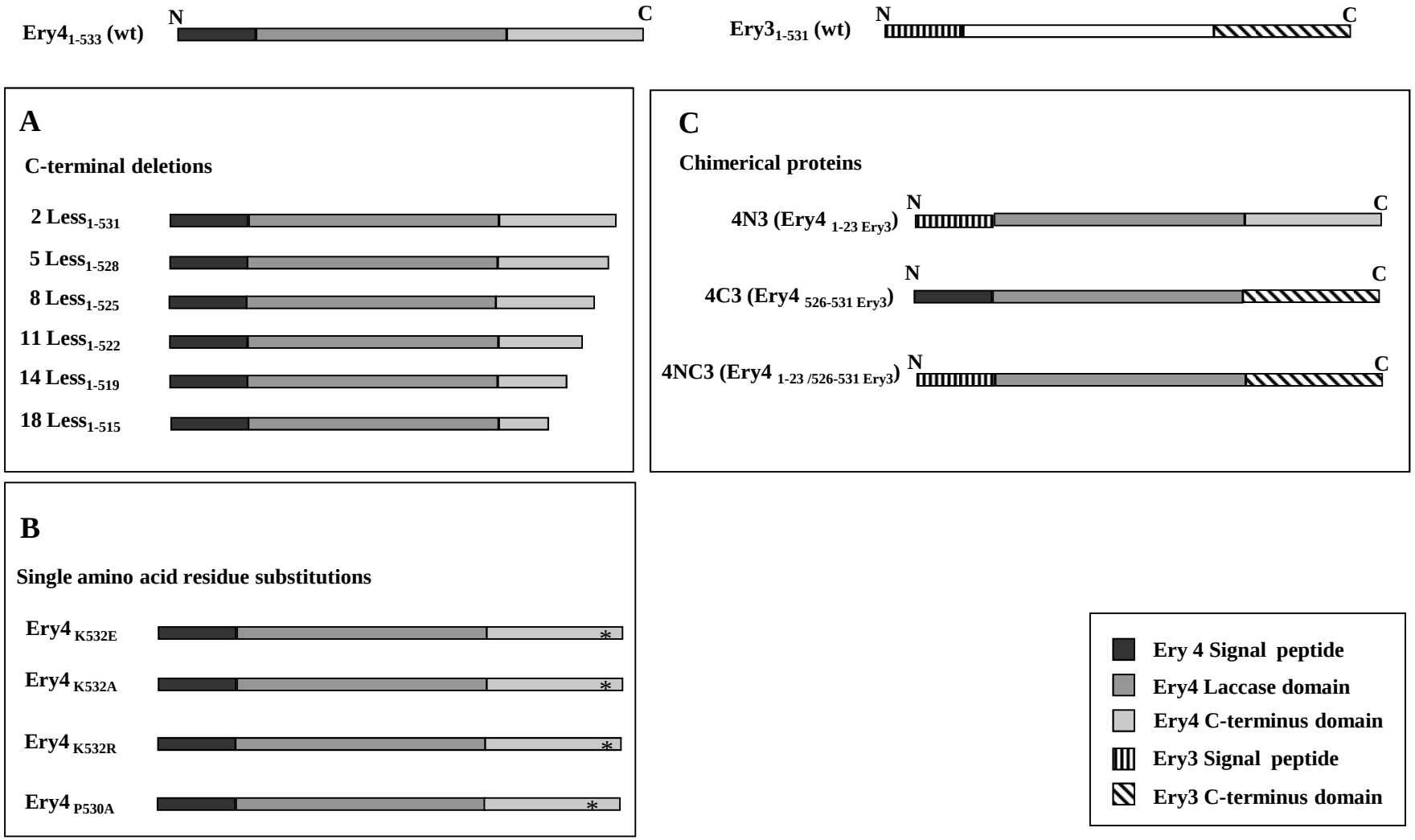

Fig. 21. Summary of Ery4 and Ery3 laccases C-terminal deletions, site-directed substitutions and chimerical manipulations. Wild type and mutant laccases were shown as N-terminal, laccase central domain and C-terminal boxes. Schematic representation of (A) Ery4 C-terminal truncation mutants; (B) single amino-acid substitutions; (C) chimerical proteins. The residue lengths, the single point mutations and the N- and C-terminal stretches of Ery3 and Ery4 were reported.

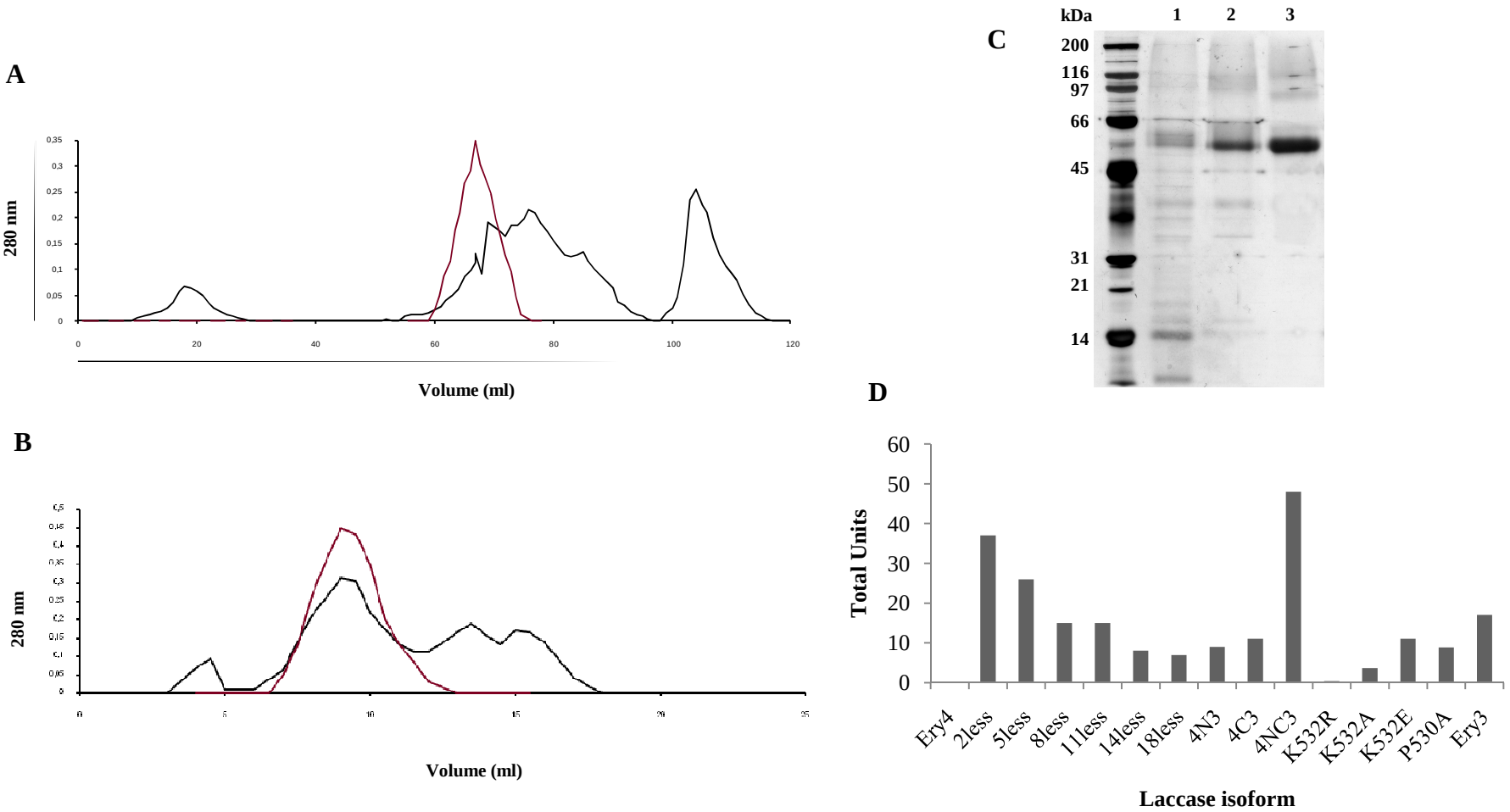

Fig. 22. Outcome of the procedures adopted for recombinant laccases purification. (A) Anion exchange chromatographic separation of laccase mutant 5Less isoform using linear NaCl gradient (red line) in a 50 mM Tris/HCl (pH 8). Absorbance at 280 nm (thick solid line), laccase activity (U ml-1) with ABTS as the substrate (blue line) are displayed. (B) Size exclusion chromatography (Sephadex g-75) equilibrated with 50 mM Tris/HCl (pH 8) that contained 50 mM NaCl. Absorbance at 280 nm (thick solid line), laccase activity (U ml^{-1}) with ABTS as the substrate (purple line). (C) SDS-PAGE gel analyses of proteins secreted by i cells transformed with pY-Ery45Less, after filtration (Lane 2), anion exchange chromatography (Lane 3) and size exclusion chromatography (Lane 4). Molecular weight marker in kDa is indicated on the left of the figure. (D) Laccase activity with ABTS of all protein isoforms after purification (total units).

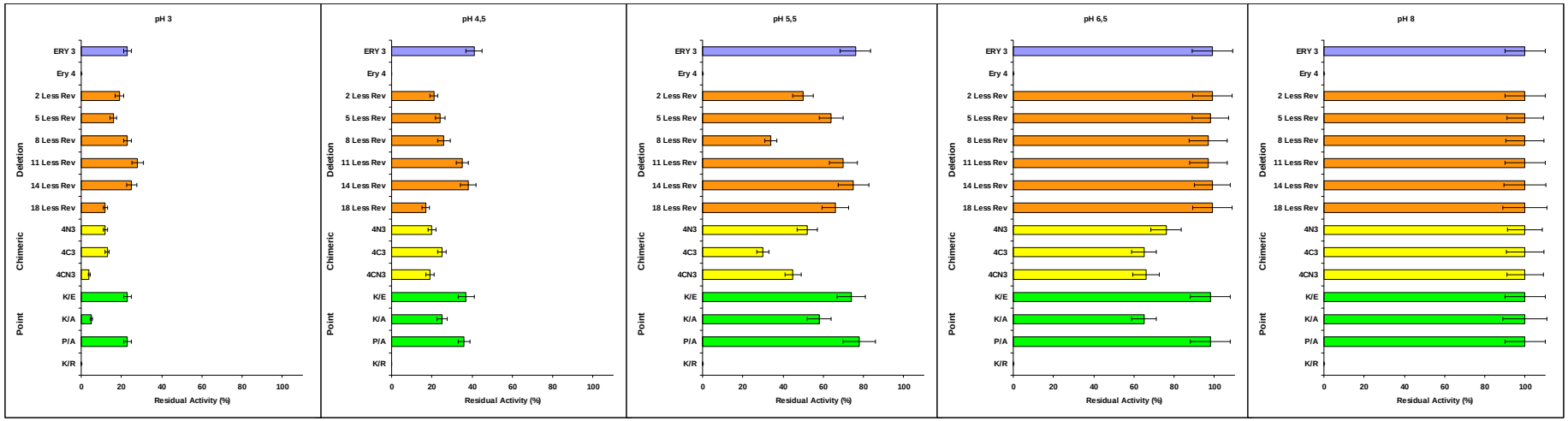

Fig. 23. pH-dependent stability of purified laccase isoforms after 60 h incubation at 25°C in 0.1 M Sodium Citrate buffer pH 3, 0.1 M Sodium Acetate buffer pH 4.5 and pH 5.5, and in 0.1 M Phosphate buffer pH 6.5 respectively. Laccase activity was reported as residual activity (%) using ABTS as substrate

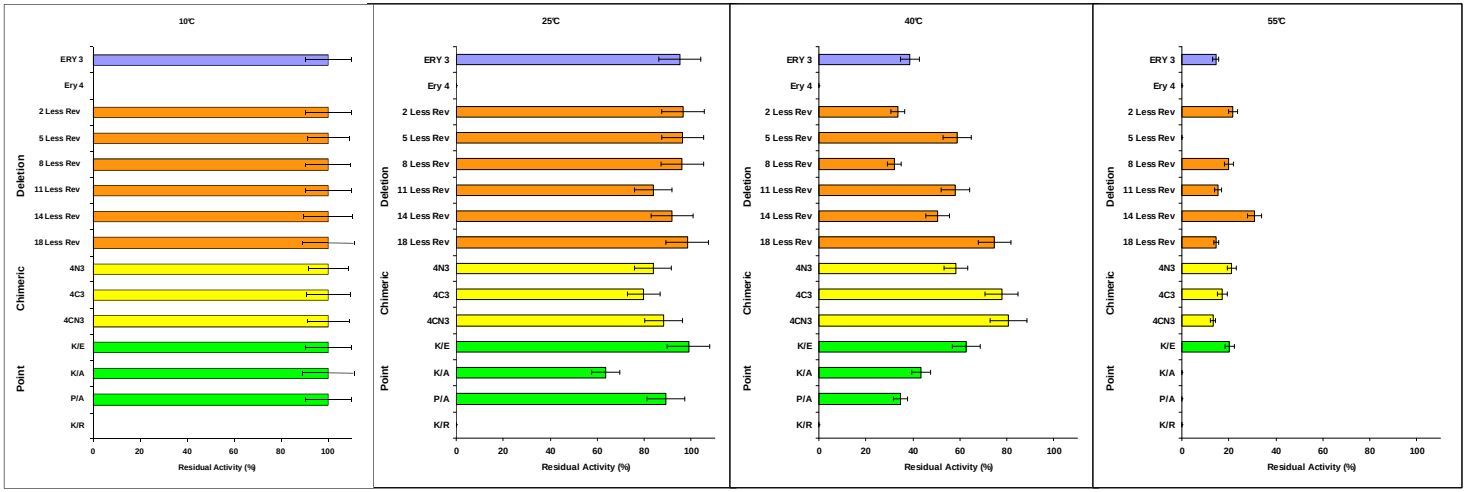

Fig. 24. Thermal stability of purified laccase isoforms after 60 h incubation in 0.1M Phosphate buffer pH 6.5 at 10, 25, 40, 55 and 70°C respectively. Laccase activity was reported as residual activity (%) using ABTS as substrate.

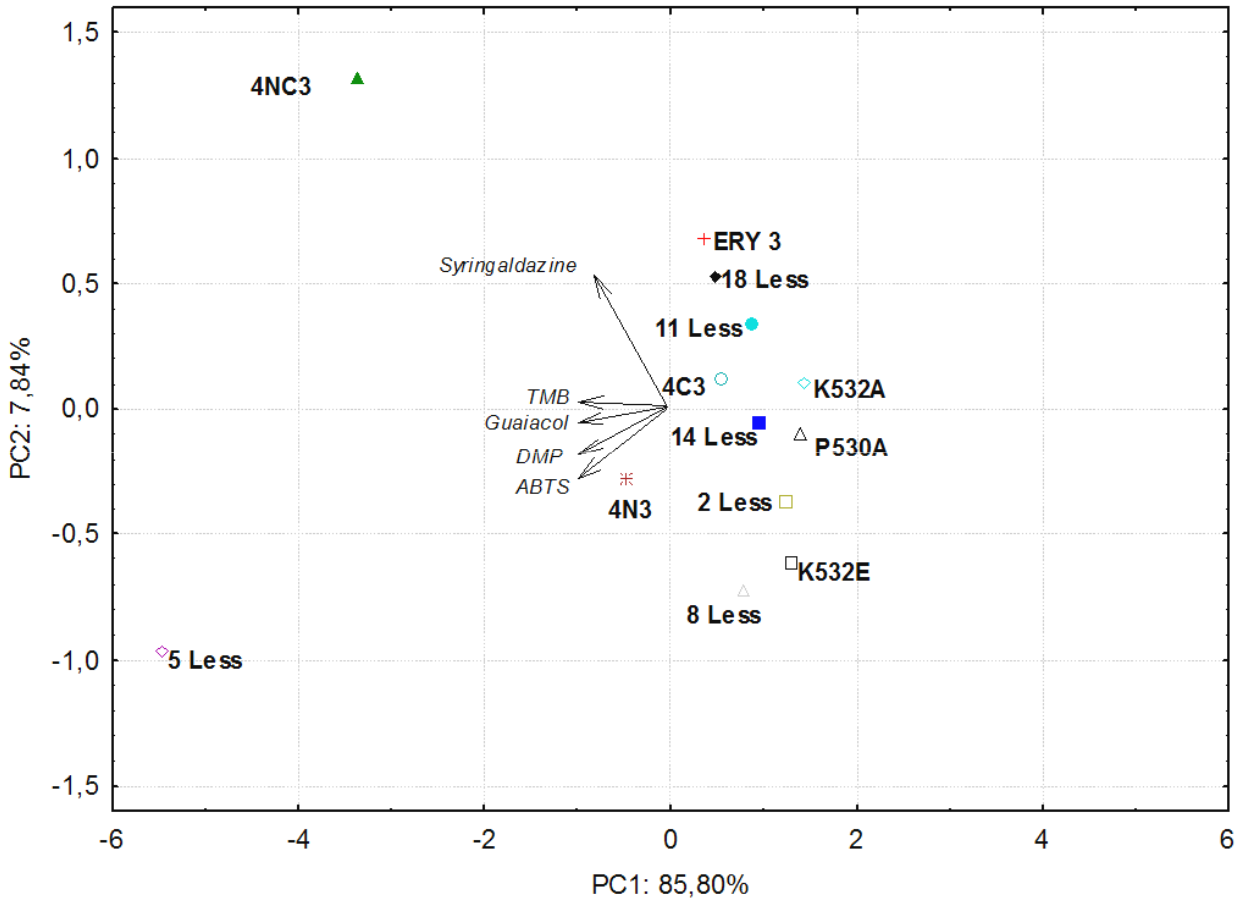

Fig. 25. Principal Component Analysis (PCA) carried out on the complete data matrix reported in Table 6. The figure is bi-plot displaying the sample scores and variable loadings in the planes formed by PC1-PC2

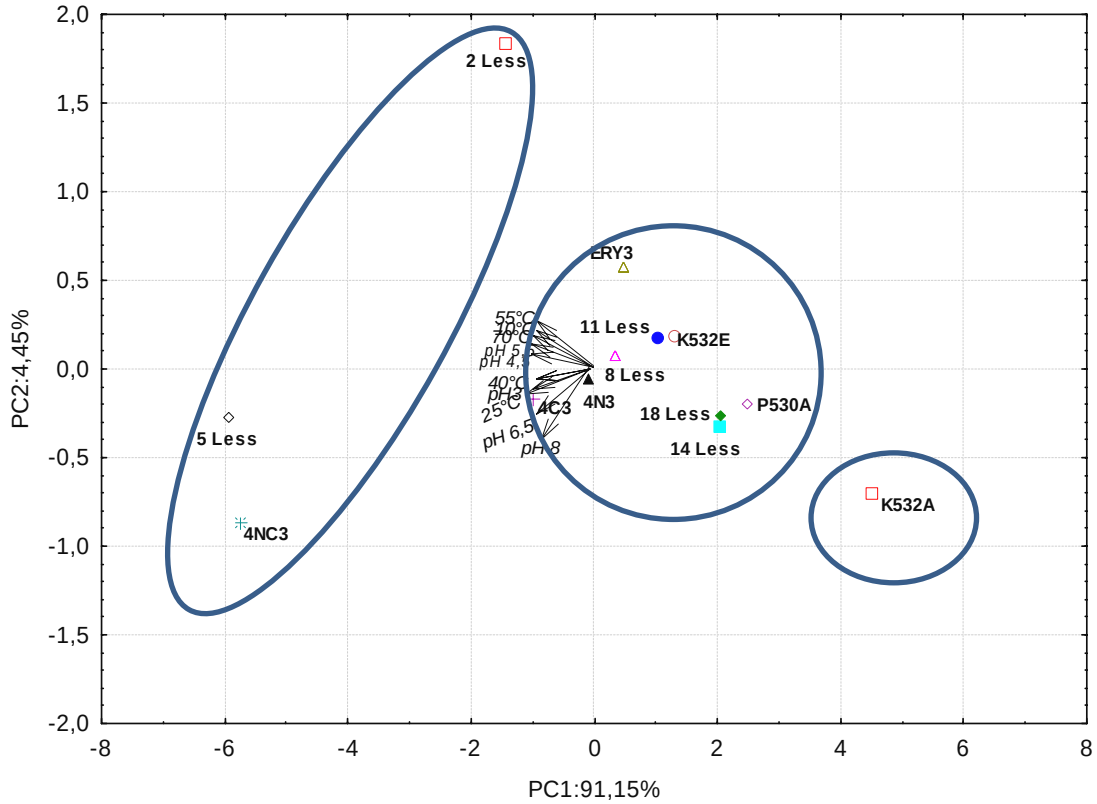

Fig. 26. Score plot of variables (pH, Temperature) and isoform in the plan made of the first two principal components (PC1 against PC2).

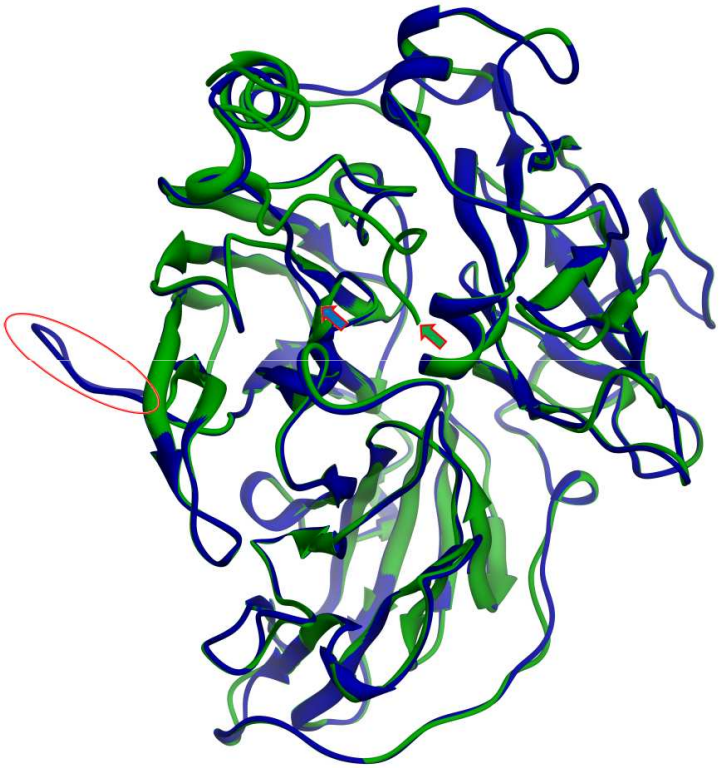

Fig. 27. 3D modelling of Ery4 and Ery3. The superimposition of the 3D models of Ery3 (blue) and Ery4 (green), with a root mean square deviaton (rmsd) equal to 0.41 Å. The superimposition display two main differences: 1) a loop in Ery3 that lacks in Ery4 (red ring); 2) the C-terminus in Ery4 is longer than Eryt3 (arrows).

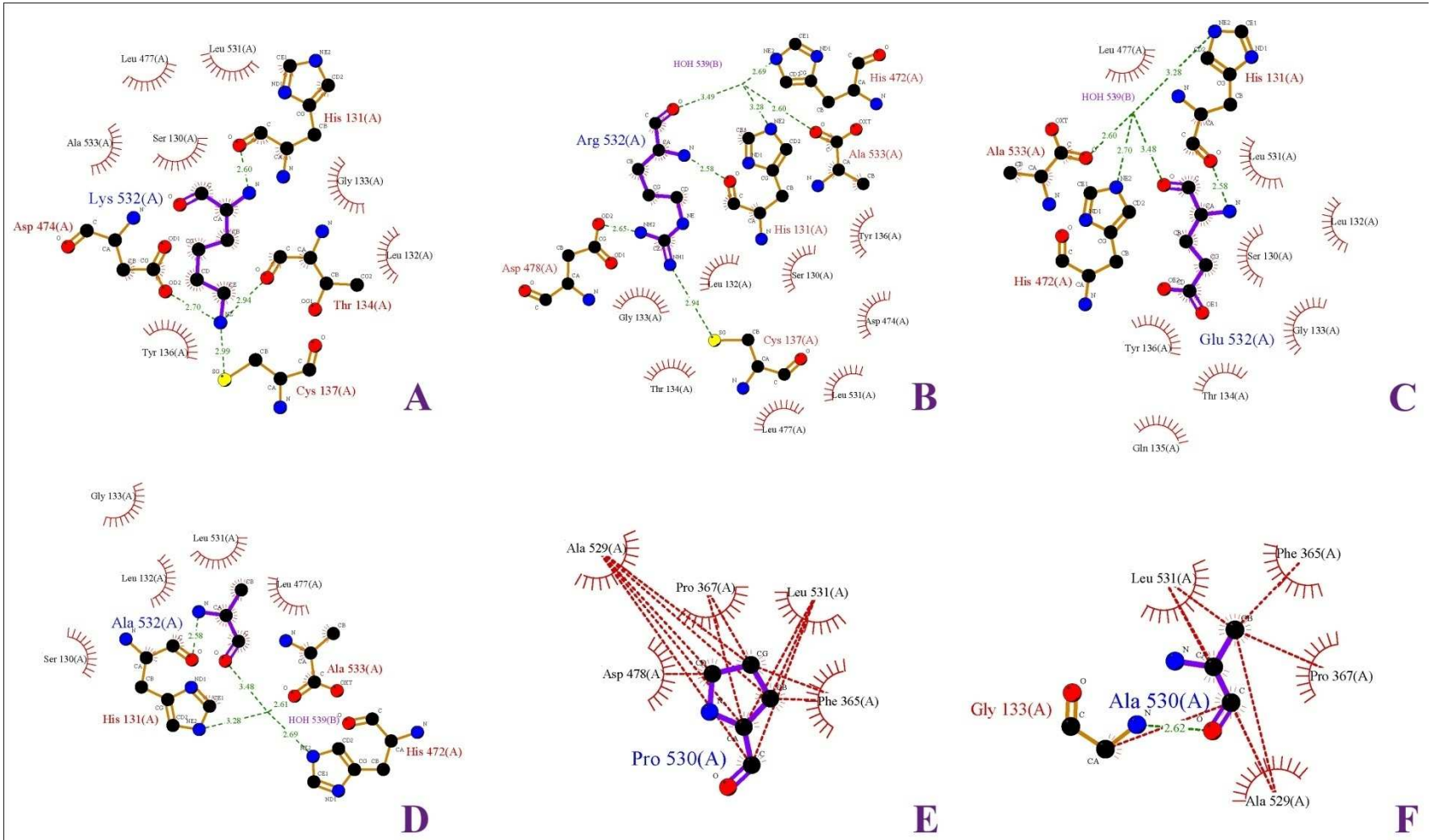

Fig. 28. 3D modelling of ERY4 and point mutated isoforms. A) The wild type K532 residue stabilized by the negative charged residues D474, H131, T134 and C137; B) K532R substitution. R532 residue interaction with the residues H131, C137. There is an increased degree of freedom for the C-terminus portion by charge neutralization due to D478 residue and the absence of interaction with T134 residue. C) K532E substitution. E532 residue has an interaction only with H131. The negative charge is not neutralized, producing a change of C-terminus portion folding and a loss of C-terminus stabilization. D) The K532A substitution. The apolar A532 residue interacts only with H131 residue by few reactivities, giving an improved degree of freedom for the C-terminus portion. E) The wild type P530 aromatic ring stabilized by D478 residue. F) The P530A substitution. The apolar A532 residue produced a more relaxed structure in the C-portion. P530 isoform showed the lost of interaction of the Proline aromatic ring with D478 residue and the new interaction with G133 residue.

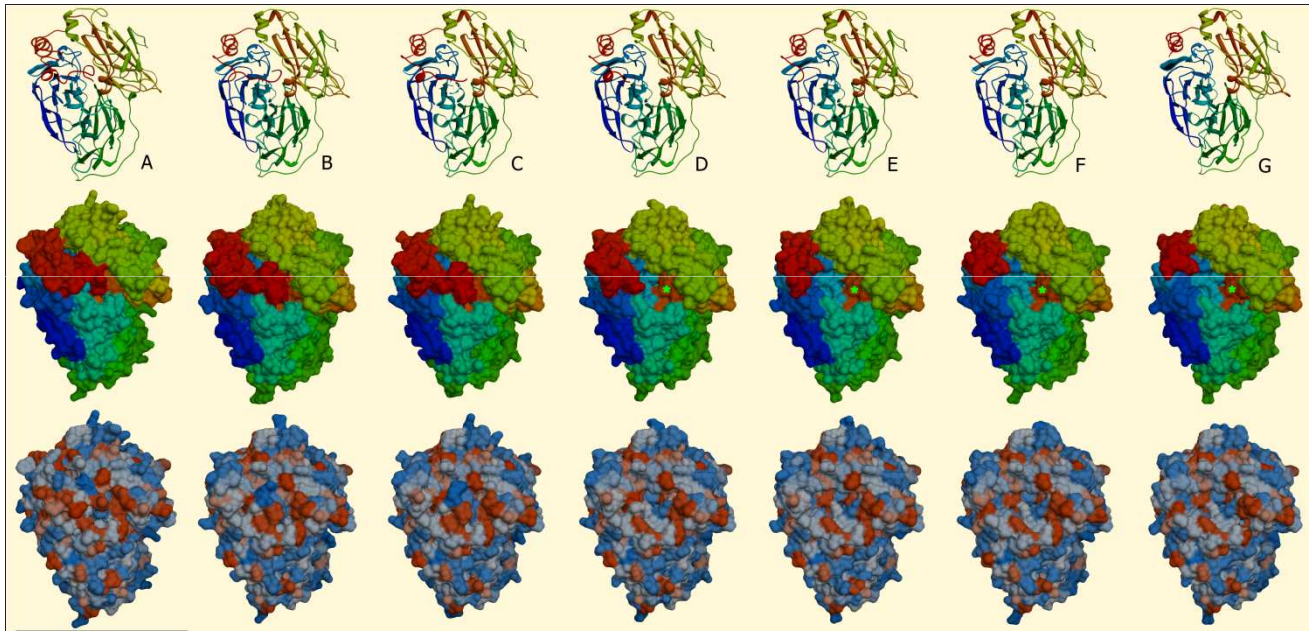

Fig. 29. Cartoon, spacefill and hydrophobic surface representation, of the 3D model of Ery4-WT (A) and the mutant isoform models obtained with progressive deletion of C-terminus portion 2-less (B), 5-less (C), 8-less (D), 11-less (E), 14-less (F), 18-less (G). Cartoon and spacefill are group colored, from N-ter (blue) to C-ter (red). This representation highlights the occupancy of the C-terminus of Ery4-WT (A) and the increased accessibility to the Cu-cluster site (*) due to the C-terminus deleted residues (B-G).

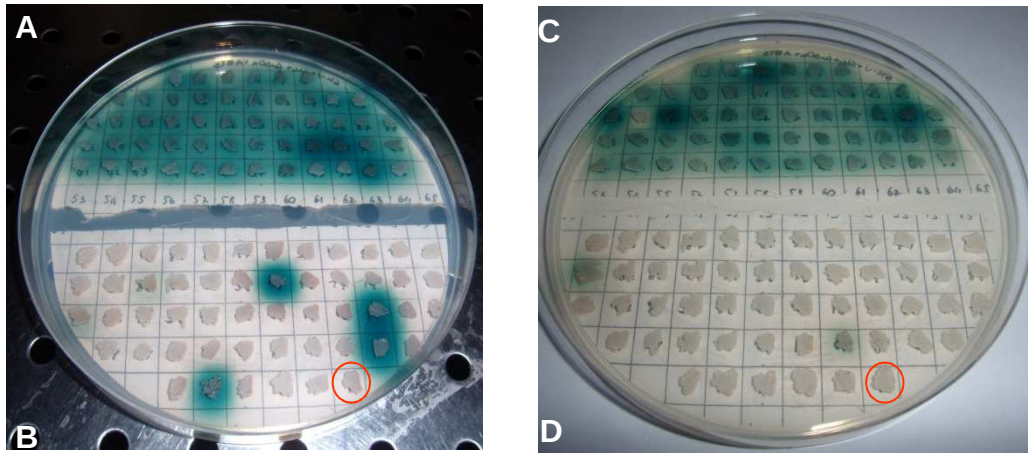

Fig. 30. Laccase enzymatic assay on plate. Recombinant CEN.PK2-1C clones, separately transformed with the vectors, p426-PIR2—5LESS (panel A), p426-FS—5LESS (panel B), p426-PIR2—4NC3 (panel C), p426-FS—4NC3 (panel D) were grown on minimal medium with glucose added as carbon source in the presence of ABTS as laccase-specific substrate for a direct colorimetric reaction. The pYES2 vector was used as control (red ring).

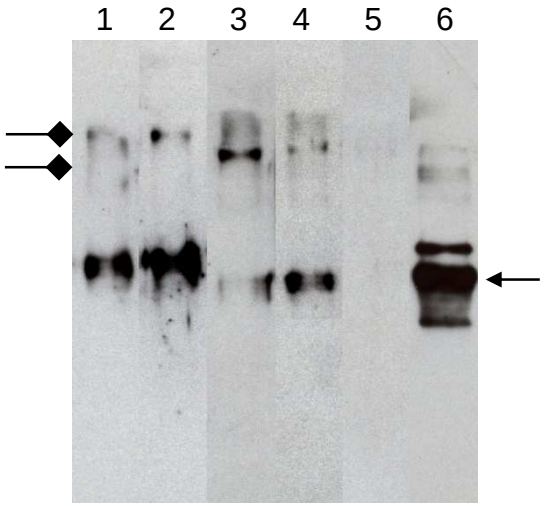

Fig. 31. Western blot analyses of purified proteins from cell wall of yeasts transformed with: FS-5less (Lane 1), FS-4NC3 (Lane 2), PIR2-5less (Lane 3), PIR2-4NC3 construct (Lane 4), with p426-GDP vector (Lane 5); *T. versicolor* laccase as positive control (Lane 6).
(⟶◆) FS-5less, FS-4NC3, PIR2-5less, PIR2-4NC3 constructs. (⟶) *T. versicolor* laccase, performing using antibodies raised against the *Pycnoporus cinnabarinus* laccase.

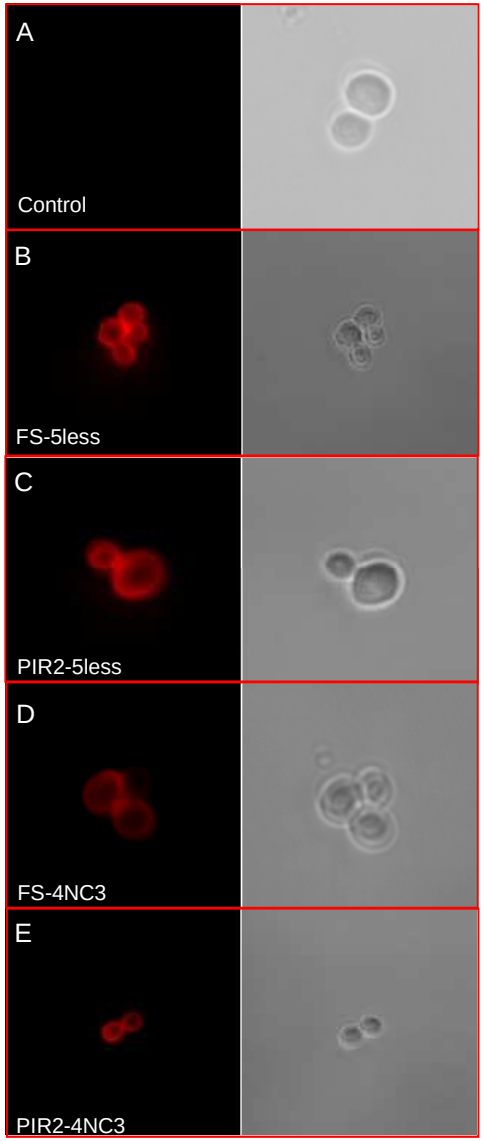

Fig. 32. Confocal laser scanning visualization, after 48 h *GPD1* promoter induction, of yeast cells over-expressing *FS-5LESS* (B), *PIR2-5LESS* (C), *FS-4NC3* (D) and *PIR2-4NC3* (E) genes. Yeast cells were also transformed with the non recombinant vector, as control (A). Cells were probed with anti-laccase serum and fluorescently stained with a goat anti-rabbit IgG conjugated with Alexa Fluor 633.

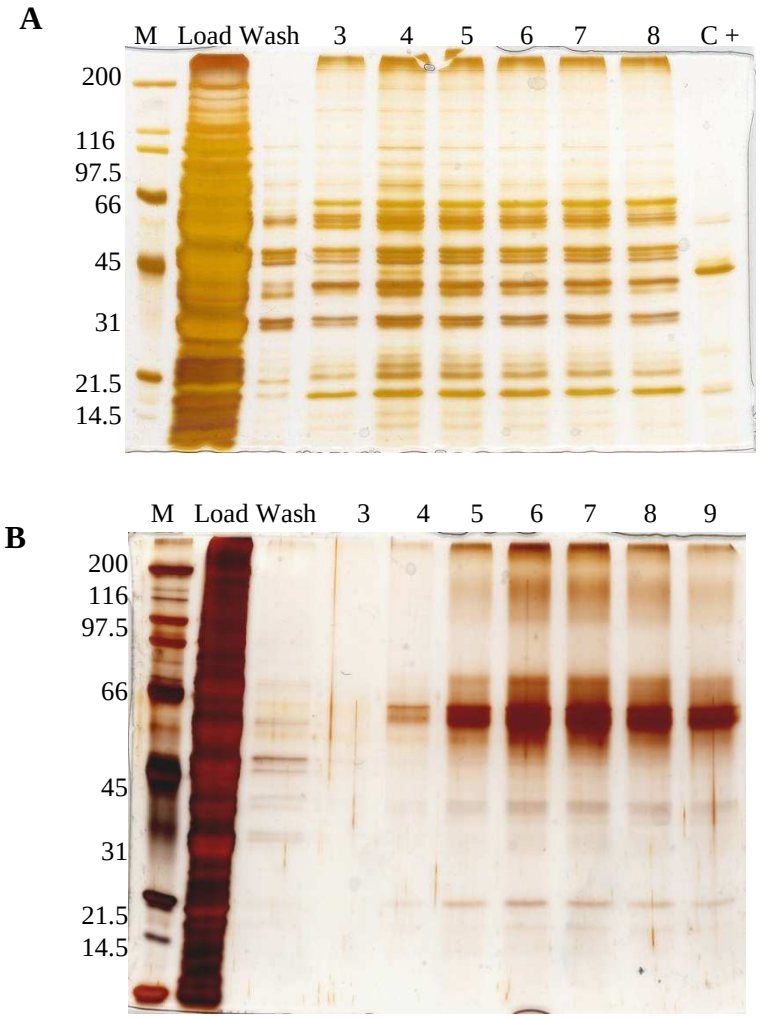

Fig. 33. Analysis of isolated tyrosinase from affinity column using 12% SDS-PAGE. A: Purification profile obtained using a Phosphate buffer containing 0.5 M NaCl. B: Purification profile obtained using a Phosphate buffer containing 0.15 M NaCl.
M: molecular markers (expressed in kDa). Load and Wash: cell lysate before and after flowing through the affinity column, respectively. Lane 3-9: fractions 3, 4, 5, 6, 7, 8 and 9 eluted from the affinity column with elution buffer and collected.

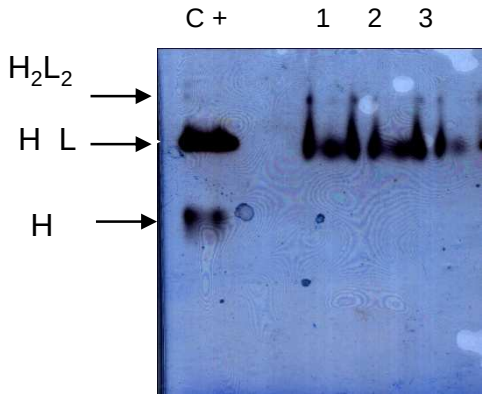

Fig. 34. Identification of tyrosinase activity and tetrameric (H_2L_2) and dimerc (HL) structure of recombinant tyrosinase on polyacrilamide native gel, stained with L-dopa solution. Commercial tyrosinase was used as positive control.
C+: commercial tyrosinase. Lane 1-3: purified tyrosinase fractions eluted from affinity column.

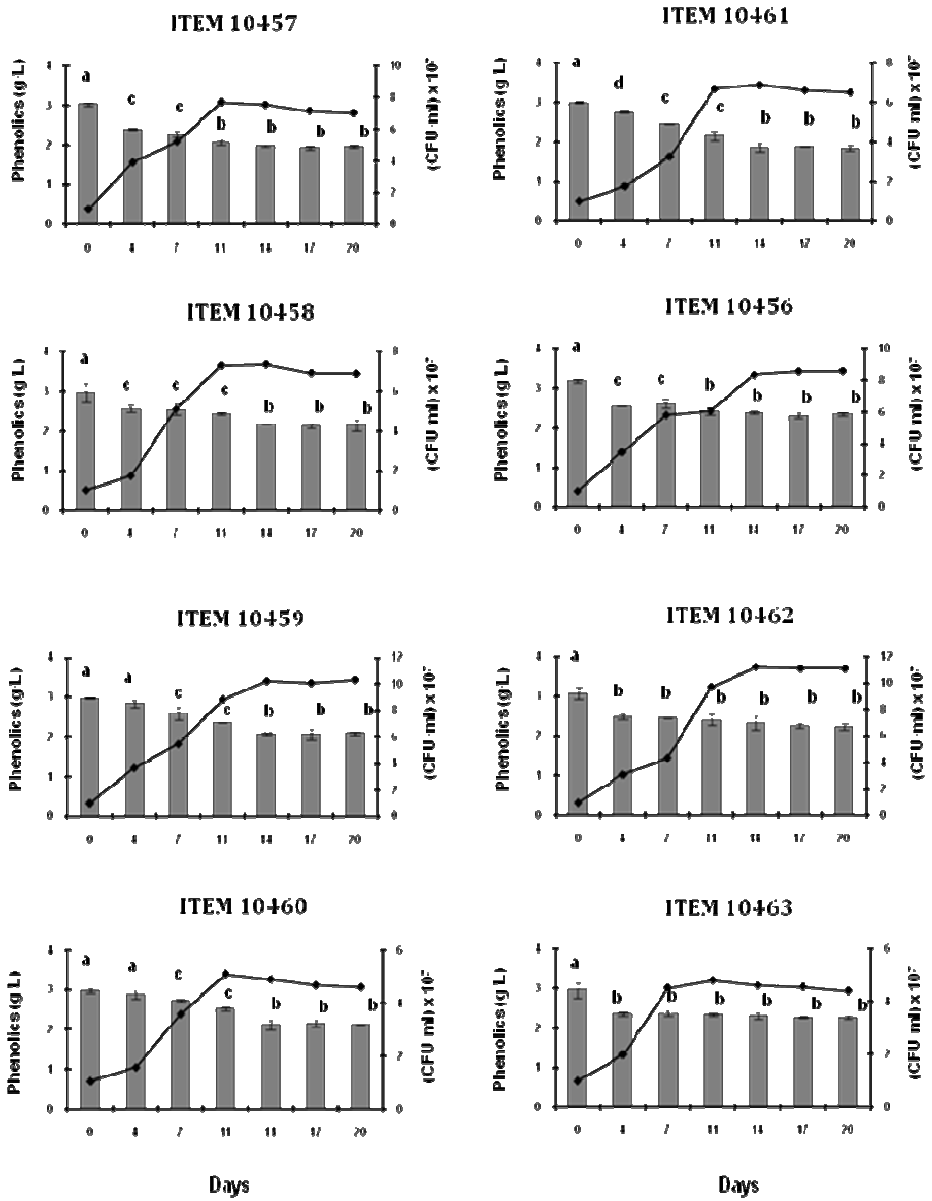

Fig. 35. Phenolic removal (grey bars) and biomass production (—◆—) during the cultivation of selected yeast strains in OMW medium. The isolate number is indicated. Letters above each bar indicate the results of Turkey's test ($P < 0.05$); values sharing letters in the same graph are not significantly different.

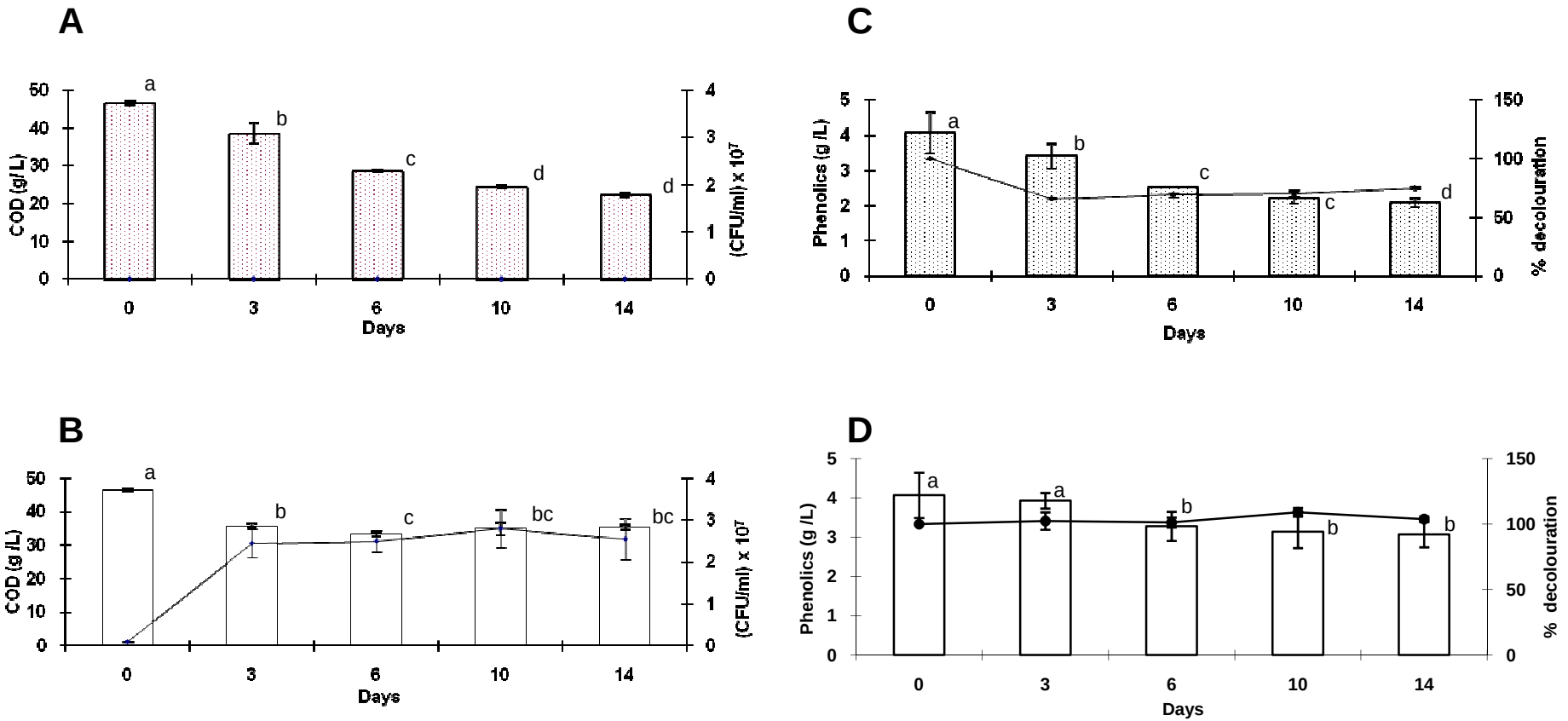

Fig.36. Time course of OMW treatment with *G.candidum* ITEM 10461 cells as freely suspended culture and immobilized in Ca-alginate gel beads. Beads were prepared and incubated by Bleve et al. (2008). Panels A and B respectively illustrate COD reduction by immobilized (dotted columns) and free cells (white columns). The cell concentration in both samples is reported (—◆—). Panels A and D respectively show the removal of phenol compounds for immobilized (dotted columns) and free cells (white columns). The percentage of remaining colour is indicated (—●—). Letters on the top of each bar indicate the results of Turkey's test ($P < 0.05$); column sharing letters in the same graph are not significantly different.

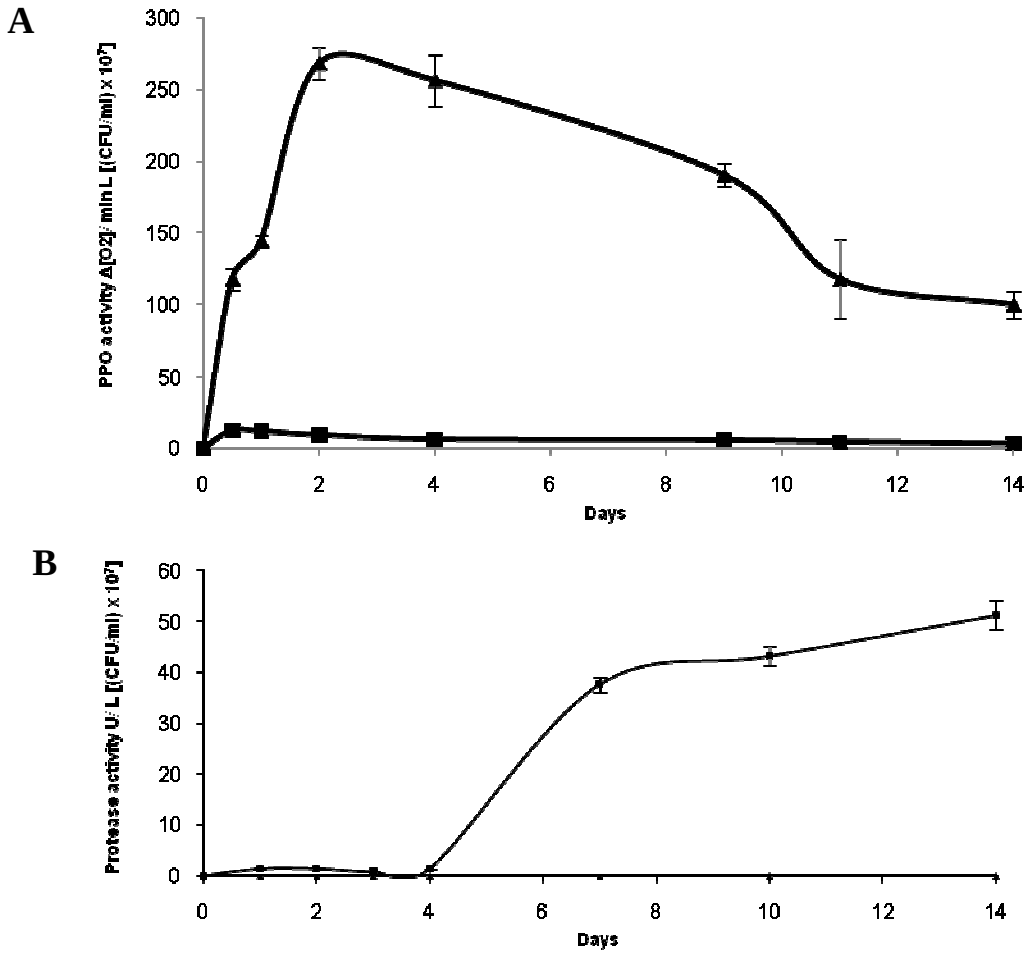

Fig. 37. (A) oxidative enzymes activity related to cell concentration in OMW treated either by free (—■—) and immobilized (—▲—) *G. candidum* ITEM 10461 cells. (B) Protease activity detected in the same OMW treated with free (—■—) and immobilized (—▲—) cells .

Tables

Tab. 1. Names and sequences of the utilized oligonucleotides. Introduced restriction sites are in bold. The underlined nt of primer ERY3SSREV anneal to the sequence of the Ery3 ORF coding for the signal sequence. The underlined nt of primer ERY4CERY3 anneal to the 3' - end of the Ery3 ORF.

Name	**Sequence**
ERY3_Eco.for	AAA**GAATTC**ATGTTYCCARGCGCACGGAT
ERY3_Not.rev	AAA**GCGGCCGC**CTAAGCTABGCCRCCTCTGTCG
ERY4_Eco	AAA**GAATTC**ATGGCGGTTGCATTCATTGC
ERY4_Not	AAA**GCGGCCGC**TCATGCCTTCAGTGGCGCA
2Lessrev_Not	AAA**GCGGCCGC**TCACAGTGGCGCATGCAGA
5Lessrev_Not	AAA**GCGGCCGC**TCAATGCAGACGGCCGAT
8Lessrev_Not	AAA**GCGGCCGC**TCAGCCGATGGCATTGGT
11Lessrev_Not	AAA**GCGGCCGC**TCAATTGGTGCCCATTAGGA
14Lessrev_Not	AAA**GCGGCCGC**TCACATTAGGAGTTTCGATGGG
18Lessrev_Not	AAA**GCGGCCGC**TCACGATGGGTTTGAGCTGTT
ERY4CERY3	AAA**GCGGCCGC**CTAAGCTATGCCACCTCTGTCCGATGGGTTTGAGCTGTTGTA
ERY3SSRev	GATGTTCAGCGTTCCGCGGGGCCCAATGCTAGCGTGAGTGCCATGTAAAAG
ERY4FUSFor	AGCATTGGGCCCCGCGGAACGCTG
ERY4FMut	GGCCGCTCGAGCATGCATCTAGAGG
ERY4K/E.rev	GCTCATGCCTCCAGTGGCGCATGCAGACGG
ERY4K/A.rev	GCTCATGCCGCCAGTGGCGCATGCAGACGG
ERY4K/R.rev	GCTCATGCCCTCAGTGGCGCATGCAGACGG
ERY4P/A.rev	GCTCATGCCTTCAGTGCCGCATGCAGACGG
PIR2_Eco.for	GGG**GAATTC**ATGCAATACAAAAAGACTTTGG
PIR2_Bam.for	GGG**GGATCC**ATGCAATACAAAAAGACTTTGG
PIR2.Hind.rev	AAA**AAGCTT**ACAGTCTATCAAATCGATAGCTT
FS_Eco.for	AAA**GAATTC**ATGACAATGCCTCATCGCTA
FS_Bam.for	AAA**GGATCC**ATGCAATGCCTCATCGCTA
FS_Hind.rev	AAA**AAGCTT**GGTGATTTGTCCTGAAGATGATG
4NC3Flag.Hind.for	AAA**AAGCTT**GACTACAAGGATGACGATGACAAGTTTCCAGGCGCACGGAT
4NC3_Xho.rev	AAA**CTCGAG**CTAAGCTATGCCACCTCTGTCCGATGGGTTTGAGCTGTTGTA

Name	Sequence
5less_Xho.rev	AAA**CTCGAG**TCAATGCAGACGGCCGAT
PPO2.his_Not.rev	AAA**GCGGCCGC**CTAAGCTABGCCRCCTCTGTCG
PPO2.Flag_Hind.for	AAA**AAGCTT**GACTACAAGGATGACGATGACAAGTCGCTGATTGCTACTGTCG
PPO2_Xho.rev	AAA**CTCGAG**TCAGTTAATAACATGCACCGC
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

Tab. 2. Name, accession number and description of laccase sequences of fungi belonging to *Pleurotus* genus used for bioinformatic analyses.

Denomination	Accession number	Description
P.os_lccK	AB089612	*P. ostreatus lccK* gene for laccase
P.os_poxA	AJ005017	*P. ostreatus poxA1b* gene for laccase
P.os_pox2	AJ005018	*P. ostreatus* mRNA for laccase (*poxa1b* gene)
P.os_pox3	AJ344434	*P. ostreatus poxa3* gene for laccase
P.sa_lac1	AJ507324	*P. sajor-caju* mRNA for laccase 1 (*lac1* gene)
P.sa_lac2	AJ507325	*P. sajor-caju* mRNA for laccase 2 (*lac2* gene)
P.sa_lac3	AJ507326	*P. sajor-caju* mRNA for laccase 3 (*lac3* gene)
P.sa_lac4	AJ507327	*P. sajor-caj*u mRNA for laccase 4 (*lac4* gene)
P.sa_lac5	AJ507328	*P. sajor-caju* mRNA for laccase 5 (*lac5* gene)
P.sp_lac1	AJ786026	*P. sapidus* mRNA for laccase1(*lac1* gene)
P.sp_lac2	AJ973225	*P. sapidus* mRNA for laccase2 (*lac2* gene)
P.os_lacA	AY450404	*P. ostreatus* laccase mRNA,
P.os_lacB	AY485827	*P. ostreatus* laccase mRNA,
P.pu_lac6	AY686700	*P. eryngii* laccase (*pel3*) gene
P.pu_lac2	AY836674	*P. pulmonarius* laccase 6 (*lac6*) mRNA.
P.er_lac3	AY836675	*P. pulmonarius* laccase 2 (*lac2*) mRNA
P.er_lac4	DQ234990	*P. eryngii* laccase (*pel4*) mRNA
N.cra	P10574	*N. crassa* laccase

Tab. 3. Oxidation activity in the presence of ABTS and protein concentration in liquid culture of recombinant laccase

	Liquid culture			*After purification*	
Laccase isoform	mU ml^{-1}	U mg^{-1}	μg ml^{-1}	Total Units	Total protein concentration (mg)
Ery4	ND	ND	ND	ND	0.14
2less	18	7.2	2.5	37	0.273
5less	33	6.6	5	26	0.150
8less	12	2.8	4.2	15	0.168
11less	10	2.5	3.9	15	0.2
14less	11	3.2	3.41	8	0.247
18less	15	3.5	4.3	6.9	0.116
4N3	11	7.3	1.5	9	0.110
4C3	16	5	3.18	11	0.335
4NC3	21	7.5	2.8	48	0.110
K532R	ND	ND	ND	ND	0.11
K532A	2.3	2.3	1.5	3.67	0.142
K532E	13	3.25	4	11	0.111
P530A	5.3	2.12	2.5	8.85	0.152
Ery3	7	1.4	4.8	17	0.077

ND, not determined.

Tab. 4. Purification yields of recombinant laccases, obtained at each purification step.

	Total volume (ml)	**Total protein (mg)**	**Total Enzyme activity (U)**	**Specific activity (U/mg)**	**Yield (%)**	**Fold**
Broth	1000	3.25 ± 1.5	26 ± 9.1	5 ± 2.5	100	1,0
Filtration	35 ± 10	2.86 ± 1,1	21 ± 6.5	7.3 ± 2.9	80.1 ± 16.8	1.46 ± 0.25
Hight Q	15 ± 5	0.935 ± 0,24	14 ± 4.2	33 ± 8.7	28.7 ± 7.34	6.6 ± 1.38
Sephadex g-75	2 ± 1	0.143 ± 0.053	8 ± 2.3	56 ± 13.4	4.3 ± 1.2	11.2 ± 2.47

Tab. 5. Kinetic parameters in the presence of ABTS of mutants and wild-type Ery3 and Ery4 expressed in *S. cerevisiae*. ND, not determined.

ABTS									
	pH 3			pH 4.5			pH 6.5		
Laccase isoform	K_m (μM)	K_{cat} ($min.^{-1}$)	K_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	K_{cat} ($min.^{-1}$)	K_{cat}/K_m (min^{-1} μM^{-1})
2Less	216	6693	30,990	813	4944	6,081	2304	304	0,132
5Less	28	18670	666,800	1136	14190	12,490	2217	1242	0,560
8Less	95	8981	94,530	1349	6989	5,180	3703	696,76	0,188
11Less	79	5174	65,490	1278	4232	3,310	4000	489	0,122
14Less	68	6373	93,720	1090	4497	4,125	5260	755,28	0,143
18Less	96	9061	94,380	1262	5879	4,657	4347	836,12	0,192
4CN3	53	12650	238,600	1277	10240	8,021	6858	1948	0,284
Ery3	41	6763	164,900	925	4631	5,006	3190	322,1	0,100
4N3	51	13921	272,900	1075	9729	9,050	7836	1977	0,252
4C3	97	5590	57,620	1298	4667	3,595	4405	832	0,189
K532E	84	5871	69,890	1278	4480	3,505	2487	330,5	0,133
K532A	58	6043	104,100	653	4201	6,434	2174	355,6	0,154
K532R	ND	ND	ND	ND	ND	ND	ND	ND	ND
P530A	105	4236	40,340	1219	3636	2,981	3703	274,7	0,074
Ery4	ND	ND	ND	ND	ND	ND	ND	ND	ND

Tab. 6. Comparison of kinetic parameters in the presence of different substrates of mutants and wild-type Ery3 and Ery4 expressed in *S. cerevisiae*.

	Syringaldazine			DMP			TMB			Guaiacol		
	pH 6.5			pH 4.5			pH 3			pH 6.5		
Laccase isoform	K_m (μM)	K_{cat} ($min.^{-1}$)	K_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	K_{cat} ($min.^{-1}$)	K_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	K_{cat} ($min.^{-1}$)	K_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1} μM^{-1})
2Less	9,9	462,94	46,76	722	4485	6,212	42	959,65	22,84	ND	ND	ND
5Less	6,6	1463	221,6	562	12790	22,760	20	2520	126	6940	98,09	0,014
8Less	11,76	379,4	32,26	778	5734	7,371	27	1209,48	44,8	ND	ND	ND
11Less	4,58	501,59	109,04	658	3098	4,709	23	651,13	28,31	ND	ND	ND
14Less	8,32	698,65	83,97	767	4114	5,364	40	870,85	21,77	ND	ND	ND
18Less	4,89	676,41	138,04	840	5049	6,011	30	951,24	31,71	ND	ND	ND
4CN3	5,38	1527	282,8	656	9239	14,080	13	1579,3	121,5	8850	77,27	0,009
Ery3	2	329,94	165	703	3801	5,406	47	902,98	19,21	ND	ND	ND
4N3	6,86	831,53	120,5	881	8631	9,797	28	1512	54	ND	ND	ND
4C3	10,79	974,79	88,6	842	4568	5,426	11	672	61,09	ND	ND	ND
K532E	15,76	377,21	23,6	1048	4444	4,240	23	750,55	32,63	ND	ND	ND
K532A	3,47	290,98	83,13	893	1196	1,340	125	986,6	7,89	ND	ND	ND
K532R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P530A	4,13	241,75	58,96	917	3058	3,335	28	602,77	21,53	ND	ND	ND
Ery4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND, not determined

Tab. 7. Effect of pH on the activity of purified laccase isoforms. Enzymatic activity was calculated using ABTS as substrate

Laccase isoform	pH 3 U/L	pH 4.5 U/L	pH 5.5 U/L	pH 6.5 U/L	pH 8 U/L
Ery4	ND	ND	ND	ND	ND
2Less	4190	2510	1040	110	0
5Less	7730	3760	1260	240	37
8Less	3720	1870	690	130	0
11Less	2880	1760	692	100	0
14Less	2810	1340	578	97	0
18Less	2960	1270	492	99	0
4N3	4780	2300	726	140	0
4C3	4000	1860	1030	176	15
4NC3	7940	3500	1310	256	42
K532E	3270	1670	662	89	0
K532A	1290	690	223	37	0
K532R	ND	ND	ND	ND	ND
P530A	2620	1450	438	64	0
Ery3	4050	2140	676	74	0

ND, not determined.

Tab. 8. Effect of temperature on the activity of purified laccase isoforms. Enzymatic activity was calculated using ABTS as substrate

Laccase isoform	**10°C U/L**	**25 C° U/L**	**40 C° U/L**	**55 C° U/L**	**70 C° U/L**
Ery4	ND	ND	ND	ND	ND
2Less	2870	4190	5290	9070	8480
5Less	3460	7730	9600	9790	10800
8Less	1810	3720	6100	5570	5400
11Less	1850	2880	5930	4810	4480
14Less	1360	2810	4060	3770	3700
18Less	1370	2960	3580	4740	3480
4N3	1880	4780	5040	5160	5570
4C3	2390	4000	5580	5680	7120
4NC3	3190	7940	8980	9740	9400
K532E	1580	3270	4290	5700	3990
K532A	771	1290	1620	1900	1860
K532R	ND	ND	ND	ND	ND
P530A	1170	2620	3420	3810	3900
Ery3	2240	4050	4720	5320	5400

ND, not determined.

Tab. 9. The apparent half-life values, $T_{1/2}$, of the Ery4 and Ery3 wild type and the mutant laccase isoforms for ABTS at 25, 40, 55°C and at pH 3, 4.5 and 5.5.

$T_{1/2}$	2Less	5Less	8Less	11Less	14Less	18Less	4N3	4C3	4NC3	K532E	K532A	K532R	P/A	Ery3	Ery4
25°C	667	820	750	176	334	1502	177	143	250	1506	81	ND	250	600	ND
40°C	45	73	44	71	60	115	71	130	150	79	45	ND	45	48	ND
55°C	38	30	34	35	43	33	38	36	35	38	30	ND	30	35	ND
pH 5,5	60	83	45	99	122	85	62	43	55	117	37	ND	138	126	ND
pH 4,5	38	39	40	46	49	36	37	39	37	48	33	ND	46	51	ND
pH 3	37	35	38	42	39	34	34	33	31	39	30	ND	38	38	ND

Data reported in hours. ND, not determined.

Tab. 10. Kinetic parameters of recombinant laccases displayed on the *S. cerevisiae* surface.

	ABTS	**DMP**	**Guaiacol**	**TMB**	**Syringaldazine**
FS 5less	1.5 mU/ml	O.1 mU/ml	0.5 mU/ml	0.06 mU/ml	ND
PIR2 5less	1.9 mU/ml	1.4 mU/ml	ND	ND	ND
FS 4NC3	ND	0.1 mU/ml	ND	ND	ND
PIR2 4NC3	ND	0.06 mU/ml	ND	0.02 mU/ml	ND

ABTS. Syringaldazine. DMP. TMB. Guaiacol have been tested at their optimal pH values. ND. not determined.

Tab. 11. Kinetic parameters of commercial and recombinant PPO2 tyrosinase.

	Commercial *A. bisporus* Tyrosinase			**PPO2 from *A. bisporus***		
	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu M^{-1}min^{-1}$)	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu M^{-1}min^{-1}$)
L- Tyrosin	0.375	175.9	469.1	0.302	683.6	2263.8
L- Dopa	1.33	934.6	702.78	1.22	8459	6713.7
Pyrocathecol	1.26	303.6	240.96	1.38	857	625.5
Pyrogallol	0.865	513.25	600.29	0.885	4776.1	5399

L-Tyrosin, L-Dopa, Pyrocathecol, Pyrogallol were tested at their optimal pH values.

Tab. 12. Yeast and mould isolated from the five OCW and OMW samples and identified by the similarity of their ITS1-5,8S-ITS2 rRNA region to sequences in the GenBank nucleotide sequence database.

Source	OCW		OMW	
	Species	Isolate nr.	Species	Isolate nr.
Casarano	*Saccharomyces cerevisiae*	19	*Geotrichum candidum*	18
	Rhodotorula mucilaginosa	5	*Saccharomyces cerevisiae*	9
	Candida membranifaciens	2	*Pichia fermentans*	3
	Geotrichum candidum	2		
	Pichia holstii	2		
Galatone	*Penicillium canescens*	15	*Geotrichum candidum*	13
	Penicillium citrinum	7	*Saccharomyces cerevisiae*	8
	Penicillium crustosum	1	*Pichia holstii*	5
	Pichia holstii	5	*Pichia fermentans*	4
	Candida michaelii	1		
	Candida incospicua	1		
Martano	*Candida tropicalis*	1	*Pichia holstii*	12
	Geotrichum candidum	5	*Geotrichum candidum*	12
	Pichia holstii	19	*Saccharomyces cerevisiae*	6
	Saccharomyces cerevisiae	5		
Scorrano	*Geotrichum candidum*	4	*Pichia holstii*	3
	Pichia fermentans	10	*Pichia fermentans*	5
	Saccharomyces cerevisiae	9	*Saccharomyces cerevisiae*	7
	Pichia holstii	7	*Geotrichum candidum*	15
Torchiarolo	*Geotrichum candidum*	3	*Geotrichum candidum*	14
	Pichia fermentans	12	*Pichia fermentans*	6
	Candida tropicalis	11	*Saccharomyces cerevisiae*	10
	Saccharomyces cerevisiae	4		

Tab. 13. Yeast strains able to use OMW as unique nutrient source were identified according to their similarity to ITS1-5.8S-ITS2 region sequences in the GenBank nucleotide sequence database. The ratio between the radial growth on 75% OMW agar plate and the radial growth of the same isolate on YEPD agar is reported. The strains and the nucleotide sequence of their ITS1-5.8S-ITS2 region were respectively deposited in the ISPA collection and EMBL Database. the corresponding accession numbers are given.

ITEM Acc. Nr.	Closest match	% similarity	EMBL Acc. Nr.	Radial growth %
10455	*Candida membranifaciens* (AY452740.1)	99	FN376411	87
10456	*Candida tropicalis* (EU288196.1)	99	FN376412	93
10457	*Geotrichum candidum* (AJ279445.1)	97	FN376413	92
10458	*Geotrichum candidum* (AJ279445.1)	97	FN376414	94
10459	*Geotrichum candidum* (AJ279445.1)	97	FN376415	92
10460	*Geotrichum candidum* (AJ279445.1)	98	FN376416	91
10461	*Geotrichum candidum* (AJ279445.1)	98	FN376417	95
10462	*Pichia fermentans* (DQ674358)	99	FN376418	88
10463	*Pichia holstii* (AY761154.1)	94	FN376419	92
10464	*Pichia holstii* (AY761154.1)	91	FN376420	84
10465	*Saccharomyces cerevisiae* (AM262829.1)	99	FN376421	91
10466	*Saccharomyces cerevisiae* (AM262829.1)	99	FN376422	90

Tab. 14. Antibacterial activity against *B. megaterium* ATCC 25848 and removal percentage of total phenols. COD and colour in olive mill wastewaters fermented with eight selected yeast isolates.

Strain	Species	Inhibition halo	Phenol (%)	COD (%)	initial colour (%)
N.T.	-	+++	0	0	100
10456	*C. tropicalis*	+	25	18	100
10457	*G. candidum*	-	31	22	94
10458	*G. candidum*	-	25	20	90
10459	*G. candidum*	+	29	20	99
10460	*G. candidum*	-	29	22	100
10461	*G. candidum*	-	30	23	97
10462	*P. fermentans*	+	26	18	97
10463	*P. holstii*	+	17	15	99

N.T. OMW not treated. - no inhibition; + inhibition halo 4mm in diameter; ++ inhibition halo 5-8 mm in diameter; +++ inhibition halo 9-12 mm in diameter.

Tab. 15. Cellulase, xylanase, lignin and manganese-dependent peroxidises, laccase and tyrosinase activity associated to the selected yeast and determined by specific plate assay.

Isolate	Species	Ce	Xy	LMp	La	Ty
10456	*C. tropicalis*	+	-	-	-	-
10457	*G. candidum*	+	+	+	+	+
10458	*G. candidum*	+	+	+	+	+
10459	*G. candidum*	+	-	-	+	+
10460	*G. candidum*	+	-	+	+	+
10461	*G. candidum*	+	+	+	+	+
10462	*P. fermentans*	-	-	-	-	-
10463	*P. holstii*	-	-	-	-	-

+ positive result; - negative result. Ce. cellulase; Xy. xylanase; LMp. lignin and manganese-dependent peroxidase; La. laccase; Ty. tyrosinase:

Appendix

Blocking buffer

5% (w/v) BSA or Dry milk
0.1% (w/v) NaN_3 (Sodium azide)
1X TS
50 µl Tween
Add distilled H_2O to 10 ml (prepare just before)

TS buffer 10X

0.002M Tris-Cl pH 7.4
0.14M NaCl
Add distilled H_2O to 200 ml

Breaking Buffer

2% (v/v) Triton X-100 (Biorad)
1% (w/v) Sodium Dodecyl Sulphate (SDS) (Biorad)
100mM NaCl (J. T. Baker)
10mM Tris-Cl pH 8.0
0,1mM EDTA

Buffer A

10mM Tris HCl, pH 8
1mM phenylmethyl-sulphonyl fluoride (PMSF)
20 µl/ml of Protease Inhibitor Cocktail (Sigma, USA)

Coomassie staining solution

50% (v/v) Methanol
0.05% (v/v) Coomassie brilliant blue R-250 (BioRad)
10% (v/v) Acetic acid
40% (v/v) distilled H_2O

DNase assay buffer

40 mM Tris-HCl, pH 8.0
10 mM $MgSO_4$
3 mM $CaCl_2$

Destaining solution

30% Methanol
10% Acetic acid
Add distilled H_2O to 100 ml

Laemmli sample buffer 4X

5 ml 1M Tris-HCl, pH 6.8
10 ml 20 % SDS
5 ml 2-mercaptoethanol (Sigma)
5 ml 0.2% bromophenol blue in methanol
10 ml glycerol
Divide in aliquots and store at –20°C

LETS buffer

200 mM LiCl
20 mM EDTA
20 mM Tris-HCl, pH 8.0
0.4% sodium dodecyl sulfate

Lysis buffer

50 mM NaH_2PO_4, pH 8
150 mM NaCl,
30 mM imidazole
10% glycerol,
1 mM phenylmethylsulfonyl fluoride
lysis buffer pH 8

PBS 10x

80 g NaCl
2 g KCl
14.4 g Na_2HPO_4
2.4 g KH_2PO_4
Adjust pH to 7.4 with HCl. Add distilled H_2O to 1 liter.

PBS-B-N

1x PBS
1% BSA
0.05% Nonidet P-40

Ponceau S solution

5 mg/ml Ponceau S
5% TCA
Add distilled H_2O to 100 ml (prepare just before)

SDS-Polyacrylamide Gel

Separating gel (12% Acrylamide, 10 ml)

3.3 ml H_2O
4.0 ml Acrylamide mix (29.2% acrylamide and 0.8% N,N'-methylene bis-acrylamide)
2.5 ml 1.5 M Tris pH 8.0
0.1 ml 10% SDS
0.1 ml 10% APS
0.004 ml TEMED (Sigma)

Stacking gel (3 ml)

2.1 ml H_2O
0.5 ml Acrylamide mix (29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide)
0.38 ml 1 M Tris pH 6.8
0.03 ml 10% SDS
0.03 ml 10% APS
0.003 ml TEMED (Sigma)

SDS Tris/Glicine Buffer 10X

30.2 g Tris-base (Sigma)
144 g Glycine
10 g SDS
Add distilled H_2O to 1 litre

Silver staining

Fixing solution

Ethanol 40 ml
Acetic acid 10 ml
Make up to 100 ml with distilled water.

Sensitizing solution

Ethanol 150 ml
Sodium acetate 34 g
Sodium thiosulphate 1g
Make up to 500 ml with distilled water.
Just before the use, add 500 µl of Glutaraldehyde solution (25% w/v) to 100ml sensitizing solution

Silver solution

Silver nitrate solution 1,25 g
Make up to 500 ml with distilled water.
Just before the use, add 40 µl of Formaldehyde (37% w/v) to100 ml silver solution

Developing solution

Sodium carbonate 12,5 g
Make up to 500 ml with distilled water.
Just before the use, add 40 µl of Formaldehyde (37% w/v) to100 ml silver solution

Stop solution

EDTA-Na_2•$2H_2O$ 7,5 g
Make up to 500 ml with distilled water.

SHA buffer

0.1 M sorbitol
0.1 M Na Hepes, pH 7.5
5 mM NaN_3

Staining solution

0.25% Comassie blue
50% Methanol
5% Acetic acid
Add distilled H_2O to 100 ml

STET Lysis buffer

8% saccharose
20 mM Tris HCl, pH 8
50 mM EDTA
0,5% Triton

TAE electrophoresis buffer

50X stock solution, pH 8.5
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M Na_2EDTA (pH 8.0)
H_2O to 1 liter

1X working solution
40mM Tris acetate
2mM EDTA

TE buffer

10mM Tris-HCl, pH 8.0
1.0mM EDTA

Transfer Buffer

100 ml SDS Tris/Glicine Buffer 10X
20% Methanol
Add distilled H_2O to 1 litre

Transformation Mix

S. cerevisiae

	For 1 transformation
50% w/v PEG 3500	240 µl
1.0M LiAc	36 µl
10 mg/ml boiled Salmon Sperm-carrier DNA	50 µl
Plasmid DNA (200-300 ng)/ PCR amplicon or linearized DNA (5-10 µg) plus Water	34 µl
Total	360 µl

X-gal Solution
(5-bromo-4-chloro-3--indolyl-β-D-galactoside)
(2% w/v) (Gibco BRL, Life Technologies)
20 mg/ml solution in dimethylformamide.
Store at -20°C

Wash buffer
50 mM NaH_2PO_4
150 mM NaCl,
30 mM imidazole
20% glycerol
0,1% Triton x100
1 mM phenylmethylsulfonyl fluoride

YEPD agar
1% yeast extract
2% peptone
2% glucose
2% agar; pH 4.5

SM medium
0.67% (w/v) yeast nitrogen base,
supplemented with auxotrophic requirement
glucose (2%, w/v) or galactose (2%, w/v)

YP medium
1% (w/v) yeast extract
2% (w/v) peptone

YPD medium
1% yeast extract
2% peptone
2% glucose

Publications

1) Bleve G., LEZZI C., Rampino P., Mita G., Perrotta C., Grieco F. (2007). Espressione in *Saccharomyces cerevisiae* dell'isoforma LAC3 di una laccasi di *Pleorotus eryngii* in forma biologicamente attiva. Atti del X Congresso dell'Associazione Italiana di Biologia Generale e Molecolare (AIBG) 20-22 Settembre, Torino, Italy, pag.105.

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