NEW OXIDATIVE ENZYMES FOR INDUSTRIAL APPLICATION

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«Preserva il tuo diritto a pensare,
perché anche pensare in modo sbagliato
è sempre meglio che non pensare affatto»

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Alla mia famiglia e a Donato

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CHAPTER 3

SUMMARY

Fungal laccases are remarkable green catalysts that have a broad substrate specificity and many potential applications in bioremediation, lignocellulose processing, organic synthesis, and more. The white-rot fungus *Pleurotus ostreatus* is able to express multiple laccase genes encoding isoenzymes with different and particularly interesting physico-chemical characteristics: POXC, POXA1w, POXA1b, POXA3a and POXA3b. Several *P. ostreatus* laccases have been successfully expressed in yeasts and the availability of established heterologous recombinant expression systems has allowed the construction of mutated, "better performing" enzymes through molecular evolution techniques. The cDNAs encoding POXA1b and POXC have been selected as "parent molecules" to guide the evolution of laccases with higher specific activity and different substrate specificities. Genetic variants were created by random mutagenesis through error prone PCR (EP-PCR) and DNA shuffling. After two round of mutations, four POXA1b mutants (1M9B, 1L2B, 1M10B and 3M7C) were selected for their improved activity against non-phenolic substrates.

New *criteria* of selection were applied in a further screening of the already avalaible 2300 mutants library, using different substrates (e.g. 2,6 dimethoxyphenol, DMP), assaying the enzyme stability and activity at different operating conditions (different pHs). Three new mutants were selected for their improved performances and characterized from a structural and functional point of view. They showed stability at pH 5 and at 60°C higher than that of the wild-type enzyme.

A new mutant was rationally designed and constructed. This new mutant, R4, contains the mutations of the two parental enzymes 1M10B and 3M7C. Catalytic and kinetic properties of R4 mutants were analyzed and compared with those of the wild-type enzyme. It showed stability at pH10 2 fold higher than that of the wild-type enzyme. This new clone was used as template for producing a new collection of 1100 mutants. Two new mutants (4M10G and 1H6C) were selected for their improved activity against ABTS. The isolated mutants were partially purified and fully characterized.

Amino acid substitutions of each mutant were identified and located in POXA1b laccase model. Analysis of possible molecular mechanisms responsible of better performances was carried out.

A parallel session of the project was aimed at overproducing POXA1b laccase and its best variant 1H6C using the filamentous fungi *Aspergillus niger* as host. The necessity to produce large amounts of enzymes for biotechnological applications and the low yields of laccases secreted from wild-type fungal organisms claims for an efficient heterologous expression of the enzymes of interest. The two laccases have been expressed in the filamentous fungi *A. niger*, obtaining an improvement of 100-fold in the enzyme production. These preliminary results indicate that *A. niger* could be a promising expression system for *P. ostreatus* laccases if compared to other expression systems.

RIASSUNTO

Nell'ultimo decennio, l'utilizzo degli enzimi in campo industriale ha avuto un rapido sviluppo. Il loro impiego, infatti, offre numerosi vantaggi rispetto ai convenzionali catalizzatori chimici, sia per la loro produzione da fonti rinnovabili, che per la loro facilità di smaltimento.

Numerose sono le applicazioni industriali in cui gli enzimi hanno brillantemente sostituito i reattivi classici: da quello della detergenza, con l'utilizzo ormai da molti anni delle proteasi e delle cellulasi, a quello tessile, con l'uso delle amilasi e delle pectinasi come alternativa ecocompatibile alla soda caustica, passando per molti altri ancora. Resta invece una certa difficoltà alla diffusione dell'utilizzo degli enzimi nel campo del risanamento ambientale. Tuttavia proprio in quest'ambito, i problemi connessi all'utilizzo dei metodi convenzionali, come l'incenerimento, per la rimozione di sostanze tossiche dall'ambiente determinano un crescente interesse verso lo sviluppo di trattamenti alternativi economici e ecocompatibili. In particolare si può considerare l'aumento di attenzione verso i funghi basidiomiceti che rappresentano un'interessante fonte di enzimi ossidativi dalle potenziali applicazioni industriali. Essi, infatti, producono una grande varietà di isoforme di tali enzimi extracellulari tra cui le laccasi, le manganese perossidasi e le lignino perossidasi, che trovano numerose applicazioni sia in processi di degradazione, che di biosintesi. Infatti, data la bassa specificità di substrato, i diversi enzimi ossidativi sono in grado di degradare, oltre a polimeri naturali quali la lignina, anche numerose molecole tossiche di natura xenobiotica, come composti fenolici e aromatici. Per quanto riguarda i processi di sintesi gli enzimi ossidativi possono essere impiegati in diversi processi quali la trasformazione di una grande varietà di molecole alifatiche/aromatiche, la funzionalizzazione di idrocarburi inerti e la sintesi di polimeri.

In particolare le laccasi, cuproproteine che catalizzano l'ossidazione di p-difenoli con la concomitante riduzione di ossigeno molecolare ad acqua, sono commercialmente utilizzate nella demolizione di materiali ligninolitici, nella produzione di etanolo, nel processo di scolorimento dei jeans DENIM e nei processi di bioremediation, per preservare l'ambiente dai danni causati dagli effluenti industriali. Questi enzimi, infatti, si sono dimostrati capaci di ossidare anche i pesticidi e gli idrocarburi aromatici policiclici e di detossificare le acque di lavorazione delle olive e del caffè.

Il fungo basidiomicete *white rot Pleurotus ostreatus*, come la maggior parte delle specie fungine, produce più di un enzima ad attività fenolo ossidasica. Cinque sono gli isoenzimi (POX-: Phenol OXidase-) fino ad ora purificati e caratterizzati: POXC, il più abbondantemente prodotto in tutte le condizioni di crescita analizzate; POXA1b, dall'insolita stabilità a pH alcalino; POXA1w, particolare per il suo contenuto in ioni metallici; ed infine gli isoenzimi POXA3a e POXA3b, atipici per la loro struttura eterodimerica.

Nonostante l'enorme attrattiva esercitata dalle potenzialità di questi enzimi, per poter utilizzare questi sistemi ossidativi in un processo industriale è di fondamentale importanza avere una sempre più completa caratterizzazione degli enzimi secreti dai funghi basidiomiceti. Date però le numerose isoforme secrete da uno stesso organismo, per facilitare questo studio bisogna tentare di esprimere in organismi diversi, singolarmente, i geni isolati dai funghi.

Allo scopo di facilitare l'impiego degli enzimi prodotti da *P.ostreatus* nel campo delle biotecnologie industriali, si è pensato quindi di potenziare le loro caratteristiche

di stabilità e affinità ottenendo una collezione di circa 2300 nuove laccasi ricombinanti. Utilizzando, infatti, come stampo il cDNA delle fenolo ossidasi POXA1b e POXC sono stati ottenuti dei mutanti in *Saccharomyces cerevisiae* mediante tecniche di evoluzione guidata e nello specifico la tecnica dell'*Error Prone* PCR (ER-PCR), la più frequentemente utilizzata tra le tecniche non ricombinative, e del DNA *shuffling*, il più tradizionale tra i metodi ricombinativi. In seguito alla messa a punto di un sistema di selezione idoneo, sono stati identificati quattro cloni con un'attività specifica maggiore rispetto a quella del *wild type* e, in tre casi, con una stabilità maggiore in funzione del pH e della temperatura. Inoltre, grazie all'analisi di modelli *in silico* della struttura tridimensionale delle proteine mutate, si è potuto dimostrare che i residui mutati presenti nelle nuove laccasi, anche se non direttamente coinvolti nella catalisi, influenzano l'efficienza e/o la stabilità dell'enzima. Ciò ha consentito di incrementare la comprensione delle relazioni struttura-funzione di questa classe di enzimi, fattore indispensabile per un loro sempre più efficiente utilizzo in processi industriali.

Questo lavoro di ricerca ha avuto come obiettivi 1) la selezione di nuove laccasi mediante nuovi *screening* della collezione di mutanti a nostra disposizione utilizzando diversi criteri di selezione, 2) lo sviluppo di nuove ossidasi mediante un approccio semi-razionale e l'ampliamento della collezione di 2300 mutanti e 3) l'espressione delle migliori laccasi selezionate in *Aspergillus niger* per avere una overespressione delle proteine di interesse e poter quindi effettuare una caratterizzazione strutturale e funzionale dettagliata degli enzimi di interesse.

1) Nuovi criteri di selezione

La collezione di mutanti a nostra disposizione è stata selezionata utilizzando due nuovi criteri: l'impiego di diversi substrati e la maggiore attività specifica in funzione del pH. In previsione di un utilizzo delle fenolo ossidasi in processi industriali è, infatti, di fondamentale importanza che esse siano utilizzabili con un più ampio intervallo di substrati e che siano stabili e soprattutto attive a condizioni estreme di pH, normalmente presenti nei processi di lavorazione.

Nell'applicazione del primo criterio è stato utilizzato un substrato fenolico quale il 2,6-dimetossifenolo. In seguito allo *screening* sono stati selezionati due mutanti, 2L4A e 3L7H, che hanno dimostrato un'attività specifica circa due volte e mezza maggiore rispetto al *wild type*. Inoltre i due mutanti mostrano un incremento della loro stabilità a pH5 (~25 giorni) due volte maggiore rispetto al *wild type* (~12 giorni).

Dalla sequenza nucleotidica dei due mutanti sono state identificate le regioni in cui erano avvenute le mutazioni: 2L4A ha subito una mutazione nella posizione 272 con una sostituzione di una glutammina (Q) in istidina (H), mentre 3L7H ha una sostituzione della serina (S) 285 in asparagina (N) e della N in posizione 328 in S. Le mutazioni sono localizzate in regioni di loop della proteina e, come evidenziato dal modello della struttura tridimensionale *in silico*, si trovano sulla superficie esterna. La mutazione Q272H del mutante 2L4A dovrebbe stabilizzare la struttura della proteina a pH5, perché permette interazioni addizionali – legame idrogeno e interazioni

elettrostatiche – tra l'anello imidazoico del residuo di Istidina 272, carico positivamente, e la catena laterale del Acido aspartico 287. Questa interazione potrebbe inoltre causare un movimento del loop dove è localizzato il residuo in posizione 272, coinvolgendolo nella formazione di una tasca di legame al substrato riducente, che influezzerebbe l'attività dell'enzima. Interessante è, inoltre, la mutazione S285N, caratteristica del mutante 3L7H, che è localizzata molto vicino al residuo Asp287. Entrambi gli amminoacidi sono collocati nel loop Gly282 – Thr289, che sembra avere un ruolo importante nella stabilità della proteina. Invece, la mutazione N328S, sempre presente nel mutante 3L7H, potrebbe avere effetto sull'attività dell'enzima, essendo localizzata in un loop che potenzialmente interagisce con il substrato.

Prove di decolorazione sono state effettuate utilizzando reflui modelli dell'industria di colore – selezionati nell'ambito del progetto europeo SOPHIED Sustainable Oxidative Processes for Healthy Industrial European Dyes – sia con i mutanti in analisi, che con l'enzima *wild type*. I nuovi cloni si sono dimostrati buoni biocatalizzatori per applicazioni di biorisanamento in quanto sono riusciti a decolorare coloranti risultati recalcitranti a precedenti trattamenti per le loro strutture (trisazo-, polyazo- stilbene-) complesse e generalmente non degradate da laccasi. Nel caso del refluo Direct yellow 106, per esempio, si è osservata una decolorazione fino al ~25% dopo soli 10 minuti di incubazione con i mutanti 2L4A e 3L7H (POXA1b 5% dec. dopo 10min).

Il secondo criterio, invece, ha visto la selezione di mutanti più attivi a pH 5. Studi precedenti, condotti presso il laboratorio dove è stato svolto questo lavoro di ricerca, avevano dimostrato che l'enzima POXA1b presenta a questo valore di pH una diminuzione della capacità degradativa dell'ABTS, substrato utilizzato per questo *screening* per la sua dimostrata sensibilità. Si è quindi proceduto alla selezione della collezione dei 2300 mutanti nelle condizioni sopra descritte, ottenendo il mutante 1L9A caratterizzato da un'attività specifica di circa tre volte maggiore rispetto al *wild type*. L'enzima, parzialmente purificato, è stato caratterizzato sia da un punto di vista cinetico, che in base alla valutazione degli effetti del pH e della temperatura. Il mutante mostra una ridotta affinità rispetto all'enzima POXA1b, sia per l'ABTS che per il DMP. Si nota invece un aumento della stabilità di circa 1.6 volte a 60°C e di circa 1.5 volte a pH 5 rispetto all'enzima *wild type*. Dalle analisi della sequenza nucleotidica è stato possibile identificare la presenza della sostituzione di una Glicina (G) con una Istidina (H) in posizione 284. Anche questa mutazione è localizzata nelle vicinanze del loop2, coinvolto nell'interazione dell'enzima con il substrato.

I risultati descritti in questa sezione hanno permesso di selezionare tre nuove laccasi con migliorate caratteristiche di stabilità a pH acidi e alla temperatura, conservando le proprietà catalitiche dell'enzima *wild type*. Inoltre nel caso dei mutanti 2L4A e 3L7H sono state effettuate delle prove di decolorazione che ci permettono al momento solo di ipotizzare il loro utilizzo in applicazioni di biorisanamento. I dati fin qui prodotti infatti, non sono sufficienti a valutare l'applicabilità di questo sistema per la conversione dei coloranti. Si rendono quindi necessarie ulteriori analisi, finalizzate alla caratterizzazione dei parametri cinetici e catalitici per una completa valutazione delle *performances* del sistema ed una stima dei vantaggi connessi all'impiego di biosistemi enzimatici immobilizzati (in termini di volumi trattati, costi e tempi di trattamento).

2) Nuove laccasi ottenute mediante approccio semi-razionale

Parallelamente alla selezione di nuovi mutanti secondo i parametri precedentemente descritti, è stato "progettato" e "costruito" un nuovo mutante, R4, dalla fusione delle mutazioni dei mutanti 1M10B e 3M7C. Questi due enzimi derivavano dalla selezione della collezione già a nostra disposizione effettuata utilizzando come substrato l'ABTS [8]. La scelta di questi due mutanti è stata dettata dalle numerose informazioni funzionali e strutturali a disposizione. Il clone 1M10B è infatti più stabile della proteina *wild type* sia in condizioni di pH alcaline che a 60°C. Il mutante 3M7C, invece, anche se mostra una stabilità simile al progenitore, possiede una mutazione nella regione del C-terminale (P494T) che analisi bioinformatiche hanno dimostrato aumentare la flessibilità della proteina, facilitando l'accesso del substrato al sito del rame T1 e incrementando l'attività specifica del clone in analisi.

Il mutante R4 mostra un incremento dell'attività specifica di circa 2.5 volte rispetto all'enzima POXA1b. Inoltre, dall'analisi dei parametri cinetici si osserva che i valori di K_M sono simili a quelli del *wild type* e dei mutanti parentali (1M10B e 3M7C) contro ABTS e SGZ, mentre sono più bassi contro DMP. L'attività specifica contro DMP e ABTS aumenta rispettivamente di 2 e 1.5 volte se confrontata con POXA1b, mentre resta inalterata rispetto ai mutanti 1M10B e 3M7C.

Un'ulteriore caratterizzazione delle proteine isolate è consistita nello studio dell'effetto della temperatura e del pH sull'attività fenolo ossidasica: la proteina R4 non mostra significative differenze, rispetto al *wild type*, nell'andamento del profilo di attività nelle condizioni analizzate.

Di particolare rilevanza è invece l'implementata stabilità del mutante R4 rispetto l'enzima *wild type* di due volte a pH10 e di 1.5 volte a 60°C. Questi dati hanno reso il mutante R4 un buon candidato per l'ampliamento della collezione di mutanti. Il clone R4 è stato, quindi, utilizzato come stampo per la generazione di una nuova collezione di mutanti mediante la tecnica non ricombinativa dell'EP-PCR.

La nuova collezione così generata si compone di 1000 mutanti. Utilizzando come substrato l'ABTS a pH3, sono stati selezionati due nuovi mutanti, 1H6C and 1M10G, con un'attività specifica di ben 4 volte maggiore rispetto a POXA1b. Inoltre essi incrementano la loro stabilità al pH in un intervallo maggiore rispetto al progenitore R4. Infatti mostrano un incremento della stabilità a pH5 di circa 3 volte rispetto al *wild type* e a R4, conservando nel caso di 1H6C la stabilità a pH10 (~60 giorni).

Per identificare i determinanti strutturali che influenzano questo miglioramento nel caso del mutante 1H6C, sono state identificate le mutazioni presenti nella proteine e mappate sul modello della struttura 3D di POXA1b. Nella posizione 148, localizzata vicino al dominio 2 adiacente al sito riducente di legame al substrato, è stata identificata una sostituzione di una Valina con una Leucina. La sostituzione della catena laterale, con un maggiore ingombro sterico nel caso della Leucina, comporta un impaccamento della regione e un avvicinamento del residuo L148 all'anello aromatico del'Istidina 208. Quest'ultimo è localizzato nel loop 204-208 presente alla fine del sito di legame al substrato dove è anche localizzato l'Asp205 coinvolto nel legame con il substrato aromatico. Questo cambiamento potrebbe quindi influenzare il legame e l'ossidazione del substrato riducente, ma anche l'interazione tra i domini 2 e 3.

Concludendo, in confronto alla laccasi *wild type* POXA1b, l'approccio semi-razionale ha permesso di sviluppare un più efficiente biocatalizzatore – con un incremento dell'attività specifica su DMP e ABTS rispettivamente di 2 e 5 volte –, che si è dimostrato essere più versatile e resistente, esibendo una attività in un intervallo più ampio di temperatura e pH e una maggiore stabilità a pH acidi (t1/2 a pH5=35 giorni), neutri (t1/2 pH7=38 giorni) e alcalini (t1/2 a pH10=62 giorni).

3) Espressione di laccasi nel fungo filamentoso *A. niger*

In collaborazione con l'Università della Provenza e del Mediterraneo, presso il laboratorio del Dr. Eric Record dell'INRA (*Institut National de la Recherche Agronomique)* si è proceduto all'espressione della laccasi POXA1b e del mutante 1H6C nel fungo filamentoso *A. niger*. La scelta del mutante 1H6C è dovuta alle interessanti caratteristiche di attività e stabilità mostrate da questo clone e sopra descritte.

L'attenzione per questo ospite è dettata dalla necessità di dover esprimere le proteine di interesse ad elevati livelli. Dati di letteratura, infatti, dimostrano che i livelli di espressione del fungo *A.niger* (10-135 mg/L) sono 2-30 volte più alti rispetto all'utillizzo di sistemi di espressione quali i lieviti (fino a circa 5 mg/L).

Record et al. (2002) riportano i primi esperimenti di espressione della laccasi Lac1 da fungo *white rot Pycnopourus cinnabarinus.* Sulla base di questi esperimenti, è stata ideata la strategia di clonaggio e di espressione delle proteine di interesse nel fungo filamentoso *A. niger*, utilizzando il vettore di espressione pAN52-4. Durante la fase di trasformazione del fungo, si è dovuto procedere alla preparazione dei protoplasti e alla co-trasformazione del costrutto (pAN52-4 + cDNA POXA1b o 1H6C) con il vettore pAB4-1 contenente il gene *pyrG* che permetteva la selezione dei corretti trasformanti. I ricombinanti esprimenti le laccasi di interesse sono state selezionate sia per la presenza di aloni verdi sulle piastre (data la presenza di ABTS nel terreno di crescita), sia mediante saggi in piastre multi pozzetto di campioni di micelio. Ottenuti i ricombinanti si è proceduto alla crescita dei funghi trasformati in colture liquide. Dopo otto giorni di crescita, i migliori cloni di rPOXA1b avevano livelli di produzione fino 400 U/ml, mentre i livelli di attività dei cloni esprimenti r1H6C raggiungevano fino a 550 U/ml. Analisi elettroforetiche mediante gel di polacrilammide SDS mostrano la presenza di una chiara banda a circa 80 KDa che, in seguito ad esperimenti di immunorivelazione utilizzando anticorpi contro la laccasi POXA1b da *P. ostreatus*, si è dimostrata essere relatva alla proteina di interesse. Le laccasi ricombinanti mostrano un incremento del peso molecolare di circa 20 KDa rispetto alla proteina *wt*, dovuto ad una blanda glicosilazione della proteina.

L'espressione della laccasi da *P. ostreatus* POXA1b e del suo migliore mutante 1H6C nel fungo filamentoso *A. niger* ha permesso di ottenere una overespressione delle proteine di interesse fino a 100 volte maggiore rispetto alla produzione delle stesse in altri sistemi di espressione (quali i lieviti *S. cerevisiae* e *K. lactis*). La purificazione di questi enzimi consentirà di avere a disposizione quantitativi di proteina tali da poter effettuare una maggiore e più completa caratterizzazione strutturale e funzionale delle proteine di interesse.

L’evoluzione naturale di un enzima resta un processo biologico fondamentale, per lo più incompreso, e l’evoluzione guidata, che si basa sugli stessi principi, risulta essere un’arte fortunata piuttosto che una scienza. Le laccasi in particolare, sono proteine estremamente conservate, con ampie regioni necessarie per la loro funzionalità, e presentano dunque relativamente poche posizioni mutabili senza che l’attività venga persa. Il presente lavoro riporta la costruzione di mutanti di laccasi mediante EP-PCR e mediante un approccio semi razionale con la successiva identificazione e caratterizzazione di sei “nuove” laccasi. I residui mutati presenti nelle nuove laccasi, anche se non direttamente coinvolti nella catalisi, influenzano l’efficienza e la stabilità dell’enzima, e consentono, dunque, di incrementare la comprensione delle relazioni struttura-funzione di questa classe di enzimi. La selezione di queste nuove laccasi consente di ipotizzare lo sviluppo di fenolo ossidasi sempre più “*directedly evolved*” per una specifica applicazione industriale.

Introduction

Healthcare, environmental protection, food security and safety and energy supply are only some of the social problems to which Biotechnology offers full or partial solutions. Biotechnology has the potential both to allow a truly sustainable development and to contribute to the value creation in all sectors of society.

Industrial Biotechnology, in Europe also known as White Biotechnology, is the modern use and application of biotechnology for the sustainable processing and production of chemicals, materials and fuels. Biotechnological processing uses enzymes, micro-organisms and plants to make products in a wide range of industrial sectors including chemicals, pharmaceuticals, food and feed, paper and pulp, textiles, energy, materials and polymers. Mankind has already benefited from biotech for a long time, but with the evolution of new technologies and a much deeper understanding of cell metabolism and materials science, many new opportunities have been identified, and others are continuing to emerge.

There are many examples of products obtained through Biotechnology, already being on the market, such as biopolymer fibres for household applications (e.g. carpeting), biodegradable plastics made from corn, biofuels, lubricants and industrial enzymes used in detergents and in the paper and food processing industries. Biotechnology also forms the basis for the production of some antibiotics, vitamins, amino acids and other fine chemicals. European companies are world leaders in a number of Industrial Biotechnology sectors. For example, they lead the development and production of industrial enzymes. Some of these enzymes are used in detergents, allowing lower washing temperatures and reducing the consumption of water and energy, and others are creating new opportunities for the production of fine chemicals via biotechnological processes. Other examples of Biotechnology applications include using plant-based renewable resources to produce biofuels such as bioethanol or biogas, which helps to reduce carbon emissions from the transport sector.

The increasing interest in the sustainability of industrial processes has also contributed to biotechnology's attractiveness. All major facets of European society and economy, including agriculture, environmental protection and manufacturing industry [1,2] are being challenged to demonstrate their sustainability.

• **Impact on the Society**

Since White Biotechnology makes industry more sustainable, it is expected that the benefits will be seen across a wide range of social dimensions: creation of knowledge driven and new jobs, development of original technology platforms as a basis for innovation, and a reduction of society's dependence on valuable fossil resources.

• **Impact on the Environment**

We have a responsibility to leave a clean and productive environment and healthy eco-systems for future generations. Biotechnology offers new ways to improve the environmental impact of industrial processes in various sectors. It can contribute to reduce energy consumption and waste production and to achieve sustainable industrial and societal development. Biomass can be exploited as a novel feedstock for efficient conversion into high added-value products such as complex intermediates for the pharmaceutical industry. Industrial biotechnology can maximize the economic value of current waste and by-product streams through new and potentially energy-saving bio-processes, at the same time reducing net carbon emissions. The wide production and use of bioproducts can therefore have a considerable impact on industry's GHG emissions. Biotechnology also has the potential to detect, monitor, prevent, treat and remove pollution.

• **Impact on the Economy**

In parallel, the economy will benefit as biotechnology enables the introduction of more efficient and less energy-intensive processes. Global industrial enzyme sales were estimated at $2.3 billion in 2003 [3]. Among the outcoming market segments that involve enzymes application, there are polymers, bulk chemicals and bio-fuels. Some recent reports (such as those by BCC Inc and Freedonia [4,5]) predict annual growth rates of nearly 5% for fermentation products (compared to 2-3% for overall chemical production) in the coming years, while others (such as the one by McKinsey & Company [6]) predict that by 2010 biobased products (products made from biobased feedstocks or through fermentation or enzymatic conversion) will account for 10-20% of sales within the chemical industry, accounting for $125 billion in value. Although numbers may differ, all studies agree that, in the future, industrial biotechnology will play an increasingly significant role in the chemical and other manufacturing industries.

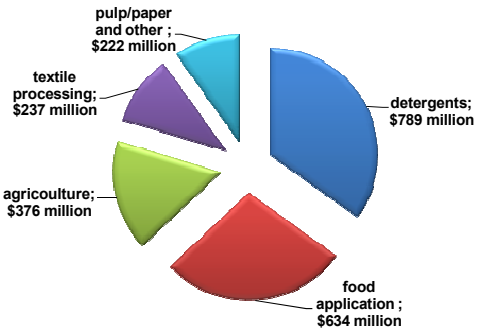

Fig.1 The main profits of global industrial enzyme in 2003 [3]

• **Impact on Agriculture**

Industrial Biotechnology can also make a major contribution to making agriculture more competitive and sustainable. The farmland of the future could produce not only sufficient food and feed as it does currently, but also chemicals, industrial raw materials and fuels. Over time, this could transform the farming and rural economies.

Industrial biotechnology is a relatively new discipline and therefore immature: there are major areas of knowledge to be still explored. That represents a bottleneck to greater exploitation, but also offers great opportunities for further research and break-through innovation. Both basic and applied sciences are essential, the first to develop our fundamental knowledge base, and the second to introduce innovative products and processes based on this knowledge.

Seven major areas of research and technology have been jointly identified by the stakeholders:

1. Novel enzymes and micro-organisms
2. Microbial genomics and bio-informatics
3. Metabolic engineering and modelling
4. Biocatalyst function and optimisation
5. Biocatalytic process design
6. Fermentation science and engineering
7. Innovative downstream processing

It is important to see these as inter-connected components in a cohesive and integrated overall programme of work. Industrial Biotechnology is a multi-disciplinary area, comprising biology, microbiology, plant sciences, biochemistry, molecular biotechnology, chemistry, bioinformatics, engineering, etc.. Good contacts and coordination among these disciplines, including the formation of multi-disciplinary project teams, are therefore crucial to create synergies to unleash Industrial Biotechnology's true potential and allow it to become a real driver of innovation and sustainability.

1. The ideal biocatalyst

For any industrial application, a good biocatalyst needs to comply with specific parameters as stability at pH and temperature, appropiate substrate specificity and so on (Fig.2) [7]. The main limit in the industrial use of enzyme is the difficulty to find a natural enzyme that is perfectly in keeping with process requirements.

As a main tool to overcome this problem, protein engineering [8] allows altering the structure of an existing protein to improve its properties. It is an important technology that increases our basic understanding of how enzymes function and have evolved. It is the key method of improving enzyme properties for applications in pharmaceuticals, green chemistry and biofuels. Different strategies of protein engineering can be exploited. Looking at individual cases can make the benefits or motivation of selecting a particular strategy for a particular problem very clear (Fig.3).

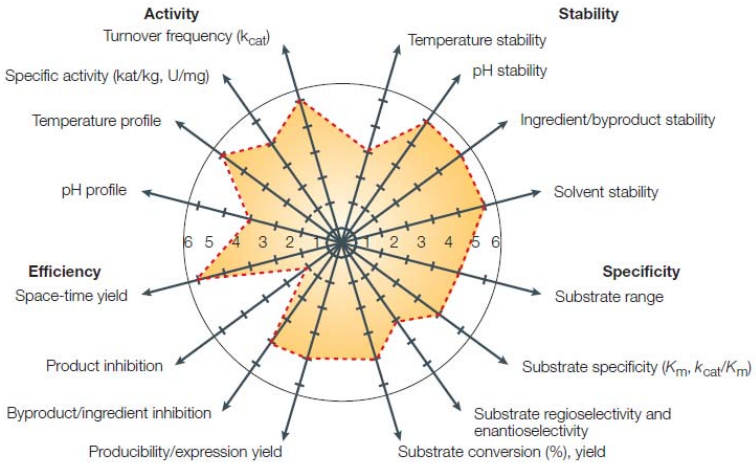

Fig.2 Multi-parameter footprint analysis. This figure illustrates the ideal biocatalyst concept. Each enzyme is ranked, from low (rating of 1) to high (rating of 6) using a specific set of criteria, to produce a multi-parameter fingerprint (shown in yellow). Criteria include *in vitro* enzyme activity, efficiency, specificity and stability. This decision matrix reveals the strengths and weaknesses of every candidate enzyme, so that the most promising candidate enzymes from diverse enzyme libraries can be selected for further process development by re-screening, protein engineering or directed evolution methods. kat, catalytic reaction rate; kcat, catalytic constant; Km, Michaelis constant; U, unit. (Lorenz & Eck, 2005)

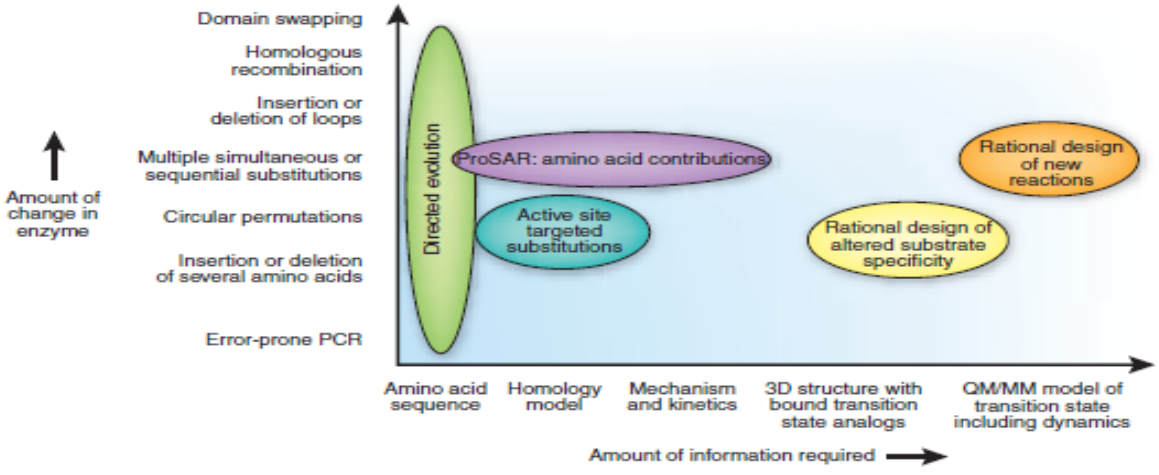

Fig.3 Protein engineering methods differ widely based on the degree that the enzyme is changed and the amount of information available for rational design. 3D, three-dimensional; QM/MM, quantum mechanics or molecular mechanics. (Kazlauskas & Bornscheuer, 2009)

Protein engineering involves three steps: designing the strategy for protein changes (engineering strategies, such as rational design or randomization), making those changes (mutagenesis) and evaluating the protein variants for improved properties (screening or selection). In each of these steps, selecting different strategies can lead to different advantages or disadvantages. For example, there is a debate between the effort devoted to rational design versus screening. At one extreme, rational design starting from X-ray crystal structures of a well understood enzyme can limit the screening effort to a few amino acid substitutions. This approach has been applied increasing the enantioselectivity of a phosphotriesterase by making substrate-binding pockets bigger or smaller as needed [9]. At the other extreme, a powerful screening method can completely eliminate the rational design step. Selection has identified protein variants with RNA ligase activity from a completely random, but very large library [10].

Identifying which protein engineering strategy is the best one and why mutations work, the protein engineering will advance more rapidly. If we compare strategies, future solutions will require less effort. It is unlikely that one strategy will prevail, as each problem differs in the goal, amount of information available and specifics of the protein. Nevertheless, some strategies will be better than others. Comparisons will also establish principles of protein engineering and increase our understanding of how enzymes work [11].

Among the numerous 'green' catalysts that can be 'modified' to improve their industrial versatility laccases represent one of these 'ecofriendly' enzymes. Their uses span from the textile to the pulp and paper industries, from food applications to bioremediation processes, therefore, attracting much interest in recent years.

2. Laccases: general features

2.1 Distribution

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper oxidase (MCO) family, along with such different proteins as plant ascorbic oxidase, mammalian ceruloplasmin or Fet3p ferroxidase from *Saccharomyces cerevisiae* [12]. These copper-containing enzymes catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water [13].

Laccase is one of the oldest enzymes so far described. Yoshida first discovered laccases in 1883 after observing that latex from the Japanese lacquer tree (*Rhus vernicifera*) hardened in the presence of air [14,15]. Later, it was found in certain other plants, in many insects, and in a variety of fungi, including yeasts (e.g., *Cryptococcus*), molds (e.g., Penicillium), mushrooms (e.g., *Agaricus*), and white-rot fungi (e.g., *Pleurotus*). It is largery widespread in ligninolytic basidiomycetes (*Phanerochaete, Trametes, Pycnoporus, Nematoloma, Sporotrichum, Stropharia*, etc.), and more than 125 different basidiomycetous laccase genes have been so far described [16]. The occurrence of laccase in prokaryotes [17] seems to be restricted to certain species (within the genera *Aquifex, Pyrobaculum, Azospirillum, Sinorhizobium, Marinomonas, Ralstonia, Streptomyces, or Bacillus*). Recently a novel polyphenol oxidase with laccase like activity was mined from a metagenome expression library from bovine rumen microflora [18].

Among the several different biological roles so far ascribed to laccases [19], they have a main role in lignin degradation, together with lignin and manganese

peroxidase [20,21]. Lignin, which provides the structural component to the plant cell wall, is a heterogeneous and complex biopolymer that consists of phenyl propanoid units linked by various non-hydrolyzable C-C and C-O bonds [22]. As a result of laccase oxidation, radicals (cationic) can be generated in lignin, and these can cause subsequent aliphatic or aromatic C–C bond cleavage and lignin depolymerization [23-27].

2.2 Biochemical features

Laccases catalyse the one-electron oxidation of four substrate equivalents, coupled to the four-electron reduction of O_2 to water. The catalysis carried out by all members of this family is guaranteed by the presence of three different copper centres, classified on the basis of their spectroscopic properties: one type-1 (T1) copper (Cu1), one type-2 (T2) and two type-3 (T3) copper ions (Cu2 and Cu3), with Cu2 and Cu3 arranged in a trinuclear cluster (TNC) (Fig.4(a)-(b)) [28].

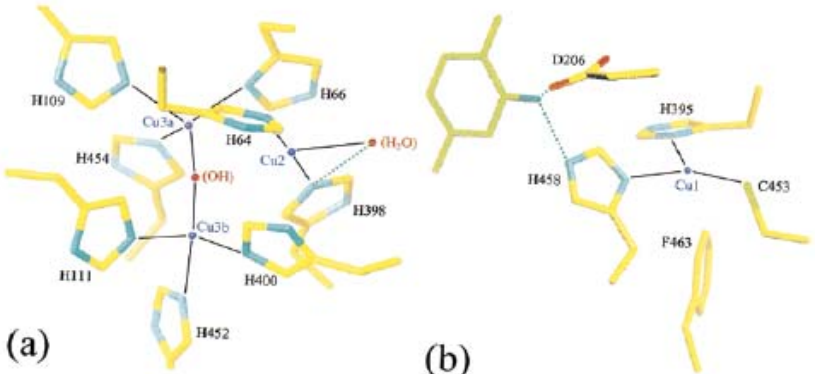

Fig.4 Laccase copper sites and their environment. (a) The trinuclear T2/T3 site. Copper coordinations are indicated with dark lines. (b) The T1 site and the neighbouring 2,5-xylidine ligand (green model). Image taken from reference [28].

Two histidines and one cysteine serve as ligands for type-1 Cu. While usually in type-1 centers of other blue copper protein, a sulfur from a methionine functions as an additional axial ligand, the fourth ligand for this copper center is not present in fungal laccases, whereas an uncoordinating Phe or Leu occupies this position [29]. Type 1 copper confers the typical blue colour to laccases, which results from the intense electronic absorption at 600nm due to the covalent copper-cysteine bond. Type 1 copper is the site where substrate oxidation of aromatic takes place. Type 2 site is characterized by the lack of strong absorption features in the visible region and by usual EPR spectra. It is strategically positioned close to the Type 3 copper, a binuclear center, spettroscopically characterized by an electron adsorption at 330nm (oxidized form). Type 2 and Type 3 copper ions form a trinuclear cluster, where reduction of molecular oxygen and release of water take place (Fig.5). Type 2 copper is coordinated by two histidines and Type 3 copper atoms by six histidines. The two copper ions of the T3 site are EPR-silent due to an antiferromagnetic coupling mediated by a bridging hydroxyl ligand. Substrates (phenols and aromatic or aliphatic amines) are oxidized by

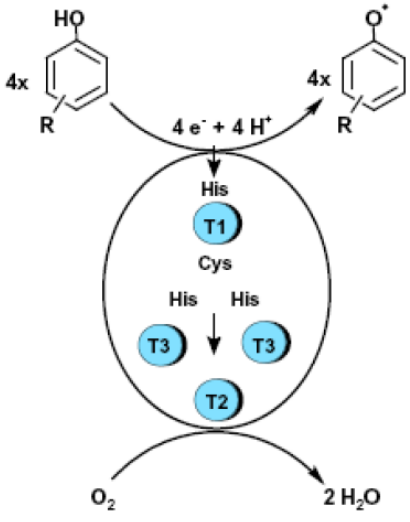

Fig.5 Catalytic cycle of laccase

the T1 copper to produce radicals that can then produce dimers, oligomers and polymers. The extracted electrons are transferred, probably through a strongly conserved His-Cys-His tripeptide motif, to the T2/T3 site, where molecular oxygen is reduced to water [30].

Despite the amount of information on laccases as well as on other blue multicopper oxidases, neither the precise electron transfer pathway nor the details of dioxygen reduction in blue multicopper oxidases are fully understood [31].

Kinetic data suggest a mechanism of reaction “two site ping-pong bi bi” type, according to which the products are released before a new substrate molecule is ligated [32].

2.3 Structural properties

Current knowledge about the structure and physico-chemical properties of fungal proteins is based on the study of purified proteins. Up to now, more than 100 laccases have been purified and characterized from fungi. Based on the literature, general characteristics of these enzymes can be drawn out. Typical basidiomycetes fungal laccase is a monomeric protein of approximately 60–70 kDa with an acidic isoelectric point around pH 4.0 (Tab.1 [33]). It seems that there is considerable heterogeneity in the properties of laccases isolated from ascomycetes, especially with respect to molecular weight.

Property	n	Median	Q_{25}	Q_{75}	Min	Max
Molecular weight (Da)	103	66,000	61,000	71,000	43,000	383,000
pI	67	3.9	3.5	4.2	2.6	6.9
Temperature optimum (°C)	39	55	50	70	25	80
pH optimum						
ABTS	49	3.0	2.5	4.0	2.0	5.0
2,6-Dimethixyphenol	36	4.0	3.0	5.5	3.0	8.0
Guaiacol	24	4.5	4.0	6.0	3.0	7.0
Syringaldazine	31	6.0	4.7	6.0	3.5	7.0
K_M (µM)						
ABTS	36	39	18	100	4	770
2,6-Dimethixyphenol	30	405	100	880	26	14,720
Guaiacol	23	420	121	1600	4	30,000
Syringaldazine	21	36	11	131	3	4,307
K_{cat} (s^{-1})						
ABTS	12	24,050	5220	41,460	198	350,000
2,6-Dimethixyphenol	12	3,680	815	6,000	100	360,000
Guaiacol	10	295	115	3,960	90	10,800
Syringaldazine	4	21,500	18,400	25,500	16,800	28,000

Tab.1 Properties of fungal laccases: n, number of observations; Q25, lower quartile; Q75, upper quartile (Baldrian, 2006)

A common feature of all plant and fungal laccases is glycosylation. The carbohydrate moiety of the majority of laccases consists of mannose, N-acetylglucosamine, and galactose and constitutes about 45% of the protein mass in laccases of plant origin and about 10-20% of fungal ones. Glycosylation of fungal laccases is one of the main problems for their heterologous production. It was proposed that in addition to the structural role, glycosylation can also participate in the protection of laccase from proteolysis and inactivation by free radicals [34].

The presence of carbohydrate chains and the heterogeneity of laccase proteins were the major problems in the crystallization of laccase for X-ray analisis for a long time. However, recently, several laccase structures from *Coprinus cinereus* (in a copper Type 2-depleted form) [35-38], *Trametes versicolor* [39,40], *Melanocarpus albomyces* [41], *Bacillus subtilis* [42], *Pycnoporus cinnabarinus* [43], *Rigidoporus lignosus* [44], *Cerrana maxima* [45], have been determined, the last seven enzyme with a full complement of copper ions. All these laccases exhibit a similar molecular architecture organized in three sequentially arranged cupredoxin-like domain. Each of them has greek key β-barrel topology, strictly related to that of small copper protein like azurin and plastocyanin and common to all the members of the MCOs family.

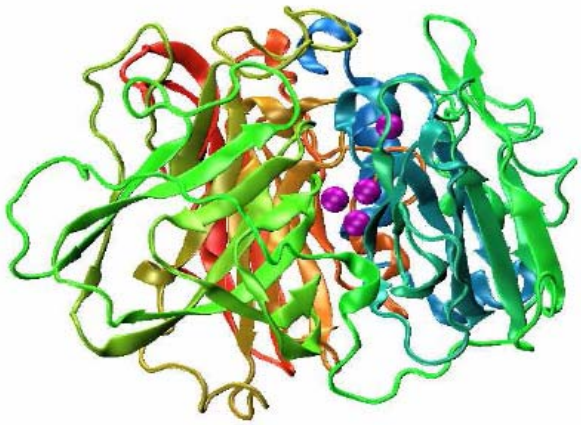

Fig.6 Robbinson representation of *Trametes versicolor* laccase X-ray structure. Domain 1 is showed in red, domain 2 in green and domain 3 in blue. Copper ions are show in vdW representation (Piotek, 2002)

The three domain are characterized by different distribution of β-sheet and elics (Fig.6). Domain 1 comprises two four-stranded β-sheets and four 3_{10}-helices. The second domain has one six-stranded and one five-stranded β-sheet. Finally, domain 3 consists of a β-barrel formed by two five-stranded β-sheets and a two-stranded β-sheets that, together with a α-helix and a β-turn, form the cavity in which the type-1 copper is located. A 3_{10}- helix between domains 2 and 3 forms part of a 40-residue-long extended loop region. The trinuclear copper cluster (T2/T3) is embedded between domains 1 and 3 with both domains providing residues for the coordination of the coppers. At the C-terminal of domain 3, three sequentially arranged α-helices complete the fold. An α-helix formed by 13 residues at the C-terminal end is stabilized by a disulfide bridge to domain 1 (Cys-85–Cys-488), and a second disulfide bridge (Cys-117–Cys-205) connects domains 1 and 2. Both N-terminal and C-terminal amino acids benefit from hydrogen bonding networks to the rest of the protein.

A question that is yet to be answered is to ascertain how these different MCO modulate their redox potential (E°) [46] at the structural level, although having very similar [47] or apparently equal copper coordination geometry [48]. Investigations into this issue are especially relevant in the case of laccases [48-50] because their cover such a wide range of E°s. Laccases are divided into “low” (500mV *versus* normal hydrogen electrode) and “high” (700-800mV) E° laccases. The high-redox potential laccases occur mainly in basidiomycetes, especially white-rot fungi [51-55], while the low-redox potential laccases seem to be widely distributed in molds [56], bacteria, insects, and plants. However, detailed studies by Klonowska et al. [57,58] have shown that the white-rot fungus *Trametes sp.* C30 possesses not only the high-redox potential laccase (LAC1) but also two additional low-redox potential laccases (LAC2 and LAC3) with minor activity.

A detailed structural comparison between a low redox potential *C. cinereus* laccase and a high E_0 *T. versicolor* laccase showed that structural differences of the

Cu1 coordination possibly account for the different E_0 values [39]. This was later confirmed by studies on *R. lignosus* laccase with a high redox potential [44]. However, more efforts will be needed to elucidate the relation between the structure of the catalytic site and the substrate preference of different laccase enzymes. These relations have important implication for laccase biotechnological implication and future manipulation of this property by protein engineering strategies.

3. Industrial applications

Laccases exhibit an extraordinary natural substrate range (phenols, polyphenols, anilines, aryl diamines, methoxysubstituted phenols, hydroxyindols, benzenethiols, inorganic/organic metal compounds and many others) which is the major reason for their attractiveness for several biotechnological applications [59-61]. Moreover, in the presence of small molecules, known as redox mediators, laccases enhance their substrate specificity. Indeed, laccase oxidizes the mediator and the generated radical oxidizes the substrate by mechanisms different from the enzymatic one, enabling the oxidative transformation of substrates with high redox potentials, otherwise not oxidized by the enzyme (Fig.7)[62].

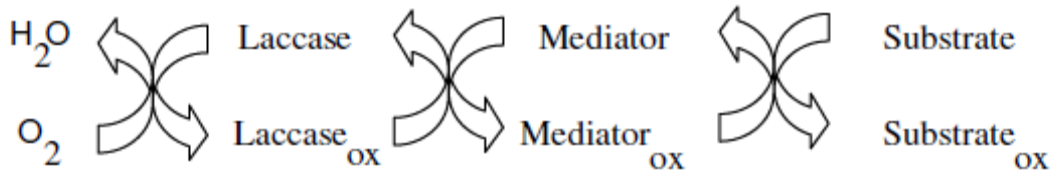

Fig.7 Expanded role of laccase oxidizing non-usual substrate by the action of redox mediator [62]

Few laccases are at present in market for textile, food and other industries, and more candidates have been developing actively for future commercialization. A vast amount of industrial applications for laccases have been proposed. Being specific, energy-saving, and biodegradable, laccase-based biocatalysts fit well with the development of highly efficient, sustainable, and eco-friendly industrial processes.

3.1 Pulp and paper industry

In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine- or oxygen-based chemical oxidants. Non-chlorine bleaching of pulp with laccase was first patented in 1994 using an enzyme treatment to obtain a brighter pulp with low lignin content [63]. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods. In spite of this new method, the pretreatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose [64-667]. Laccases are able to delignify pulp when they are used together with mediators [68]. The mediator is oxidized by laccase and the oxidized mediator molecule further oxidizes subunits of lignin that otherwise would not be laccase substrates [69,70]. Furthermore, the application of laccases in pulp-kraft bleaching may result in higher pulp yields and energy savings. Most of studies have been patented about the use of laccase mediator system (LMS) in the pulp-kraft bleaching processes [67,71-75]. More recently, the potential of this enzyme for cross-linking and functionalizing ligninaceous compounds was discovered [76,77]. Finally, laccases can be used for binding fiber-, particle- and paper-boards [78-80].

3.2 Textile industry

Laccase is used in commercial textile applications to improve the whiteness in conventional bleaching of cotton and the biostoning process [81]. Potential benefits of the application include chemicals, energy, and water saving. In 1996 Novozyme (Novo Nordisk, Denmark) launched a new industrial application of laccase enzyme in denim finishing: DeniLite®, the first industrial laccase and the first bleaching enzyme acting with the help of a mediator. Laccase also can be used in situ to convert dye precursors for better, more efficient fabric dyeing [82,83]. Laccases find potential applications for cleansing, such as cloth- and dishwashing [84]. Laccase may be included in a cleansing formulation to eliminate the odor on fabrics, including cloth, sofa surface, and curtain, or in a detergent to eliminate the odor generated during cloth washing [85,86]. Lantto et al. [87] found that wool fibers can be activated with LMS. Therefore, the use of laccase for anti-shrink treatment of wool seems very attractive.

3.3 Food industry

Many laccase substrates, such as carbohydrates, unsaturated fatty acids, phenols, and thiol-containing proteins, are important components of various foods and beverages. Their modification by laccase may lead to new functionality, quality improvement, or cost reduction [84]. Sometimes, O_2 is detrimental to the quality or storage of food/beverage because of unwanted oxidation. Laccases may be used as O_2-scavengers for better food packing [88]. The flavor quality of vegetable oils can be improved with laccase by eliminating dissolved oxygen [89]. Laccase can also deoxygenate food items partly or entirely derived from extracts of plant materials. Cacao was soaked in solutions containing laccase, dried and roasted in order to improve the flavor and taste of cacao and its products [90]. Various enzymatic treatments have been proposed for fruit juice stabilization, among which it can be found the use of laccase [91,92]. Laccases are added to the dough used for producing baked products, to exert an oxidizing effect on the dough constituents and to improve the strength of gluten structures in dough and/or baked products [93]. Wine stabilization is one of the main applications of laccase in the food industry as an alternative to physical-chemical adsorbents [94,95]. Musts and wines are complex mixtures of different chemical compounds, such as ethanol, organic acids (aroma), salts and phenolic compounds (color and taste). Polyphenol removal must be selective to avoid an undesirable alteration in the wine's organoleptic characteristics. Laccase presents some important features when used for the treatment of polyphenol elimination in wines, such as stability in acid medium and reversible inhibition with sulphite [96]. Laccases are also used to improve storage life of beer. Haze formation in beers is a persistent problem in the brewing industry. Nucleophilic substitution of phenolic rings by protein sulphydryl groups may lead to a permanent haze that does not re-dissolve when warmed. A laccase has recently been commercialized (Suberzyme®) for preparing cork stoppers for wine bottles [97].

3.4 Bioremediation

Laccases have many possible applications in bioremediation [98]. Laccases may be applied to degradation of various substances such as undesirable contaminants, by-products, or discarded materials. Laccase may be applied to degrading plastic waste having olefin units [99]. Laccase may also be used to eliminate odor emitted from places such as garbage disposal sites, livestock farms, or pulp mills [100]. Also, they could be used for decolorizing dye house effluents that are hardly decolorized

by conventional sewage treatment plants [101,102]. In addition to dye house effluents, laccases can decolorize waste waters from olive oil mills [103] and pulp mills [104] by removing colored phenolic compounds. Another potential environmental application for laccases is the bioremediation of contaminated soils, as laccases and LMS are able to oxidize toxic organic pollutants, such as various xenobiotics, PAHs, chlorophenols, and other contaminants [105-109]. Phenolic compounds are present in wastes from several industrial processes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others [110]. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants [111-114].

Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil. LMS has been extensively study in the oxidation of recalcitrant PAHs, main components of several ship spills. In this sense, LMS is being included in several enzymatic bioremediation programs [115].

3.5 Organic synthesis

Recently, increasing interest has been focused on the application of laccase as a new biocatalyst in organic synthesis [116]. Laccase provided an environmentally benign process of polymer production in air without the use of H_2O_2 [117,118].

Laccase-catalyzed cross-linking reaction of new urushiol analogues for the preparation of “artificial urushi” polymeric films (Japanese traditional coating) was demonstrated [119]. It is also mentioned that laccase induced radical polymerization of acrylamide with or without mediator [120]. Laccases are also known to polymerize various amino and phenolic compounds [121-123]. Recently, to improve the production of fuel ethanol from renewable raw materials, laccase from *T. versicolor* was expressed in *S. cerevisiae* to increase its resistance to (phenolic) fermentation inhibitors in lignocellulose hydrolyzates [124]. The enzymatic preparation of polymeric polyphenols by the action of laccases has been investigated extensively in the past decades as a viable and non-toxic alternative to the usual formaldehyde-based chemical production of these compounds [125,126].

3.6 Pharmaceutical sector

Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents. Due to their specificity and bio-based nature, potential applications of laccases in the field are attracting active research efforts. Laccase can be used in the synthesis of complex medical compounds as anesthetics, antiinflammatory, antibiotics, sedatives, etc. [127], including triazolo(benzo)cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline [128].

One potential application is laccase-based *in situ* generation of iodine, a reagent widely used as disinfectant [129,130]. Also, laccase has been reported to possess significant HIV-1 reverse transcriptase inhibitor activity [131].

A novel application field for laccases is in cosmetics and deodorants for personal-hygiene products, including toothpaste, mouthwash, detergent, soap, and diapers.

3.7 Nanobiotechnology

Nanoscience has grown rapidly in the last decade. Recently, more attention is focused on the applications of nanotechnologies. The high potential impacts of nanotechnology almost cover all fields of human activity (environmental, economy,

industrial, clinical, health-related, etc). Nanostructured materials (nanoparticles, nanotubes, and nanofibers) have been used extensively as carrying materials for biosensoring, and biofuel cells.

Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, realtime analysis and operation simplicity [132]. A number of biosensors containing laccase have been developed for immunoassays, and for determination of glucose, aromatic amines and phenolic compounds [133-137]. Laccase catalysis can be used to assay other enzymes. Laccase covalently conjugated to a biobinding molecule can be used as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical, or nucleic acid-detection assays. The bioreporter applications are of interest for the high-sensitivity diagnostic field. In addition to biosensors, laccases could be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems [136,137].

4. Heterologous expression of fungal laccases

Biotechnological applications require large amounts of enzymes. Laccases secreted from wild-type fungal organisms may not be suitable for commercial purposes mainly because the low yields and undesirable preparation procedures (such as presence of toxic inducers) are not economically advantageous. However, recent advances in bioreactor design and culture conditions have significantly increased the production yields [138].

Heterologous expression should be better suited for large-scale production, because of the potential of expressing different laccases in one selected optimised host. Laccases, like other oxidative enzymes, are difficult to express in non-fungal systems. There is a considerable variability of production yields with respect both to cDNAs expressed (originating from the same organism) and to the host used for the heterologous expression. As a fact, LCC1 laccase from *T. versicolor* was heterologously expressed in *Pichia pastoris* [139] and not in *S. cerevisiae* [140]. Moreover, when *S. cerevisiae* was used as host, it was possible to note different level of expression between cDNAs originating from the same organism [140,141]. Such “selectivity” in expression may reflect the inability of yeast to process different laccases post-translationally with the same efficiency [141]. Furthermore, these variable yields hinder forecast of the most suitable host, or the most promising laccase to express.

Another current challenge for industrial applications is to improve the catalytic properties of natural laccases. For this purpose, the yeast *S. cerevisiae* has already been successfully used for directed evolution studies of a fungal laccase [142].

In order to get new insights into the structure/function/stability relationships of laccases, and with the aim to use directional evolution to create improved laccases with desirable physicochemical characters like a higher redox potential, a neutral or alkaline optimal pH, and a thermostability, recombinant expression of laccases in heterologous systems is fundamental.

4.1 Laccase engineering

Rational design and directed evolution are the two most common ‘protein engineering’ systems.

Based on laccase structures, over the last decade several residues in the neighbourhood of the catalytic copper ions have been subjected to site-directed

mutagenesis to determine the parameters that define the catalytic activity and the E° of fungal laccases [143,144].

To overcome many of the limitations of the rational design, and in the absence of enough structural information, directed molecular evolution represents a promising alternative. This methodology recreates in the laboratory the key events of natural evolution (mutation, recombination and selection) doing in such a manner those more efficient enzymes, even with novel functions, can be tailored. Diversity is mimicked by inducing mutations and/or recombination in the gene encoding a specific protein. Afterwards, the best performers in each generation are selected and further used as the parental types for a new round of evolution. The process is repeated as many times as necessary, enhancing exponentially the targeted features, until a biocatalyst with the desired traits is obtained: stability at high temperature or in organic solvents; improved catalytic activities; higher specificity; etc. A thorough understanding of efficient and reliable highthroughput screening methodologies is a prerequisite for the design and validation of this type of experiments [145]. A key query result of *smart* laboratory evolution is the improvement of several enzymatic properties at the same time (e.g. stability and activity). The first successful example of directed laccase evolution reported came from Arnold's group [142]. They carried out the functional expression of a thermophilic laccase in *S. cerevisiae* by directed evolution: after ten rounds of laboratory evolution and screening, a 170- fold improvement of the total enzymatic activity along with better performances at high temperatures was achieved.

Besides methods that involve iterative steps of random mutagenesis and/or DNA recombination, semi-rational studies – which take advantage from both protein structure and combinatorial libraries constructed by saturation mutagenesis – are being employed successfully. This approach consist in the 'rational' fusion of the identified serendipitous mutations. It can be employed to analyze the interaction of mutations selected by random methods and fused in order to evaluate and identify their possible interactions and synergies and to have structural information that are not immediately obtained with the classic methods.

5. Laccases from *Pleurotus ostreatus*

The white-rot fungus *P. ostreatus* is able to express multiple laccase genes encoding isoenzymes with relevant properties considering both industrial applications and structure-function studies. Amount of each specific enzyme produced depends on culture conditions. So far, five isoenzymes secreted by the mycelium have been purified and characterized: POXC [146], POXA1w [147], POXA1b [148], and, more recently, the two strictly related isoenzymes POXA3a and POXA3b [149]. POXC is the most abundantly produced in all growth conditions tested so far; POXA1w shows peculiar differences with regard to metal ions content, containing two zinc atoms, one iron atom, and only one copper atom per molecule; POXA1b is the most stable at alkaline pH and shows activity inside the cell or on the cell wall [150]; POXA3a and POXA3b are heterodimeric laccases [149]. Studies on laccase encoding genes have also led to the identification of four different genes and of the corresponding cDNAs, *poxc* (previously named *pox2*) [151], *pox1* (which codes for a laccase isoenzyme not identified yet) [152], *poxa1b* and *poxa3* [148,149].

P. ostreatus was shown to be able to decolorize industrial dye. First experiments were performed using a representative antraquinonic dye, RBBR, in solid and liquid culture and the key role played by laccases in this process has been demonstrated

[153]. Successively, decolourization experiments with *P. ostreatus* whole cells and crude laccase mixtures were achieved for biodegradation of model waste water from textile industry [154,155].

For the relevant roles in biotechnological applications of this fungus and in particular of its laccases, heterologous expressions was performed. *P. ostreatus* POXC and POXA1b laccases were expresses in two yeasts, *Kluyveromyces lactis* and *Saccharomyces cerevisiae* [156]. Both transformed hosts secreted recombinant active laccases, although *K. lactis* was much more effective than *S. cerevisiae*. The availability of the established recombinant expression systems allows the development of new oxidative catalysts through molecular evolution techniques.

POXA1b was used as a scaffold to carry out directed evolution [157], using Errore Prone PCR and DNA shuffling methods. Mutated cDNAs were expressed in *S. cerevisiae*. After two generation of mutation, a library of 2300 variants of POXA1b laccase was obtained. The collection was screened by assaying activity towards the non phenolic 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate, and four evolved forms with increased activity were selected [157].

6. The thesis

The development of new bio-based processes using laccases requires deeper understanding of the structure/function relationships and the 'invention' of better performing molecules, more suited to industrial applications.

In this context, this research has been aimed to develop new laccases from *P. osreatus* for industrial application.

The main subjects which this work dealt with are:

1) New criteria of selection were applied in a further screening of the already available 2300 mutants library, using different substrates (2,6 dimethoxyphenol, DMP) and assaying the enzyme stability and activity at different operating conditions (different pHs). Characterizations, from structural, catalytic and thermodymamic points of view of the three new selected mutants is reported.

2) A new mutant was rationally designed and constructed. This new mutant, R4, contains the mutations of the two parental enzymes (1M10B and 3M7C) previously selected [157]. On the basis of improved properties of stability, this new clone was used as template for producing a new collection of 1100 mutants. Two new mutants were selected for their improved activity against ABTS. Thermodynamic and catalytic characterization of these mutants is described.

3) A new system for overproduction by recombinant expression was developed. POXA1b and his best variant 1H6C have been expressed in the filamentous fungi *A. niger*, obtaining an improvement of 100- fold in the enzyme production*.

* Work carried out in collaboration with Dr. Eric RECORD of the Institut National de la Recherche Agronomique (INRA) - University of Science of Luminy, Marseille (FRANCE)

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Chapter 1

1. Introduction

Laccases are intensely studied for their potential uses in industrial processes. They generally work under mild conditions: room temperature and atmospheric pressure, with water as solvent [1-4]. For industrial use, the current challenge is to obtain both enhanced expression levels and improved laccases with desirable physicochemical characters such as a higher redox potential, optimal activity at neutral or alkaline pH, and thermostability [5]. Strategies to obtain such variants include natural biodiversity screening and optimization of nature-derived scaffolds.

Mutagenesis (rational or random) is often used to generate laccase variants. In their pioneering work, Xu et al. [6] have reported significant changes in pH optimum, KM and kcat for triply mutated fungal laccases. A similar improvement factor was also reported for variants found in simple libraries of *in vitro* randomly generated mutants from *Formes lignosus* [7] or *Pleurotus ostreatus* [8].

P. ostreatus laccases are a heterogeneous set of phenol-oxidases. In particular, POXC – the most abundantly produced isoenzyme in all growth conditions tested so far – and POXA1b – the most stable at alkaline pH – were successfully heterologously expressed in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* [9]. The availability of established recombinant expression systems allows the development of new oxidative catalysts through molecular evolution techniques.

Random mutagenesis experiments on *poxc* and *poxa1b* cDNAs have been performed, using error prone PCR (EP-PCR), the most frequently used non recombinative method, and DNA shuffling, the most traditional among recombinative methods. After transformation into *S. cerevisiae*, a first library of 1100 clones has been obtained and screened. The POXA1b mutant named 1M9B selected from the screening of the first library, is more active toward ABTS but less stable with respect to POXA1b in all the analyzed conditions. This mutant has been used as a template for a second round of EP-PCR. From the library of 1200 clones obtained, three mutants have been selected (1L2B, 1M10B, and 3M7C), partially purified, and characterized. These mutants, besides improved activity toward phenolic and nonphenolic substrates, exhibit stability higher than that of POXA1b in all the conditions analyzed.

Aim of this work has been to select new laccases with improved performances of stability and activity at different operating conditions. The collection of 2300 mutants of POXA1b laccase was screened using different *criteria* of selection. The first strategy is based on the selection of laccases more active than the wild type in the degradation of phenolic substrate as 2,6 dimethoxyphenol (DMP) – widely used as a substrate of laccases catalysis and activity determination in aqueous solution.The second strategy is the selection of mutants with improved catalytic efficiency against ABTS – an excellent substrate for its low detection limit and stability in aqueous solution – at pH5. Previously studies are shown that the activity of POXA1b already decreases at pH5.

The development of new bio-based processes using laccases requires deeper understanding of the structure/function relationship of native enzymes and the ‘finding’ of novel an improved molecules that are better suited for industrial applications. The selections of these ‘better performing’ laccases through two news strategies of screening and their characterization from a structural and functional

point of view could give informations about the molecular determinants in the mechanism of functioning of laccases form *P. ostreatus*.

2. Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation

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ORIGINAL ARTICLE

Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation

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Abstract

Aims: To select better performing laccase variants among the 2300 randomly mutated variants of *Pleurotus ostreatus* POXA1b laccase to develop improved laccase-based biocatalysts.

Methods and Results: Screening of collections of 2300 randomly mutated variants of POXA1b was performed by assaying activity towards the phenolic substrate 2,6-dimethoxyphenol. Two new variants endowed with higher enzyme activity than the wild-type laccase were characterized, and their ability to decolourize industrial dyes with complex trisazo-, polyazo- and stilbene-type structures, in the absence of mediators, was demonstrated. One of the mutants (2L4A) was also proved to be highly stable at both acidic and alkaline pH values (displaying a half-life of around 1 month at the pH levels of both 5 and 10).

Conclusions: In comparison with the wild-type laccase, the new selected 2L4A mutant shows a significant increase in stability at acidic pH, whilst storing its high stability at alkaline pH. This variant also represents a more versatile enzyme with respect to both the variety of xenobiotics degraded and the operative conditions.

Significance and Impact of the Study: This work represents the first example of improvement of a basidiomycete laccase for industrial effluents bioremediation by directed evolution.

Introduction

Laccases (*p*-diphenol: dioxygen oxidoreductases, EC 1.10.3.2) are multi-copper proteins using molecular oxygen to oxidize a wide range of aromatic compounds (Thurston 1994; Ullah *et al.* 2000). These enzymes contain two distinct metal active sites: the T1 single copper site and the T2/T3 trinuclear copper site. Ligands for copper binding at the T1 centre are two histidines and one cysteine, and ligands at the T2/T3 cluster are eight histidines (Bertrand *et al.* 2002; Hakulinen *et al.* 2002; Piontek *et al.* 2002). The T1 active site is close to the external surface where organic substrates (mainly phenols or arylamines) are oxidized releasing one electron to the T1 copper ion. The electrons taken from the substrates are transferred, through two intramolecular electron transfer pathways (Kyritsis *et al.* 1993) – formed by CuT1 coordinating Cys and by two His residues coordinating the two CuT3 – from the T1 copper ion to the second active site, where the oxygen molecules bind, and are reduced to water molecules.

Owing to their high nonspecific oxidation capacity, laccases are useful biocatalysts for several biotechnological applications, such as pulp bleaching in paper industry, textile dye decolourization, detoxification of environmental pollutants and organic synthesis (Mayer and Staples 2002). For these applications, the most suitable laccase for each specific purpose should be found and produced. Many efforts are being devoted to the modification of enzyme activities to meet the needs of green chemistry industry (Chica *et al.* 2005; Alcalde *et al.* 2006) and to create tailored biocatalysts by protein engineering of wild-type

enzymes (Bornscheuer and Pohl 2001). Directed evolution mimics the processes of Darwinian evolution combining random mutagenesis and/or recombination with screening for enzyme variants with desired improvements, not requiring information about how the enzyme structure relates to function. The development of a high-throughput screening methodology is the main limitation of directed evolution, because many mutants must be screened to select a suitable variant (Williams *et al.* 2004).

The laccase family of the basidiomycete fungus *Pleurotus ostreatus* represents a variegated group of enzymes (Pezzella *et al.* 2009), having relevant roles in biotechnological applications of this fungus (Palmieri *et al.* 2005a,b; Faraco *et al.* 2009a,b). Within *P. ostreatus* laccase family, POXA1b (Giardina *et al.* 1999) was considered a suitable platform for the molecular evolution strategy for different reasons. An active form of POXA1b is expressed in *Saccharomyces cerevisiae* (Piscitelli *et al.* 2005). This laccase is the most thermostable isoenzyme among *P. ostreatus* laccases ($t_{\frac{1}{2}}$ at 60°C = 3 h) and exhibits a notably high stability at alkaline pH ($t_{\frac{1}{2}}$ at pH 10 = 30 days). POXA1b has been already shown to be an appropriate scaffold to carry out directed evolution (Festa *et al.* 2007), coherently with the original theory (Bloom *et al.* 2006) that supports the direct relationship between thermostability, mutational robustness and evolutionary capacity. Thus, thermostable enzymes should be more susceptible to evolution because they are better able to tolerate functionally beneficial but destabilizing mutations. As a matter of fact, when a library of 2300 randomly mutated variants of POXA1b laccase was screened by assaying activity towards the nonphenolic 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate, evolved forms with increased activity were selected (Festa *et al.* 2007).

To enlarge the panel of laccase-based biocatalysts, new selection rounds have been performed on the POXA1b mutants library, by assaying activity towards the phenolic 2,6-dimethoxyphenol (DMP) substrate. Two new POXA1b variants, endowed with higher enzyme activity than the wild-type enzyme on DMP, were characterized, and their ability to degrade dye with a complex stilbene-type structure, as well as the dyes with trisazo and polyazo structures decolourized by the wild-type enzyme, was verified. One of the new mutants selected for improved activity was also proved to be highly stable at both acidic and alkaline pH values.

Materials and methods

Library screening

Single clones grown on plate were picked and transferred into 96-well plates, containing 30 μl of selective medium per well. Plates were incubated at 28°C, 250 rev min^{-1} for 24 h. After 24 h, 130 μl of selective medium was added to each well, and the plates were incubated at 28°C, 250 rev min^{-1} for 24 h. Thirty microlitres of each culture was transferred to a new 96-well plate to measure the OD_{600} value. The plates were then centrifuged for 10 min at 1500 ***g***, 4°C, and a suitable volume of supernatant was transferred to a new 96-well plate to perform laccase assay. Phenol oxidase activity was assayed at 25°C using 2 mmol l^{-1} ABTS in 0·1 mol l^{-1} sodium citrate buffer, pH 3·0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36 000 M^{-1} cm^{-1}), using Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). Enzyme activity was expressed in international units (U).

Cultures were also performed in shaken flasks. Precultures (10 ml) were grown in selective medium at 28°C on a rotary shaker (150 rev min^{-1}). A volume of suspension sufficient to reach a final OD_{600} value of 0·5 was then used to inoculate 250-ml Erlenmeyer flasks containing 50 ml of selective medium, and cells were then grown in a rotary shaker. Optical density and laccase activity determination were daily assayed.

DNA sequencing

Sequencing by dideoxy chain termination method was performed by the Primm Sequencing Service (Naples, Italy), using specific oligonucleotide primers.

Protein purification

Preparative cultures were performed in 1-l flasks containing 200 ml of medium. Culture media were harvested on the optimal laccase production day (sixth day), cells were sedimented by centrifugation at 1600 ***g*** at 4°C for 15 min. Secreted proteins were filtered through Whatmann sheet. The sample was concentrated using Amicon stirred cells with PM30 ultrafiltration membranes (Millipore, Billerica, MA, USA). Most secreted proteins were precipitated by the addition of $(NH_4)_2SO_4$ up to 100% saturation at 4°C and centrifuged at 10 000 ***g*** for 40 min. The supernatant from ammonium sulfate precipitation (containing laccases) was loaded onto a 26/60 Superdex 75 prep grade (GE Healthcare Bio-Sciences, AB, Uppsala, Sweden) column equilibrated with 0·15 mol l^{-1} NaCl, 50 mmol l^{-1} Na-phosphate buffer pH 7. The active fractions were pooled and concentrated on an Amicon PM-30 membrane. Protein concentrations were determined by the Bradford method using the Bio-Rad Protein assay (Bio-Rad) and bovine serum albumin as standard.

Laccase activity assays

Laccase activity was assayed at room temperature, using ABTS, DMP and syringaldazine (SGZ) as substrates as previously described (Palmieri *et al.* 1997). For activity measurements at various pH values, laccase activities were assayed using DMP and ABTS as substrates in citrate-phosphate buffer at pH ranges 3·0–6·2 and 2·5–5·5, respectively. pH stability was determined at 23°C using ABTS as substrate in citrate-phosphate buffer adjusted at pH 3 and 5, in phosphate buffer adjusted at pH 7·0 and in Tris–HCl buffer adjusted at pH 7 and 10. The residual activities were determined daily using the standard activity assay. To explore the effect of temperature on mutants, assays were performed at temperature from 20 to 80°C, at 10°C intervals. For thermostability measurements, laccase solution was incubated at 60°C in 0·1 mol l^{-1} Na phosphate buffer, pH 7, and samples were withdrawn at 20-min intervals. Remaining enzyme activities were measured using the standard assay procedures. All the assays were performed in three replicates.

Decolourization assays

Batch decolourization experiments on textile industrial acid and direct dyes were performed with wild-type and mutant forms of POXA1b laccase. Then, 0·1 U ml^{-1} of crude preparation of laccases was incubated with dyes (125 ppm) at room temperature. Decolourization of each dye was assayed at different times (10 min, 2, 24 h) of enzyme incubation as decrease in absorption at wavelength of maximum absorbance of the dye.

Results

Screening of mutant laccase libraries

New screening rounds were run on the collections of 2300 randomly mutated variants of POXA1b laccase, as previously prepared (Festa *et al.* 2007). A library of 1100 clones prepared by error prone PCR (EP-PCR) experiments on *poxa1b* cDNA had been screened by assaying activity towards ABTS, allowing selection of the mutant 1M9B. This mutant shows a single mutation (L112F), leading to an enzyme more active but less stable with respect to the wild-type enzyme (POXA1b) in all the analysed conditions. 1M9B was subsequently used as a template for a second round of EP-PCR, generating a second-generation library of 1200 clones.

Both the first- and second-generation mutant collections were screened by assaying activity towards the phenolic DMP substrate, to isolate enzyme variants, which are more efficient in degrading phenolic compounds and thus further enlarging biotechnological applicability of POXA1b-derived laccases. Several mutants were selected using multi-well plate screening, having activity towards DMP higher than that of the control (yeasts expressing POXA1b wild type), and, among these, the three 1L2B, 1M10B and 3M7C variants – already selected in the previous screening experiments (Festa *et al.* 2007) – were found again. The second screening, following growth rate and activity production of the clones in multi-well plates for 3 days, allowed us to confirm twelve mutants out of the previously isolated ones, including 1L2B, 1M10B and 3M7C. The nine new mutant clones were then grown in shaken flasks for 6 days for further analyses. The mutants 2L4A and 3L7H from the second-generation collection showed 2·5-fold higher laccase-specific activity (39 and 37 mU per OD at the fifth day, respectively) compared to that of POXA1b wild type (14·5 mU per OD at the fifth day).

Characterization of mutated and wild-type laccases

After partial enzyme purification, catalytic parameters of the two selected mutants 2L4A and 3L7H were determined for the nonphenolic ABTS substrate and the two phenolic SGZ and DMP substrates, and compared with those of the POXA1b wild type and of the 1M9B parental mutant (Table 1).

The 3L7H and 2L4A mutants show Michaelis constant (K_M) values similar to those of the wild-type and the parental mutant towards DMP and SGZ, whilst their affinity towards ABTS is (two and threefold, respectively) lower. The specific activities of the new mutants towards ABTS show an increase of around 2- and 1·5-fold with respect to the wild-type protein and parental mutant, respectively. The mutants also exhibit 1·5-fold increase in specific activity towards DMP. On the other hand, no significant change in the ability of oxidizing SGZ was detected for the new mutants with respect to the wild-type enzyme, whereas the parental mutant shows a specific activity value lower than that of wild-type protein towards this substrate.

The effect of temperature on the activity of recombinant laccases towards ABTS was studied at pH 3·0 (Fig. 1a). The 2L4A mutant shows maximal activity at the temperature of 70°C, 10° higher than the optimal temperature of the wild-type enzyme and the parental mutant.

The effect of pH on the activity of recombinant laccases was analysed both towards ABTS and DMP. Experiments conducted with ABTS showed that the mutant's pH activity profile is similar to that observed for the recombinant wild-type laccase (Fig. 1b). The activity of all wild-type and mutated laccases decreased in a monotonic manner on varying the pH from 3 to 6, and,

Table 1 Catalytic parameters of the wild-type protein and selected mutants

Substrate	ABTS		DMP		SGZ	
Laccases	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})
wt	0·08 ± 0·01	175 ± 1	0·57 ± 0·02	160 ± 1	0·05 ± 0·01	24·1 ± 0·6
1M9B	0·07 ± 0·02	276 ± 3	0·40 ± 0·01	150 ± 1	0·04 ± 0·01	8·7 ± 0·5
2L4A	0·24 ± 0·01	400 ± 2	0·38 ± 0·01	231 ± 2	0·07 ± 0·03	33·3 ± 0·5
3L7H	0·16 ± 0·01	328 ± 1	0·41 ± 0·01	240 ± 1	0·04 ± 0·04	20·6 ± 0·8

ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); DMP, 2,6-dimethoxyphenol; SGZ, syringaldazine.

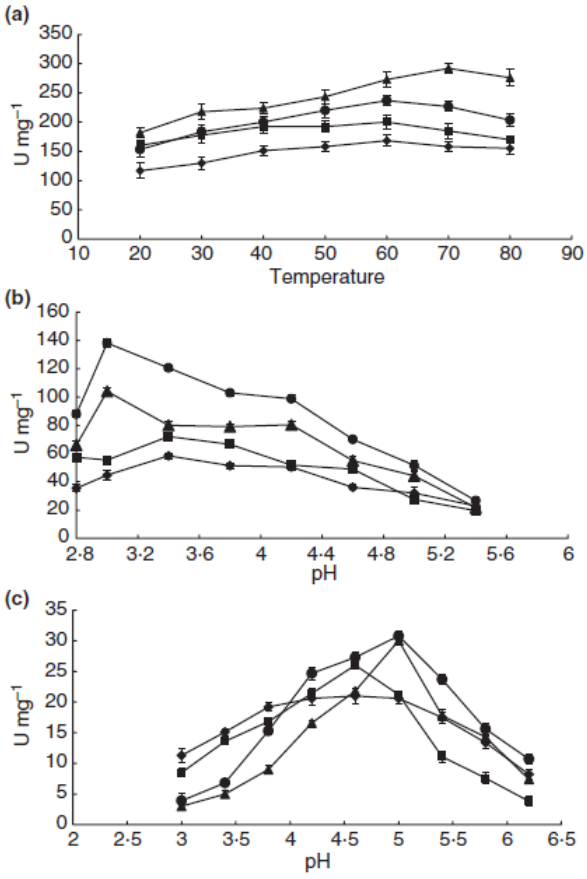


Figure 1 Effect of temperature on the activity of wild-type and mutated laccases towards 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate (a) and effect of pH on the activity of wild-type and mutated laccases towards ABTS (b) and 2,6-dimethoxyphenol (c) as substrates. (◆) POXA1b wild-type; (■) 1M9B mutant; (●) 3L7H mutant and (▲) 2L4A mutant.

compared to the wild-type enzyme and the parental mutant, both the new mutants show just a slight shift of the optimal pH towards lower values.

When the influence of pH on the oxidation of DMP was examined, a bell-shaped profile of the activity was observed as a function of pH for all the laccases tested, whether mutated or not (Fig. 1c). However, all the mutants showed sharper profiles than that of wild-type enzyme. Whereas no shift of the profile with respect to the wild-type enzyme was observed for 1M9B, both 2L4A and 3L7H mutants showed a slight shift of the maximum activity towards higher pH values when compared to the parental mutant, their maximum activity being at pH 5. Moreover, the activity loss of the 3L7H mutant at higher pH levels is less rapid than that of the parental mutant (Fig. 1c).

The stability of the enzymes with respect to temperature at 60°C at neutral pH was studied. Both the new mutants proved to be more stable than the wild-type enzyme and even more than the parental mutant, showing an increase in half-life up to more than twice the $t_{\frac{1}{2}}$ value of 1M9B (Fig. 2a). Stability of the laccases in different buffers and pH values was also analysed (Fig. 2b). At pH 5, both the two new mutants show higher stability than both the parental mutant and the wild-type enzyme, displaying up to a fourfold higher half-life with respect to 1M9B. At the other tested pH conditions, the 2L4A mutant mostly stores the stability properties of the wild-type enzyme, whilst 3L7H was proved to be less able to retain its activity than POXA1b.

The effect of the organic solvents ethanol and acetonitrile on activity of the selected variants was also assessed using ABTS as substrate (Fig. 3). The selected mutants display tolerance towards cosolvents similar to the wild-type laccase. Moreover, it was verified that, in the presence of organic cosolvents, the stability of the new variants is higher than that of the POXA1b wild-type – and even more than that of the 1M9B parental mutant – in most of the analysed pH conditions (Table 2).

Sequence analyses of the selected mutants led to the identification of the nucleotide substitutions producing the mutations reported in brackets (besides the L112F mutation of the parental mutant): 2L4A (Q272H), 3L7H (S285N; N328S).

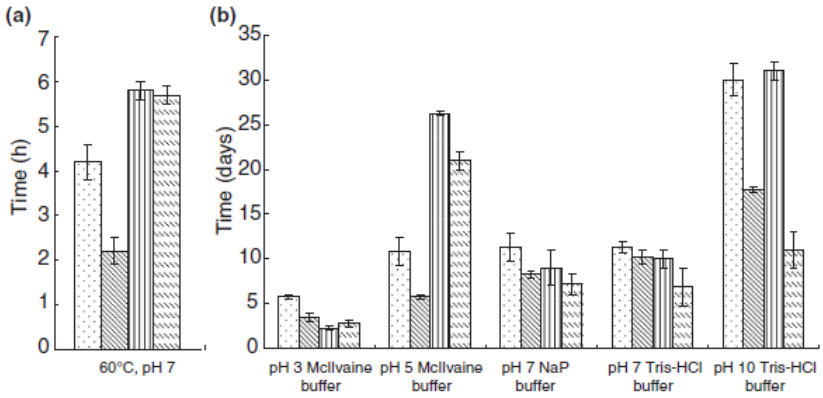


Figure 2 Half-life of the wild-type protein and selected mutants at 60°C (a) and at different pH values (b). (□, Wt; ▨, IM9B; ▯, 2L4A and ▧, 3L7H).

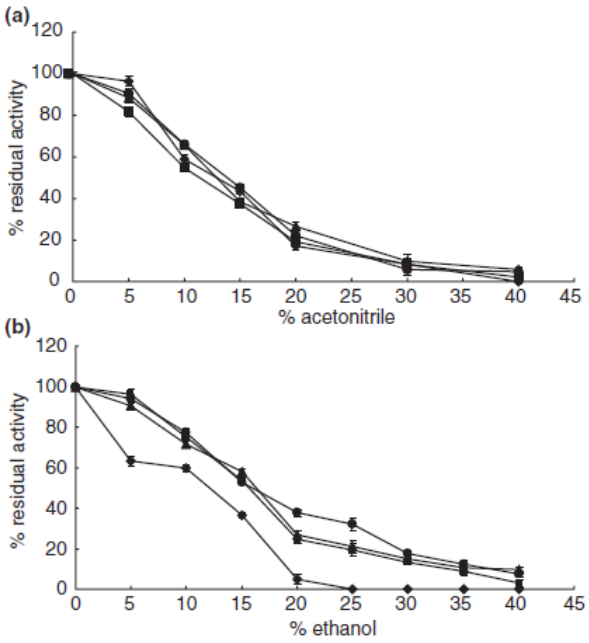


Figure 3 Effect of the organic cosolvents acetonirile (a) and ethanol (b) on the activity of wild-type and mutated laccases towards 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) as substrate. (◆) POXA1b wild-type; (■) 1M9B mutant; (●) 3L7H mutant and (▲) 2L4A mutant.

Decolourization ability of the new mutants and of the wild-type laccase

The new selected mutants were also evaluated for their abilities to decolourize synthetic industrial dyes (Table 3). These dyes had been selected in the frame of European Union Sixth Framework Program funded project SOPHIED (contract NMP2-CT2004-505899) on the basis of discharged amounts by textile industry, economic importance and representativeness of chemical structures. Decolourization of dyes by the evolved 2L4A and 3L7H mutants was assayed in comparison with that of the POXA1b wild type. In Table 3, the maximum percentage of decolourization provided by each tested enzyme and the time required to obtain it are reported. In addition to keeping the decolourization abilities displayed by the POXA1b wild type, both the mutated enzymes exhibited capability of decolorizing the recalcitrant stilbene-type Direct Yellow 106 dye, giving 20–25% decolourization after just 10 min of incubation.

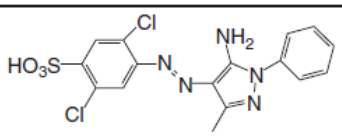
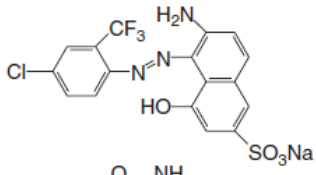
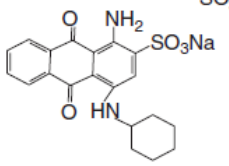
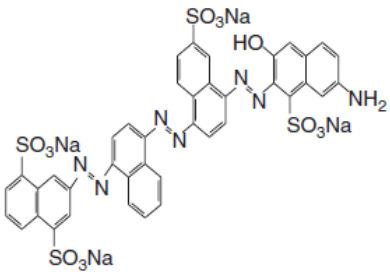
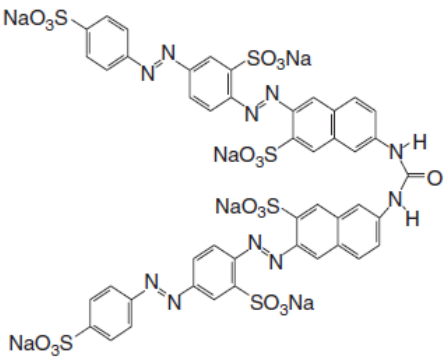
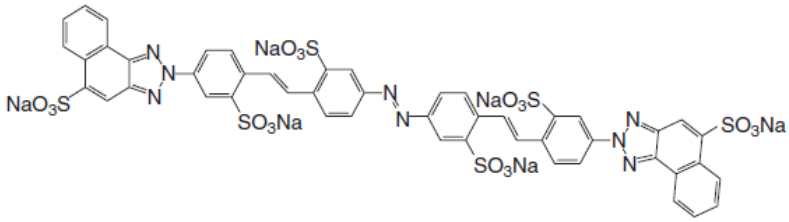
Discussion

The assortment of improved variants of the POXA1b laccase from the basidiomycete *P. ostreatus* was enlarged by screening the collections of 2300 directed evolved POXA1b variants for their ability in degrading the pheno-

Table 2 Half-life of the wild-type protein and selected mutants in the presence of cosolvents at different pH conditions

	$t_{\frac{1}{2}}$ (h)							
Laccases	pH 5, Aceto-nitrile 20%	pH 7, Aceto-nitrile 20%	pH 10, Aceto-nitrile 20%	pH 7, Aceto-nitrile 40%	pH 5, Ethanol 20%	pH 7, Ethanol 20%	pH 10, Ethanol 20%	pH 7, Ethanol 40%
wt	72	360	31·2	0·8	144	28·8	48	1
1M9B	76·8	288	24	0·5	103·2	24	24	0·8
2L4A	103·2	624	24	1·8	182·4	48	72	1·5
3L7H	110·4	528	24	1	96	48	107·52	1·5

Table 3 Chemical structures of dyes used in the study, maximum decolourization percentage provided by POXA1b wild-type and mutated forms 2L4A and 3L7H and the time required to obtain it

Dye	Structure	λ_{max}	Time	Decolourization (%) POX A1b	2L4A	3L7H
Acid Yellow 49		402	24 h	17·0	29·0	29·0
Acid Red 266		307, 500	24 h	20·0	23·0	23·0
Acid Blue 62		595, 637	2 h	14·0	18·6	15·2
Direct Blue 71		582	2 h	22·0	17·0	22·7
Direct Red 80		542	2 h	43·5	42·4	44·1
Direct Yellow 106		419	10 min	5·0	24·0	21·0

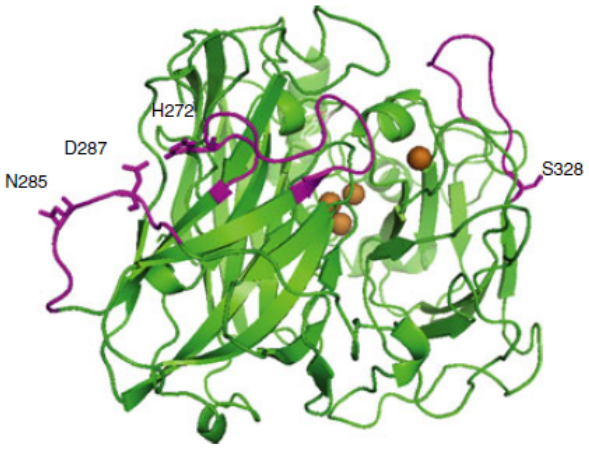


Figure 4 Ribbon representation of POXA1b 3D structure model, with the mutated residues (L112F; Q272H; N328S; S285N) and the D287 residue in stick rendering, and with highlighted the Gly282–Thr289loop, the loop containing His272 and the loop containing N328S. Trinuclear coppers and T1 copper are highlighted in van der Waals representation.

lic DMP substrate. The two new selected 2L4A and 3L7H mutants show higher specific activity than the wild-type enzyme and the parental mutant towards phenolic (DMP) and nonphenolic (ABTS) substrates and were proved to be able to work in a range of pH wider than that of the parental enzymes (Fig. 1b–c). Within the panel of wild-type and mutated POXA1b enzymes (Festa *et al.* 2007), a higher stability in a variety of environmental conditions was demonstrated as the most remarkable property of the 2L4A mutant, whilst a specific activity similar or slightly lower than that of the previously selected mutants was measured for the new mutants. Although narrowly connected during natural evolution, activity and stability are enzymatic features that are modulated by different principles and therefore their joint improvement is not easily achieved (Bloom *et al.* 2006). It is therefore worth noting that the results we achieved by directed evolution identified a laccase variant showing high activity whilst improving stability. Whilst the previously selected second-generation mutants recovered stability of the wild-type enzyme, which decreased in the first-generation mutant 1M9B, a further improvement in stability properties was achieved with the new 2L4A mutant. As a matter of fact, this enzyme was proved to be a thermostable laccase variant that, besides storing the high stability of the wild-type enzyme at alkaline pH values (10), increases its stability at acidic pH (5), with a half-life of around 1 month in both the pH conditions.

To investigate possible structural determinants of the improved properties of the new selected mutants, analysis of the effect of the mutations on the POXA1b 3D structure model (Festa *et al.* 2007) was performed. In Fig. 4, mutations identified in the new mutants are shown on the 3D structure model. Inspection of the protein model suggested that the Q272H mutation, characteristic of the 2L4A mutant, stabilizes the protein structure at pH 5, because it allows additional interactions – hydrogen bond and electrostatic interactions – between the positively charged imidazolic ring of the His272 and the side chain of Asp287. This interaction could also cause a movement of loop where it is located at the 272 position, involved in the formation of the reducing substrate-binding pocket (Larrondo *et al.* 2003), thus affecting enzyme activity. Interestingly, the S285N mutation, characteristic of the 3L7H mutant, is located very close to the Asp287 residue, both aminoacids belonging to the Gly282–Thr289 loop that would seem to have an important role for the protein stability. On the other hand, the N328S mutation, found in 3L7H, could have an effect on the enzyme activity, being located in a loop potentially interacting with the reducing substrate.

The high stability exhibited by the new 2L4A mutant in several operative conditions increases its biotechnological potential, in view of the fact that a significant limitation for laccase application in bioremediation of colour industry wastewaters and detoxification of environmental pollutants is the enzyme stability under process conditions. In fact, these processes are mostly carried out at extreme conditions in which laccases generally unfold, thereby losing their activity.

Moreover, the new variant was proved to represent a helpful biocatalyst for environmental applications, having the ability of decolourizing recalcitrant dyes. Previous decolourization experiments with *P. ostreatus* whole cells and with a crude fungal laccase preparation, mostly composed by the POXC isoenzyme (Faraco *et al.* 2009a), allowed us to distinguish the tested dyes (Table 3) in three groups: a group of dyes decolourized with high efficiency, including the anthraquinone Acid Blue 62 dye; a group of dyes slowly decolourized, consisting of the Direct Blue 71 (trisazo) and Acid Red 266 (azo dye) dyes; and a group of dyes resistant to POXC laccase decolourization, such as the Direct Red 80 (polyazo), Direct Yellow 106 (stilbene) and Acid Yellow 49 (azo dye) dyes.

Interestingly, all the tested rPOXA1b wild-type and mutated enzymes are able to decolourize the Direct Blue 71 and Acid Red 266 dyes, and also the Direct Red 80 and Acid Yellow 49 dyes. Moreover, the new variants show a further expansion of dye degradation specificity, differing from the wild-type enzyme for their ability in decolourizing the recalcitrant Direct Yellow 106 dye too. It is worth noting that the analysed laccases show the ability to decolourize dyes with complex trisazo-, polyazo- and stilbene-type structures, generally not degraded by laccases,

whose preferred substrates are anthraquinonic dyes, whilst degradability of the more resistant azo dyes depends on their different aromatic substitution patterns (Chivukula and Renganathan 1995; Kandelbauer *et al.* 2004).

In conclusion, this work allowed us to develop a new laccase variant as a more durable and more versatile biocatalyst, with respect to both the varieties of xenobiotics degraded and the operative conditions, thus representing a helpful 'evolved form' of the laccase enzyme (Baldrian 2006). The new enzyme variant represents a good candidate both for applications at alkaline pHs—such as bioremediation of textile wastewaters and of wastewaters produced by chemical plants in the manufacturing organic molecules of commercial interest (Alloway and Ayres 1998)—and for those requiring slightly acidic pHs—such as xenobiotics transformation (Ceylan *et al.* 2008; Zhang *et al.* 2008), organic synthesis and enzymatic polymerization (Aktaş and Tanyolaç 2003; Kunamneni *et al.* 2008).

Moreover, whilst most studies use laccase-mediator systems in the degradation of aromatic xenobiotics (Johannes *et al.* 1998; Soares *et al.* 2001; Kang *et al.* 2002; Camarero *et al.* 2005; Chhabra *et al.* 2008), with possible consequent toxic effects –because of the presence of mediators or their derivatives – this study avoids this drawback applying protein engineering to laccase isoenzyme to produce mutants suitable for industrial applications.

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3. Selection of variants of POXA1b from *Pleurotus ostreatus* more active at different pH

3.1 Results and Discussion

The collection of 2300 mutants obtained by random mutagenesis of POXA1b laccase was screened in microtiter plate using ABTS as substrate at pH5. No positive clones were obtained from the collection of the first generation, whereas the results of analysis of the second generation libraries were reported in the table 1. To further verify the results from the first screening, the activity of the 51 positive clones were analysed for three days in multiwell plates. Seven of the fifty-one mutants from the first screening were further selected. In the last step of the screening procedure, the selected seven mutants were analysed in shaken flasks. One clone, 1L9A, was selected on the basis of its ability to over express laccase activity (~3 and 1.5 fold in comparison with the yeast expressing wild type protein and 1M9B parental mutant, respectively).

Libraries from second round		**Clones selected in each step**		
		First screening in multiwell plates	**Second screening in multiwell plates for three days**	**Third screening in shaken flasks**
EP-PCR (*1m9b*)	500 mutants (frequency: 0÷3 mut/ kbase)	35 mutants	5 mutants	1 mutant: **1L9A**
	700 mutants (frequency: 3÷7 mut/ kbase)	16mutants	2 mutants	0

Table 1 Summary of obtained mutants and their curtailment

Purification of wild-type and mutant laccases

The enrichment of the recombinant protein was carried out. Time course of growth – measured as OD600nm – and laccase activity production was monitored. Mutants and wild type expressing yeast growth curves do not differ significantly. Therefore the higher specific activity observed for the mutants (Fig. 1) can be attributed to more effective enzymes production. Culture broths collected at the 6th growth day were used for laccase purification. The recombinant POXA1b protein and the 1M9B and 1L9A mutants were purified by ammonium sulphate selective precipitation followed by gel filtration chromatography Superdex 75 prep grade as described in Materials and Methods.

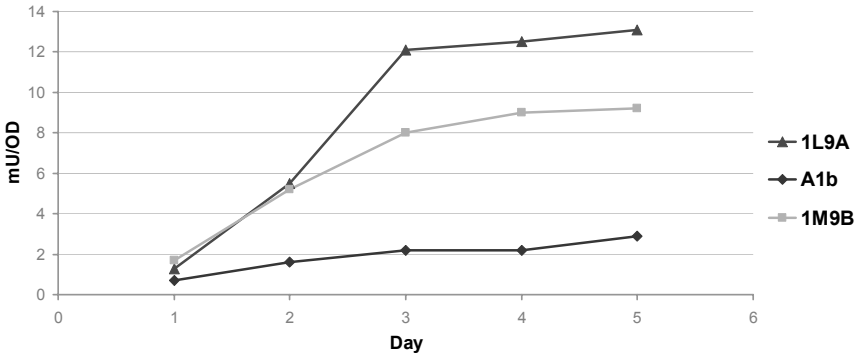


Fig.1 Laccases's specific activity production in preparative cultures with ABTS in Na-citrate pH5

Similar amounts of total protein were obtained in all cases. The active fractions were pooled and concentrated on an Amicon PM-30 membrane. In the Table 2 yields after protein purification are given, obtained following the described procedures (see Materials and Methods).

	Purification step	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (U%)	Purification fold
Wild Type	Broth	45	3.1	14.5	100	1
	$(NH_4)_2SO_4$ and Sec*	29	0.2	145.0	64.4	10
1M9B	Broth	105	3.6	30.0	100	1
	$(NH_4)_2SO_4$ and Sec*	46	0.2	230.0	43.8	7.6
1L9A	Broth	238	3.8	62.3	100	1
	$(NH_4)_2SO_4$ and Sec*	146	0.3	521.4	61.3	8.4

*Laccase activity was recovered from supernatant of 100% ammonium sulphate precipitation

Table 2 Wild type and mutant proteins purification from *S. cerevisiae* cultures

Characterization of wild-type and mutant laccases

Kinetic and catalytic parameters for three different substrates : 1) 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) at pH 3; 2) 2,6-dimethoxyphenol (DMP) at pH 5; 3) syringaldazine (SGZ) at pH 6 were determined, as reported in Table 3.

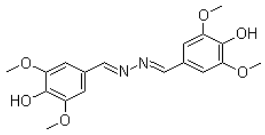
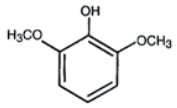
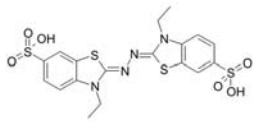
	SUBSTRATE					
	ABTS		**DMP**		**SGZ**	
Laccases						
	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})
wt	0.08±0.01	167±1	0.57±0.03	160±1	0.05±0.01	18.3±0.6
1M9B	0.07±0.02	230±1	0.39±0.01	148±1	0.04±0.01	8.7±0.5
1L9A	0.25±0.02	255±2	0.40±0.05	234±2	0.02±0.01	11.2±0.5

Table 3 Kinetic and catalytic parameters of wild type and mutant proteins

The mutant shows a higher specific activity towards ABTS (~1.8 fold) than that of wt protein, thus confirming the reliability of the screening procedure. The 1L9A enzyme shows specific activities higher than that of wt protein also towards DMP, suggesting that improvement of the activity is not substrate dependent, although the specific activities towards SGZ remain low, like those of wild type and native proteins from *P. ostreatus* [10,11].

In general slight differences have been observed in the Michaelis-Menten constant (K_M), toward all substrates, of mutant selected compared with that of the wild-type protein. In particular, 1L9A mutant has reduced affinity for ABTS and DMP compared with that of wild-type.

Effect of pH and Temperature on laccase activity

The effect of pH on the activity of laccases towards DMP has been studied in the pH range 3.0÷6.4. In general, a bell-shaped profile of the activity as a function of pH was observed for all the tested laccases. The optimum reaction pH of laccases for DMP oxidation was higher around pH values of 4-5.5.

In particular, the mutant 1L9A shows its maximum activity at value of pH 4.5 (fig.2).

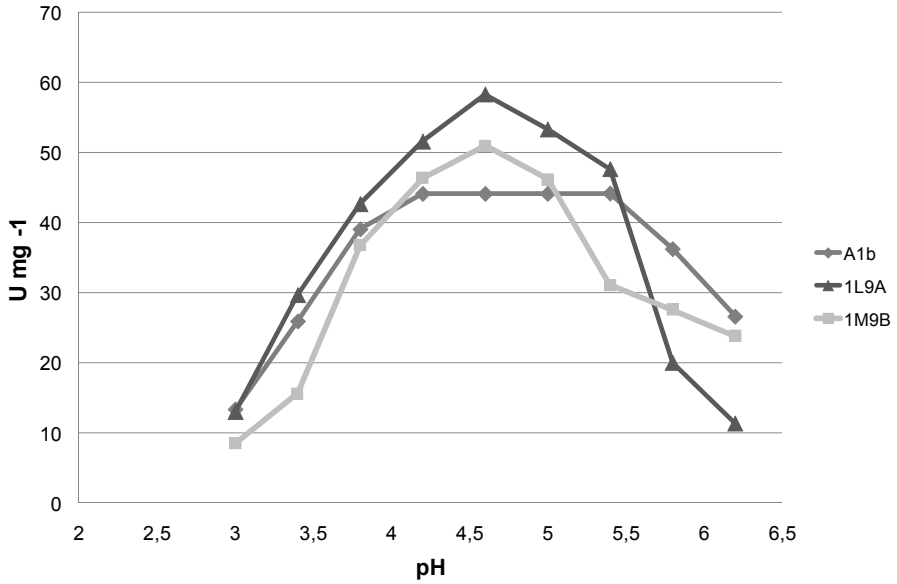


Fig.2 Effect of pH on the activity of wild-type and mutated laccases.

Optimal reaction temperature was determined by varying the reaction temperature, by 10°C increments, in the range 20–70°C. POXA1b and 1M9B mutants show their maximum activity at 60°C , whereas 1L9A at lower temperature (50°C) (fig.3).

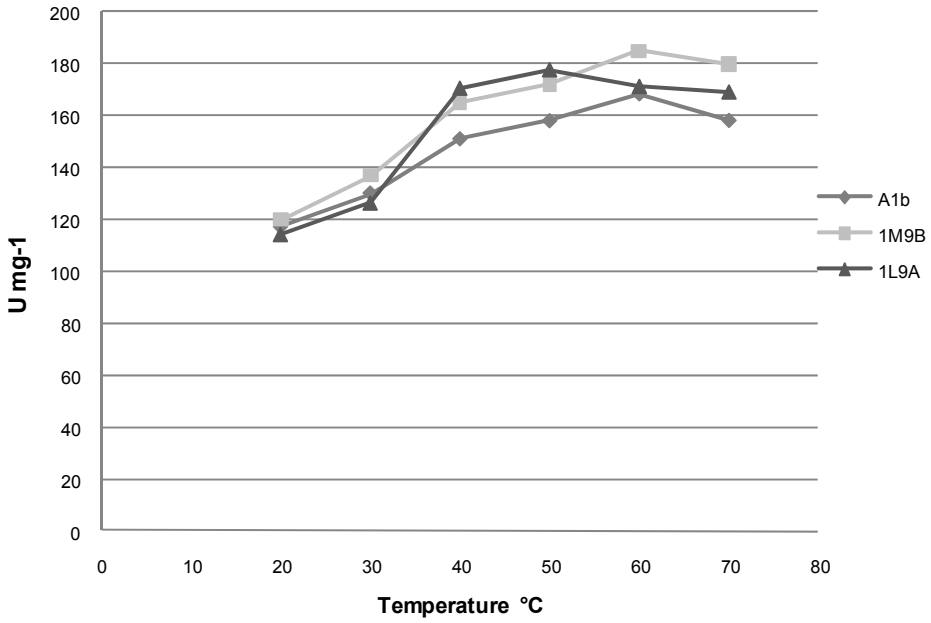


Fig. 3 Effect of temperature on the activity of wild type and mutated

Stability at pH and Temperature

Laccase stability at different pH values has been examined by pre-incubating the enzymes at various pH values varying from 3.0 to 10.0 and by measuring the residual activity under standard assay conditions.

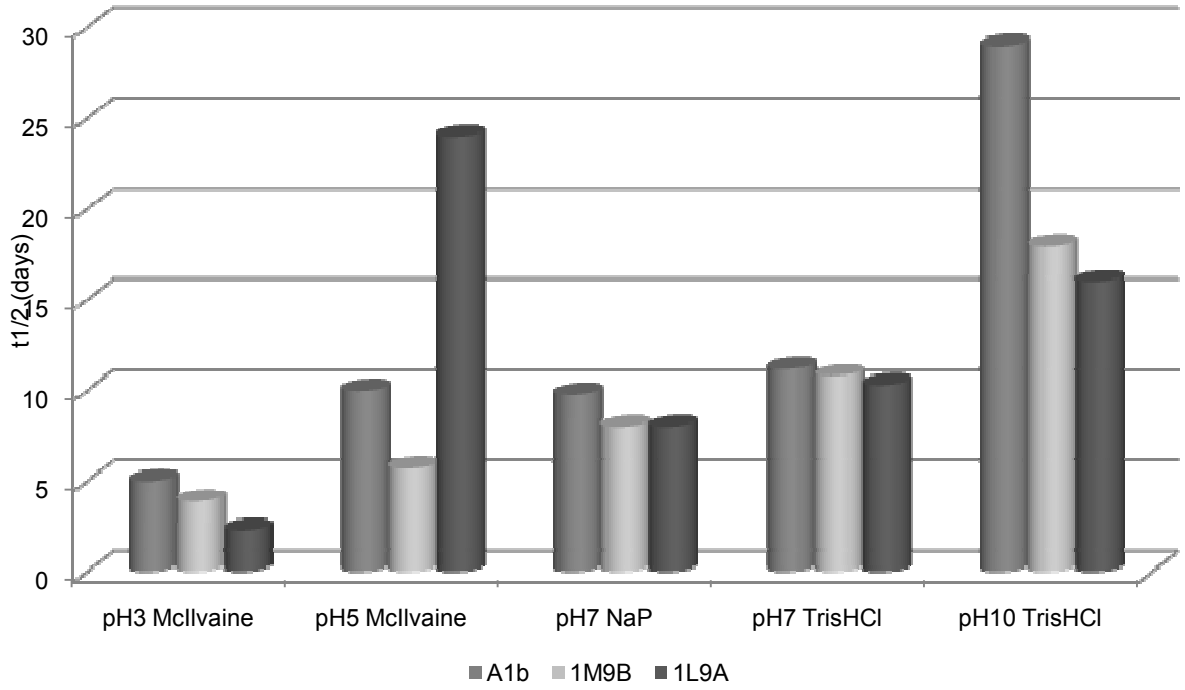


Fig.4 Stability of POXA1b and mutated laccases 1M9B and 1L9A at different pH values.

	t1/2 (Days)				
Laccases	pH3, McIlvaine buffer	pH5, McIlvaine buffer	pH7, McIlvaine buffer	pH7, Tris-HCl buffer	pH10, Tris-HCl buffer
wt	5	10	9,8	11,2	29
1M9B	3,9	5,7	8	10,8	18
1L9A	2,3	13,2	8	10,3	16

Table 4 Protein half life values at different pH values

As shown in Figure 4, an unusual behavior was observed for 1L9A mutants. In particular, at pH 5 it shows a t1/2 values more than 1.5-fold with respect to that of POXA1b, in addition at alkaline pH the mutants loose drastically the high characteristic stability of POXA1b (Fig. 4 and Table 4). At neutral pH POXA1b and its mutants are similarly stable.

In order to determine their thermostability, all enzymes were incubated at 60° at neutral pH and cooled down to room temperature prior to measuring the residual activity using ABTS as substrate. The mutant appeared to be reasonably stable with respect to temperature; in particular 1L9A displays similar stability (1.2-fold) compared with that of wild type (Table 5) and higher (2.5 fold) respect to the 1M9B parental mutant.

Laccases	$t_{1/2}$ (hours)
wild type	4.5
1M9B	2.2
1L9A	5.4

Table 5 Proteins half life values at 60°C

Structural characterization

Nucleotide sequences of the mutant revealed point mutations which resulted in the aminoacidic substitutions reported in Table 7. The observed mutations do not justify, on the basis of *S. cerevisiae* codon usage (http://www.yeastgenome.org/community/codon_usage.shtml) any variation of the expression level of the mutants respect to the wild type protein.

Mutants	Codon substitutions			Aminoacid substitutions
1L9A	CTT (11%)	→	TTT(53%)	L112F
	GGT(61%)	→	GGA(15%)	conservative
	CGC(4%)	→	CAC(40%)	R284H

*Percentage of codon usage is reported between brackets.

Table 6 Mutations found in mutants sequences

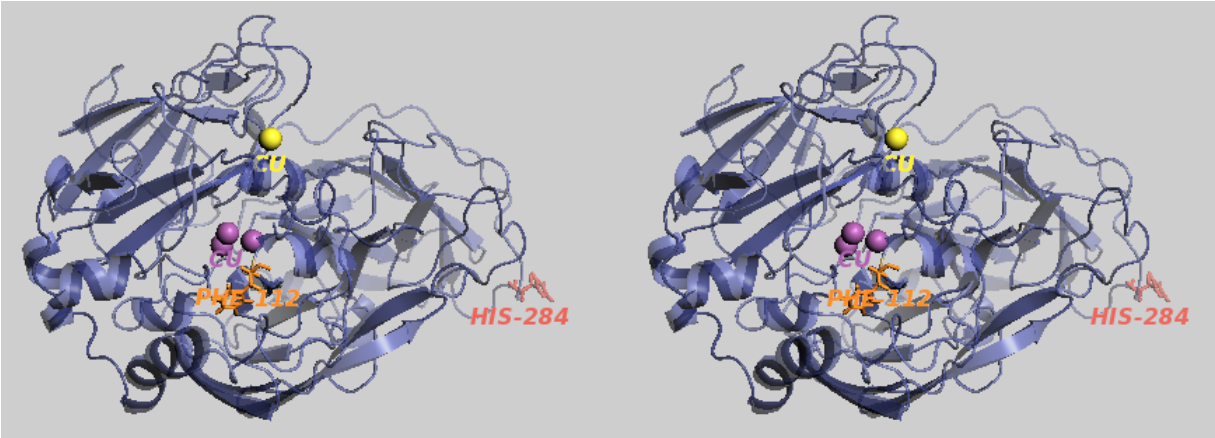


Fig. 5 Ribbon representation of POXA1b 3D structure model (stereo view), with the mutated residues (L112; R284) in stick rendering. Copper T1 and coppers T2 and T3 are represented in yellow and in purple, respectively.

To locate the substitutions in protein tertiary structure, models of mutant were constructed *in silico* replacing the original amino acids with the new residues in POXA1b 3Dmodel. The mutation L112F, common to parental 1M9B mutant, is located in a laccase consensus region, named L2 by Kumar et al, 2003 [12] (Fig 5). The other mutation R284H is indeed located very close to the Asp287 residue, both aminoacids belonging to the Gly282–Thr289 loop that would seem to have an important role for the protein stability [13].

The obtained results have highlighted that 1L9A shows a diminished affinity for the substrate (**Table 3**) and a similar stability under all tested condition except for the stability at pH5, condition that we used for the screening. These data suggest that the presence of aromatic residue in the position 284 could create a rearrangement of the loop II, responsible for substrate binding specificity, that influence the stability of the protein. A similar condition was present in the mutant 3L7H [13] that presents a mutation in the same region (S285N) and shows less affinity for the substrates and similar or less (e.g. pH10) stability under all tested condition except for pH5 (t1/2 21 days pH5). These data support our hypothesis that the region of loop II is important for protein stability.

3.2.Materials and Methods

Microorganism

Bacteria. The *Escherichia coli* strain Top 10 (F-mcrA D (mrr-hsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 D (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG) was used in all DNA manipulation. The medium used for growing *E. coli* was the rich medium Luria-Bertani (LB). Bacterial cultures were conducted at 37°C. Bacterial cells competent to chemical transformation were obtained from cells grown on SOB medium.

LB
Bacto tryptone (Difco) 1% (*w/v*)
Yeast extract (Difco) 0.5% (*w/v*)
NaCl 1% (*w/v*)
$MgSO_4$
Selective medium was supplemented with 100 µg ml^{-1} of ampicillin; solid medium contained agar 15% (*w/v*) (Difco).

Competent cells preparation and transformation. Cells were picked up from a solid culture and grown in 5 ml of SOB medium at 37° C for 16 hours on a rotary shaker (250 rpm). The culture was then diluted 1:100 in the same medium and grown up to an optical density at 600 nm of 0.6-0.7. Growth is stopped by putting the culture on ice for 20 min., and cells are sedimented by centrifugation at 3000 x g at 4°C for 10 min. Cells were washed twice by incubation with cold $CaCl_2$ 0.1 M for 20 min on ice. Cell pellet was then resuspendend in $CaCl_2$ 0.1 M, and Glycerol 15% was added to the suspension. After vigorous mixing, the suspension was aliquoted and promptly frozen in dry-ice/acetone bath. Competent cells was thawed and incubated with transforming DNA plasmid (up to 100 ng) for 30 min. on ice. Mixture was then shocked by incubation at 42°C for 90 sec. and on ice for the same time. Cells were spread on selective solid LB medium after 1 hour incubation at 37 °C in LB medium.

Mini-preparation of plasmid DNA (alkaline lysis method). Bacterial cells grown on solid LB medium were inoculated at 37°C over night in 3 ml of selective LB on a rotary shaker (250 rpm). 1.5 ml of culture was centrifuged for 30 sec. at 13000 x g at room temperature. The cellular pellet was resuspended in 100 µl of cold GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8). 200 µl of 0.2 N NaOH, 1% SDS were added to the cellular suspension. After a vigorous shaking, the solution was incubated on ice for 5 min to denaturate the cellular proteins. Then 150 µl of K-acetate 5M pH 5.2, were added, and the solution was incubated on ice for 5 min to allow the precipitation of genomic DNA. The suspension was centrifuged for 5 min at 13000 x g at room temperature. The supernatant, containing plasmidic DNA, was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The plasmidic DNA was then precipitated by adding 2.5 volume of 100% EtOH at room temperature and rinsed with cold 70% EtOH. The plasmidic DNA was resuspended in 50 µl of TE pH 8 (10 mM Tris HCl pH 8, 1 mM EDTA), RNAasi A (150 µg ml^{-1}) and incubated at 37°C for 30 min.

The yeast Saccharomyces cerevisiae. The *Saccharomyces cerevisiae* strain used for heterologous expression was W303-1A (MAT ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100). The medium used for growing wild-type S. cerevisiae was

the rich medium YPD (1% Yeast extract (Difco), 2% Bacto tryptone (Difco), 2% Glucose). Cultures were conducted at 28°C. Solid medium contained 15% of agar (Difco). Transformed yeast was grown in a selective medium:

Selective medium SD
Yeast nitrogen base w/o AA (Difco) 6.7 g/l
Casaminoacids (Difco) 5 g/l
Glucose 2% (*w/v*)
Adenine 30 mg/l
Tryptophane 40 mg/l
Succinate buffer pH 5.3, 50 mM
Copper sulphate 0,6 mM

Plate assay was performed on solid selective medium supplemented with enzyme substrate: 0.2 mM ABTS or 0.1 mM DMP (2,6–dimethoxy-phenol).

Yeast cultivation and laccase production

- *Growth in multi-well plates*. Single clones were picked and transferred into 96-well plates containing 30 µl of selective medium (SD) per well. Plates were incubated at 28°C, 250 rpm for 24 h. After 24 h 130 µl of SD was added to each well and the plates were incubated at 28°C, 250 rpm for 24 h. Thirty microliters of each culture was transferred to a new 96-well plate to measure the OD600 value. The plates were then centrifuged for 10 min at 1,500 *x g*, 4°C and a suitable volume of supernatant was transferred to a new 96-well plate to perform laccase assay.

- *Growth in shaken flasks*. Precultures (10 ml) were grown on selective SD medium at 28° C on a rotary shaker (150 rpm). A volume of suspension sufficient to reach a final OD600 value of 0.5 was then used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of selective medium and cells were then grown on a rotary shaker. Culture aliquots (1 ml) were daily collected, centrifuged at 12000 g for 2 min at 4°C and assayed for optical density and laccase activity determination. Preparative cultures were performed in 1L flasks containing 200 ml of medium.

Mini-preparation of plasmid DNA. Yeast cells were grown in 3 ml of liquid SD medium at 28°C over night on a rotary shaker (150 rpm). 1.5 ml of culture was harvested for 5 min. at 5000 x g at room temperature, and the pellet was re-suspended in 100 µl of cold STET (8% sucrose, 50 mM Tris-HCl pH 8, 50 mM EDTA, 5% Triton X-100). Cells were lysated by adding 0.2 g of 0.45 mm glass beads and vigorously vortexing. Following steps were: addition of another 100 µl of STET, briefly vortexing and incubation in a boiling water bath for 3 min. The suspension was then cooled on ice and centrifuged for 10 min. at 5000 x *g* at 4°C. To 100 µl of the supernatant was added 2.5 M ammonium acetate: the sample was incubated at -20°C for 1 h. and centrifuged for 10 min. at 12000 x *g* at 4°C. At this stage, residual chromosome DNA, large RNA species, and the putative impurities which inhibit *E.coli* transformation are precipitated. To 100 µl of the supernatant were added 200 µl of ice-cold ethanol, and DNA was recovered by centrifugation. The pellet was washed with 70% ethanol and re-suspended in 20 µl of water. 10 µl of this solution were used to transform competent bacteria.

Protein manipulation

Protein purification. Culture media were harvested on the optimal laccase production day, cells were sedimented by centrifugation at 1600 g at 4°C for 15 min. Secreted proteins were filtered throw whatmann sheet. The sample was concentrated with Amicon stirred cells with ultrafiltration membranes with cut-off 30000 NMWLC. Then, most secreted proteins were precipitated by the addition of $(NH_4)_2SO_4$ up to 100% saturation at 4°C and centrifuged at 10000 g for 40 min. The supernatant from ammonium sulphate precipitation was loaded onto a 26/60 Superdex 75 prep grade (Amersham Biosciences) column equilibrated with 0,15M NaCl 50 mM Na-phosphate buffer pH7. The active fractions were pooled and concentrated on an Amicon PM-30 membrane.

Protein determination. Protein concentration was determined using the BioRad Protein Assay (BioRad), with BSA as standard.

Assay of laccase activity. Culture aliquots were collected and cells were removed by centrifugation (12000 *g* for 2 min 4°C).

Laccase activity in the culture supernatant was assayed at room temperature, monitoring the oxidation of ABTS at 420 nm (ε_{420}= 3.6 x 10^4 M^{-1} cm^{-1}): the assay mixture contained 2 mM ABTS, 0.1 M Na-citrate buffer, pH 3.0.

Laccase activity towards DMP was assayed in a mixture containing 1 mM DMP and the McIlvaine's citrate-phosphate buffer adjusted to pH 5. Oxidation of DMP was followed by an absorbance increase at 477 nm (ε_{477}= 1.48 x 10^4 M^{-1} cm^{-1}).

Laccase activity towards syringaldazyne (SGZ) was assayed in a mixture containing 0,1 mM SGZ and 0,2 M Na-posphate buffer adjusted to pH 6.0. Oxidation of SGZ was followed by an absorbance increase at 526 nm (ε_{526}= 6.5 x 10^4 M^{-1} cm^{-1}).

Kinetic parameters determination. Laccase activity in the culture supernatant was assayed utilizing increasing substrate concentrations (ABTS, 2,6-DMP and SGZ), until obtaining the complete saturation of the enzyme. K_M values were estimated using the software GraphPad Prism, on a wide range of substrate concentrations. The values of ΔA/min according to values of concentration of the substrate have been brought back in diagram and have been determine the parameters to you of the curve of Michaelis-Menten, for that substrate.

Effect of pH and temperature

The effect of pH on laccase activity towards 2,6-DMP was measured using a McIlvaine's citrate-phosphate buffer adjusted to different pH level in the range 3.0-6.2. The effect of temperature on laccase activity was measured in the temperature range 20-70°C in 50 mM Na-phosphate buffer adjusted to pH 7.0.

Stability at pH and temperature

Phenol oxidase stability at 60°C was measured in 50mM Na-phosfate buffer adjusted to pH 7.0. Stability at pH values was measured using a McIlvaine's citrate-phosphate buffer adjusted at pH 3 and 5; 50 mM Na-phosfate buffer adjusted at pH 7.0; Tris-HCl buffer adjusted at pH 7 and 10.

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5. Conclusions

The work described in Chapter 1 has been aimed at selecting new oxidative enzymes by screening of a collection of 2300 mutants obtained by random mutation of laccase POXA1b from *P. ostreatus.* Two *criteria* were used: a different substrate – the phenol substrate 2,6-DMP – and a different pH of selection – ABTS at pH5 –. The choice of these two different strategy of screening was explored to select new laccases with improved performances of stability and activity at a different operating conditions.

Three new laccases were obtained 2L4A, 3L7H (selection with DMP) and 1L9A (selection with ABTS at pH5). These new variants endowed with higher enzyme activity than the wild-type laccase were characterized showing a significant increase in stability at acidic pH (pH5). One of the mutants (2L4A) was also proved to store its high stability at alkaline pH. This variant also represents a more versatile enzyme with respect to both the variety of xenobiotics degraded and the operative conditions. In fact, decolouration experiments of industrial dyes with complex trisazo-, polyazo- and stilbene-type structures, in the absence of mediators, were performed using 2L4A and 3L7H mutants and their ability to decolorize these model waste water was demonstrated.

The structural and computational analyses to investigate some of the reasons, at a molecular level, for the enhanced activity shown by specific mutations were performed. The identified mutations were proved, although not directly involved in catalysis, to influence the efficiency and the stability of the enzyme, increasing our knowledge of structure-function relationships in this class of enzymes.

In this chapter two main goals were pursued:

- the identification of mutations in region of the protein proved to influence the stability of the enzymes. These data produced informations to understand enzyme structure/function relationships;

- the improvement of a basidiomycete laccase for industrial effluents bioremediation by directed evolution. The laccase POXA1b is known for its high stability at alkaline pH and for this reason it has been chosen to generate a collection of mutants by directed evolution. Thanks to the described screening, three new enzymes were selected for their improved properties.

Chapter 2

1. Introduction

Semi-rational mutagenesis is a new ‘protein engineering’ system that takes advantage from protein structure and combinatorial libraries constructed by saturation mutagenesis. This approach consist in the ‘rational’ fusion of the identified serendipitous mutations. It can be employed to analyze the interaction of mutations selected by random methods and fused in order to evaluate and identify their possible interactions and synergies and to raise structural information that are not immediately obtained with the classic methods.

Previously, the *in vitro* evolution of POXA1b laccase has been described, leading to enzyme variants characterized by increased specific activity as well as improved stability. Among the selected POXA1b mutants, 1M10B and 3M7C enzymes were chosen in this study to develop an improved laccase-based bio-catalyst, by a semi-rational approach based both on computational design and directed evolution.

The 1M10B mutant shows more durable activity than the wild-type enzyme, displaying improved stability both at 60°C and at neutral and alkaline pH values. On the other hand, the 3M7C mutant stores stability properties of POXA1b, while improving its catalytic efficiency. Besides the mutation L112F present in the parental mutant, the two additional mutations, responsible for the features of the 1M10B mutant, are located in the N-terminal region of the protein (K37Q, K51N), whereas only a further substitution is found in the C-terminal tail of the 3M7C mutant (P494T). Molecular dynamic simulations on 3D model structure of 3M7C had shown the latter mutation to affect flexibility of the protein, increasing the accessibility of the T1 copper site and thus leading to an improved activity of the enzyme.

Aim of this chapter has been the ‘design’ and ‘construction’ of a chimeric laccase, R4, and the improvement of collection of POXA1b mutants. The new variant R4 was obtained by joining the mutations identified in the 1M10B and 3M7C. Catalytic and kinetic properties of R4 mutant were analyzed and compared with those of the wild-type enzyme. It shows stability at pH10 2 fold higher than that of the wild-type enzyme. This new clone was used as template for producing a new collection of 1100 mutants. Two new mutants (1H6C and 1M10G) were selected for their improved activity against ABTS. Thermodynamic and catalytic characterization of these mutants is described.

2. A semi-rational approach to engineering laccase enzymes

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Article Type: Research Papers

Keywords: oxidases; semi-rational mutagenesis; chimeric enzyme; directed evolution; enzyme stability

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Abstract: In order to develop improved laccase-based bio-catalysts, semi-rational mutagenesis of the laccase POXA1b from Pleurotus ostreatus was performed through a combination of directed evolution with elements of rational enzyme modification. The R4 chimeric laccase was prepared by joining mutations of previously selected POXA1b random variants. The chimera construction provided an enhancement of stability features, making the novel enzyme R4 more appropriate as scaffold for directed evolution. A library of 1000 randomly mutated variants of R4 was prepared and screened for the ability of oxidizing 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). One of the variants selected for improved activity was also proved to show higher stability than R4 at pH 5, and to retain its high stability at pH 7 and 10. In comparison with the POXA1b wild-type laccase, the semirational approach allowed us to develop a more efficient bio-catalyst, rising specific activity on ABTS up to around 5- fold. The new variant was also proved to be both more versatile and more durable than the wild-type enzyme, exhibiting higher activity in wide temperature and pH ranges and higher stability at acidic (t1/2 at pH 5=35 days), neutral (t1/2 at pH 7=38 days) and alkaline (t1/2 at pH 10=62 days) pH values.

A semi-rational approach to engineering laccase enzymes

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Short running title: Semi-rational laccase mutants

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Abstract

In order to develop improved laccase-based bio-catalysts, semi-rational mutagenesis of the laccase POXA1b from *Pleurotus ostreatus* was performed through a combination of directed evolution with elements of rational enzyme modification. The R4 chimeric laccase was prepared by joining mutations of previously selected POXA1b random variants. The chimera construction provided an enhancement of stability features, making the novel enzyme R4 more appropriate as scaffold for directed evolution. A library of 1000 randomly mutated variants of R4 was prepared and screened for the ability of oxidizing 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). One of the variants selected for improved activity was also proved to show higher stability than R4 at pH 5, and to retain its high stability at pH 7 and 10. In comparison with the POXA1b wild-type laccase, the semi-rational approach allowed us to develop a more efficient bio-catalyst, rising specific activity on ABTS up to around 5- fold. The new variant was also proved to be both more versatile and more durable than the wild-type enzyme, exhibiting higher activity in wide temperature and pH ranges and higher stability at acidic ($t_{1/2}$ at pH 5=35 days), neutral ($t_{1/2}$ at pH 7=38 days) and alkaline ($t_{1/2}$ at pH 10=62 days) pH values.

Keywords: oxidases; semi-rational mutagenesis; chimeric enzyme; directed evolution; enzyme stability

Introduction

Laccases (p-diphenol: dioxygen oxidoreductases, EC 1.10.3.2) are multi-copper enzymes oxidizing aromatic compounds, with concomitant reduction of molecular oxygen to water [1,2]. Laccases exhibit a wide range of natural substrates –including phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindols, benzenethiols, inorganic/organic metal compounds and many others which is the main reason for their relevance in several biotechnological applications. Besides their natural substrates, laccases are also able to oxidise non-phenolic structures having higher redox potentials, in the presence of small molecules acting as redox mediators [3]. Thus, laccases and laccase-mediator systems find potential application in several sectors such as delignification and biobleaching of pulp, detoxification of industrial wastewaters and pollutants, textile dye decolourisation, construction of biosensors and biofuel cells [4-6]. A critical point for the successful application of laccase-based biocatalysts is the protein engineering of these enzymes with the aim of improving enzymatic features such as thermostability and stability under harsh operating conditions -*e.g.* extreme pH values.

The laccase family of the basidiomycete fungus *Pleurotus ostreatus* represents a variegated group of enzymes [7], having relevant roles in biotechnological applications of this fungus [8-11]. Within *P. ostreatus* laccase family, POXA1b [12] has been proved to be able of degrading dyes with complex trisazo and polyazo structures, generally not transformed by other isoenzymes [13]. Moreover, POXA1b is the most thermostable isoenzyme among *P. ostreatus* laccases (t1/2 at 60°C = 3h)

and exhibits a greatly high stability at alkaline pH (t1/2 at pH 9=30 days), that increases its relevance for bioremediation of industrial effluents.

We have previously described the *in vitro* evolution of POXA1b laccase [14]. A collection of 1100 random mutated forms of POXA1b has been prepared by error prone PCR (EP-PCR) and expressed into *Saccharomyces cerevisiae*. The 1M9B mutant, more active but less stable than the wild-type enzyme, has been selected and then used as a template to obtain a second generation library of further 1200 clones. This library has been successfully screened leading to enzyme variants characterized by increased specific activity as well as improved stability [13,14].

Among the selected POXA1b mutants, the 1M10B and 3M7C enzymes [14] were chosen in this study to develop an improved laccase-based bio-catalyst, by a semirational approach based both on computational design and directed evolution. Synthesis of a chimeric laccase joining mutations of 3M7C and 1M10B variants was performed to combine their improved features, followed by *in vitro* evolution of the novel enzyme.

Materials and methods

Strains, media, and plasmids

The *Escherichia coli* strain Top 10 (F-mcrA D (mrrhsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1araD139 D (ara–leu) 7697 galU galK rpsL (StrR) endA1 nupG) was used in all DNA manipulations. *E. coli* was grown in Luria–Bertani (LB) medium (in g L^{-1}: 10 bacto tryptone, 10 NaCl, 5 yeast extract), supplemented, when required, with 100 µg mL^{-1} of ampicillin.

The *S. cerevisiae* strain used for heterologous expression was W303-1A (MATade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100). The plasmid used for *S. cerevisiae* expression was B-pSAL4 [17] carrying URA3 gene for auxotrophic selection. *S. cerevisiae* was grown on a selective medium [6.7 g L^{-1} yeast nitrogen base without amino acids and ammonium sulfate, 5 g L^{-1} casamino acids, 30 mg L^{-1} adenine, 40 mg L^{-1} tryptophan, 50 mM succinate buffer (pH 5.3), 20 g L^{-1} glucose].

Chimeric cDNA construction

To obtain the chimeric laccase cDNA *r4* the 3'terminal portion (from the nucleotide 453) of the cDNA coding for 3M7C was ligated to pSAL4 vector containing the 5'terminal portion (the first 5' terminal 452 nucleotides) of 1M10B encoding cDNA, after KpnI digestion of pSAL4 vectors containing the two cDNAs.

Transformation of S. cerevisiae

The *r4* cDNA was transformed in *S. cerevisiae* using a lithium acetate protocol [15]. The cell pellet was resuspended in 1ml sterile water and centrifuged at 12000 x g for 10 sec. The cell pellet was then incubated in 1 ml of 0.1 M LiAc for 5 min at 30 °C. Cells were sedimented by centrifuging at 12000 x g for 10 sec, resuspended in a mixture containing: 240 µl of PEG (50% w/v), 36 µl LiAc 1 M, 25 µl SS-DNA (2.0 mg/ml), transforming DNA plasmid (up to 5 µg), 45 µl of H_2O and incubated at 42° C for 20 min. Cells were then centrifuged, resuspended in H_2O, and spread on selective medium. Plate assay was performed on solid selective medium supplemented with the enzyme substrate: 0.2 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or 1mM 2,6-dimethoxyphenol (DMP). The plates were incubated inverted for 4 days at 28°C and checked for the development of green or orange colour, corresponding to ABTS and DMP oxidation, respectively.

Construction of a random mutant library

Random mutations were introduced with low, medium, and high frequency of mutation, into the R4 encoding cDNA using GeneMorphTM PCR Mutagenesis Kit

(Stratagene, La Jolla, CA). EP-PCR was performed with primers POXA1bfw ATAAAAGCTTGAATTCATGGCGGTTGCATTCG and POXA1brev TAAGGATCCAAGCTT TTATAATCATGCTTC.

The cDNAs resulting from EP-PCR on *r4* cDNA were cloned in B-pSAL4 expression vector, digested with SmaI and BglII restriction enzymes, by using homologous recombination expression system of *S. cerevisiae*. Yeast transformation was done by using the lithium acetate protocol, as above described. The cells were spread on selective medium supplemented with 0.6 mM $CuSO_4$ and 0.2 mM ABTS and the plates incubated upside down for 4 days at 28°C.

Library screening

Single clones grown on plate were picked and transferred into 96-well plates containing 30 µl of selective medium per well. Plates were incubated at 28 °C, 250 rpm for 24 h. After 24 h, 130 µl of selective medium were added to each well and the plates were incubated at 28°C, 250 rpm for 24 h. Thirty µl of each culture were transferred to a new 96-well plate to measure the OD600 value. The plates were then centrifuged for 10 min at 1500 g, 4 °C, and a suitable volume of supernatant was transferred to a new 96-well plate to perform laccase assay. Phenol oxidase activity was assayed at 25 °C using 2mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}), using Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA). Enzyme activity was expressed in international units (IU).

Cultures were also performed in shaken flasks. Pre-cultures (10 ml) were grown on selective medium at 28 °C on a rotary shaker (150 rpm). A volume of suspension sufficient to reach a final OD600 value of 0.5 was then used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of selective medium and cells were then grown on a rotary shaker. Optical density and laccase activity determination were daily assayed.

DNA sequencing

Sequencing by dideoxy chain-termination method was performed by the Primm Sequencing Service (Naples, Italy) using specific oligonucleotide primers.

Protein purification

Preparative cultures were performed in 1 litre flasks containing 200 ml of selective medium. Culture media were harvested on the optimal laccase production day (6^{th} day), cells were sedimented by centrifugation at 1600 x g at 4 °C for 15 min. Secreted proteins were filtered through Whatmann sheet. Samples were concentrated using Amicon stirred cells with PM30 ultrafiltration membranes (Millipore, Billerica, MA). Most secreted proteins were precipitated by the addition of $(NH_4)_2SO_4$ up to 100% saturation at 4 °C and centrifuged at 10,000 x g for 40 min. The supernatant from ammonium sulfate precipitation (containing laccases) was loaded onto a 26/60 Superdex 75 prep grade (GE healthcare Bio-Sciences, AB, Uppsala, Sweden) column equilibrated with 0.15 M NaCl, 50 mM Na-phosphate buffer pH 7. The active fractions were pooled and concentrated on an Amicon PM-30 membrane. Protein concentrations were determined by the Bradford method using the BioRad Protein Assay (BioRad), and bovine serum albumin (BSA) as standard.

Laccase activity assays

Laccase activity was assayed at room temperature, using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,6-dimethoxyphenol (DMP), and syringaldazine (SGZ) as substrates as previously described [16]. For activity measurements at various pH values, laccase activities were assayed using DMP and

ABTS as substrates in citrate-phosphate buffer at pH ranges 3.0-6.2 and 2.5-5.5, respectively. pH stability was determined at 23 °C using ABTS as substrate in citratephosphate buffer adjusted at pH 3 and 5, in phosphate buffer adjusted at 7.0, and in Tris-HCl buffer adjusted at pH 7 and 10. Daily, the residual activities were determined by the standard activity assay. To explore the effect of temperature on mutants, assays were performed at temperature from 20-80 °C, at 10 °C intervals. For thermostability measurements, laccase solution was incubated at 60 °C in 0.1M Na-phosphate buffer, pH 7, and samples were withdrawn at 20 min intervals. Remaining enzyme activities were measured by the standard assay procedures.

All the assays were performed on three-replicates in at least two independent experiments.

Results

1. Construction of chimeric laccase cDNA

The cDNAs coding for 1M10B and 3M7C second generation mutants –previously selected from the collections of 2300 randomly mutated variants of *P. ostreatus* POXA1b laccase [14]- were used to prepare the chimeric laccase cDNA named *r4*. The 5'terminal region of 1M10B cDNA -coding for the first 150 amino-acids- and the 3'terminal portion of 3M7C cDNA -coding for the last 363 amino-acids- were joined generating the *r4* chimeric cDNA coding for a laccase containing the substitutions identified in 1M10B (K37Q, K51N) and in 3M7C (P494T), besides the substitution L112F introduced with the first generation mutation and present in both the selected mutants.

2. Heterologous recombinant expression and characterization of the chimeric laccase

The heterologous expression of the chimeric cDNA was performed in the yeast *S. cerevisiae*, by using the heterologous expression system previously developed for the *P. ostreatus* POXC, POXA1b and POXA3 laccases [17,18]. Growth curves of the recombinant yeast clones expressing the chimeric laccase and the parental mutants did not differ significantly, and analysis of activity production showed that the R4 chimera maintains the same specific activity production of the parental mutants (data not shown).

After partial enzyme purification, catalytic parameters of R4 were determined for the non phenolic ABTS substrate and for the two phenolic SGZ and DMP substrates, and compared with those of the POXA1b wild-type and of the 1M10B and 3M7C parental mutants (Table I). No variation of Michaelis constant (K_M) values towards ABTS and SGZ was observed following the chimera construction, whilst affinity of the chimeric enzyme towards DMP increases with respect to the parental mutants.

Since recombinant proteins have been only partially purified, specific activities values (Umg^{-1}) were estimated using the total protein concentration in the analysed enzyme solution, taking into account that similar amounts of total proteins were obtained in all cases. For all the analysed substrates, the specific activity of the chimera displays similar values to those of the parental mutants, while the comparison with the wild-type protein shows an increase of around 3- and 2- fold towards ABTS and the phenolic substrates, respectively.

When the effect of temperature on the activity of R4 on ABTS (Fig. 1A) was investigated, no significant differences were observed with respect to the parental mutants. Analysis of pH– activity profile performed using both ABTS (Fig. 1B) and

DMP (Fig. 1C) as substrates also showed similar trends for the parental mutants and the chimeric laccase.

The new enzyme was proved to be more stable than the wild-type laccase and even more than the parental mutants at 60 °C (Fig. 2A). Moreover, the chimera shows more durable activity both at pH 7 and at pH 10, exhibiting a half-life of around 1 month and half at pH 7 and 2 months at pH 10 (Fig. 2B).

3. Construction and screening of mutant laccase libraries

Random mutations were introduced into *r4* cDNA by EP-PCR, using three different PCR conditions to yield low, medium, and high average of mutation frequency (0–3 mut/kbases, 3–7 mut/kbases and more than 7 mut/kbases, respectively). A total of 1000 transformants were obtained by *S. cerevisiae* transformation with pSAL4 vector and mutated cDNAs.

When this new collection was screened by assaying activity towards ABTS, several mutants having higher activity than that of the controls (yeasts expressing R4 and POXA1b wild-type) were selected by multi-well plate screening. Following growth rate and activity production of the clones in multi-well plates for 3 days, five out of the previously isolated mutants were confirmed as positive. These five clones were then grown in shaken flasks for 6 days for further analyses. The 4M10G and 1H6C mutants showed higher laccase activity specific production (99 mU/OD at the 6^{th} day) than that of R4 (50 mU/OD at the 6^{th} day) and of the POXA1b wild-type (25 mU/OD at the 6^{th} day) (data not shown).

4. Characterization of random mutated laccases

Catalytic parameters of the two 4M10G and 1H6C selected mutants were determined for the non phenolic ABTS substrate and the two phenolic SGZ and DMP substrates, and compared with those of the POXA1b wild- type and of the parental R4 enzyme (Table II). As far as measured values of K_M are concerned, only in the case of 4M10G a decrease of affinity for ABTS was revealed.

Both the selected mutants show a (1.5- and 5- fold) increase of specific activity towards ABTS (with respect to the chimera and the wild type enzyme, respectively) and retain the activity values shown by R4 towards DMP and SGZ.

When the effect of temperature and of pH on the activity of the new mutants was analysed (Fig. 3), the 1H6C mutant displayed higher activity levels than R4 in wide ranges of pH and temperature.

Analysis of stability properties at different pH values (Fig. 4A) and at 60 °C (Fig. 4B) revealed for both the new mutants a stability increase at pH 5 in comparison with R4, whilst the improvement of stability properties observed in the parental enzyme at pH 10 and at pH 7 was shown only by 1H6C (Fig. 4A), that also displays a slightly higher thermo-stability (Fig. 4B) than the parental enzyme.

Sequence analyses of the selected mutants led to the identification of the nucleotide substitutions producing the mutations V126I for the 4M10G variant and V148L for the 1H6C mutant, besides the mutations of the parental R4 enzyme.

Discussion

The R4 chimeric laccase was obtained by joining the mutations identified in the 1M10B and 3M7C second generation mutants, previously selected from the collections of 2300 randomly mutated variants of *P. ostreatus* POXA1b laccase [14]. The 1M10B mutant shows more durable activity than the wild-type enzyme, displaying improved stability both at 60°C and at neutral and alkaline pH values. On the other hand, the 3M7C mutant stores stability properties of POXA1b, while

improving its catalytic efficiency. Besides the mutation L112F present in the parental mutant, the two additional mutations, responsible for the features of the 1M10B mutant, are located in the N-terminal region of the protein (K37Q, K51N), whereas only a further substitution is found in the Cterminal tail of the 3M7C mutant (P494T). Molecular dynamic simulations on 3D model structure of 3M7C had shown the latter mutation to affect flexibility of the protein, increasing the accessibility of the T1 copper site and thus leading to an improved activity of the enzyme [14]. A role for the C-terminal tail of POXA1b in affecting both the catalytic performance and stability properties of the enzyme was inferred through site directed mutagenesis [19] These results are consistent with the role already reported for the C-terminal tail of other fungal laccases [20,21], and with results of directed evolution experiments on laccase from the ascomycete *Myceliophthora thermophila* whose C-terminal tail was found as a targeted sequence [22,23].

To develop an improved POXA1b variant combining the increased catalytic efficiency of 3M7C with the improved stability of 1M10B, the 5'terminal region of 1M10B and the 3'terminal portion of 3M7C cDNA were joined generating the chimeric cDNA R4 coding for a laccase containing all the identified substitutions (L112F, K37Q, K51N, P494T). Joining these mutations does not affect specific activity of the laccase with respect to the parental mutants (Tab I). The main improvement due to the chimer construction is an increased stability at 60 °C , and even more at neutral and alkaline pH values (Fig. 2).

The increased stability of R4 makes it a more appropriate scaffold to carry out directed evolution, taking into account the direct relationship between stability, mutational robustness, and evolutionary capacity [24]. In fact, more stable enzymes should also be more susceptible to evolution since they have higher ability to tolerate functionally beneficial but destabilizing mutations. Therefore, directed evolution of the chimera was chosen as strategy to improve its catalytic performances. Both the new random mutants of R4, selected for enhanced activity against ABTS, 1H6C and 4M10G, were also proved to display higher stability than R4 at pH 5, showing 3 and 2 fold higher half life, respectively. Although narrowly connected during natural evolution, activity and stability are enzymatic features that are modulated by different principles and, therefore, their joint improvement is not easily achieved [24]. It is therefore worth noting that the results we achieved by directed evolution identified laccase variants that showed high activity as well as stability improvement.

Among the selected mutants, 1H6C proved to be the best variant as far as both activity and stability properties are concerned. This enzyme shows a further increase of specific activity on ABTS and exhibits the ability to retain this activity up to 60 °C (Fig. 3A) and in a wide pH range (2-5) (Fig. 3B). This variant also stores the improvements obtained by the chimera with respect to the activity towards DMP and SGZ and stability features at pH 10 and at pH 7.

In order to investigate structural determinants of the improved properties of the new mutant 1H6C with respect to the parental enzyme R4, its mutations were mapped on the 3D model previously obtained for POXA1b [14] and adopted to locate the mutations characteristic of the R4 chimera (Fig 5A).

Position 148 is located in a closely packed region of the domain 2 [25] adjacent to the reducing substrate binding site (Fig. 5B), that is located in a cleft between the domains 2 and 3. The substitution of V with L, increasing the size of the side chain, could further increase the packing of this region. Moreover, the close contact of the leucine side-chain with the aromatic ring of Y208 could change the conformation of the loop 204-208 forming the bottom of the reducing substrate binding site where the

D205 involved in the interaction with the aromatic substrate is located [25]. This change could, in turn, influence the oxidation rate of the reducing substrate but also the interaction between the domains 2 and 3.

In conclusion, in comparison with the POXA1b wild-type laccase, the semi-rational approach allowed us to develop a more efficient bio-catalyst -with a 2- and 5- fold higher specific activity on DMP and ABTS, respectively-, that was proved to be both more versatile and more durable, exhibiting activity in wider temperature and pH ranges and higher stability at acidic ($t_{1/2}$ at pH 5=35 days) neutral ($t_{1/2}$ at pH 7=38 days) and alkaline ($t_{1/2}$ at pH 10=62 days) pH values.

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	SUBSTRATE					
	ABTS		**DMP**		**SGZ**	
Laccases	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})
Wild-type	0.08±0.01	155±1	0.57±0.03	140±1	0.05±0.01	27.5±0.6
1M10B	0.10±0.01	421±2	0.35±0.02	281±2	0.04±0.02	24.3±0.6
3M7C	0.08±0.01	454±2	0.24±0.02	337±2	0.06±0.01	26.9±0.9
R4	0.13±0.01	420±2	0.15 ±0.01	280±2	0.03±0.01	45.8±0.5

Table I Catalytic parameters of the wild-type POXA1b, parental and chimeric mutants

	SUBSTRATE					
	ABTS		**DMP**		**SGZ**	
Laccases	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})	K_M (mmol l^{-1})	Specific activity (U mg^{-1})
wt	0.08±0.01	155±1	0.57±0.03	140±1	0.05±0.01	27.5±0.6
R4	0.13±0.01	420±2	0.15±0.01	280±2	0.03±0.01	45.8±0.5
1H6C	0.11±0.01	700±1	0.15±0.02	313±2	0.02±0.01	46.8±0.5
4M10G	0.23±0.01	750±2	0.13±0.01	270±1	0.01±0.01	43.8±0.2

Table II Catalytic parameters of the wild-type protein, chimeric and new selected mutants

Figure legends

Fig. 1 Effect of temperature on the activity of wild type and mutated laccases towards ABTS as substrate **(A)** and effect of pH on the activity of wild type and mutated laccases towards ABTS **(B)** and DMP **(C)** as substrates (♦, wild-type POXA1b; ■ mutant 3M7C; ● mutant 1M10B; ▲ R4)

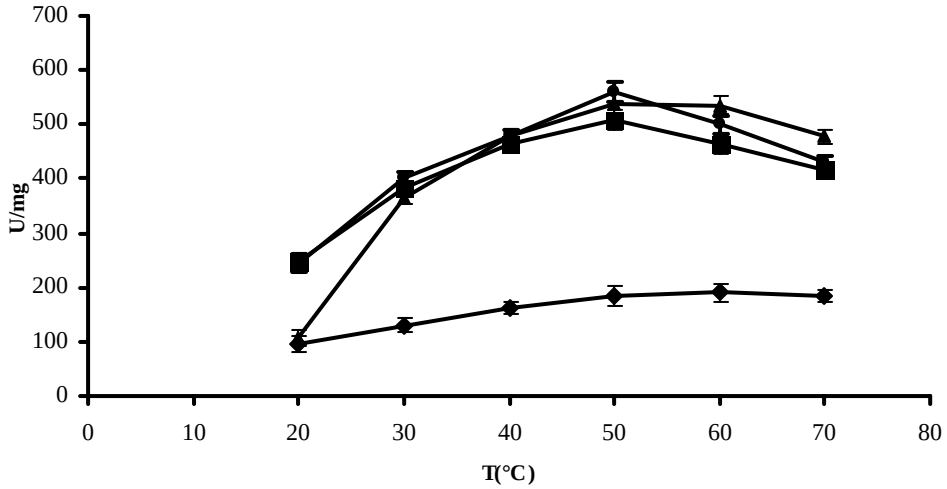
Fig. 2 Half-life of the wild-type protein and mutants at 60 °C (**A**) and at different pH values (**B**)

Fig. 3 Effect of temperature on the activity of wild type, random mutated and chimeric laccases towards ABTS as substrate (A) and effect of pH on the activity of wild type, random mutated and chimeric laccases towards ABTS (B) and DMP (C) as substrates (♦ chimeric laccase R4; ■ mutant 4M10G; ●wild-type POXA1b; ▲ mutant 1H6C)

Fig. 4 Half-life of the chimeric and random mutants at different pH values (**A**) and at 60 °C (**B**)

Fig. 5 3D structure model of POXA1b with (A) representation of the mutated residues of R4 (L112F, K37Q, K51N, P494T) in stick rendering; (B) close-up of the region surrounding the mutation (L148) in 1H6C: copper ions (orange), and the residues Y208 (pink) and L148 (cyan) are highlighted in van der Waals representation, whilst the residues of the loop 204-207 (yellow) are represented in stick rendering.

A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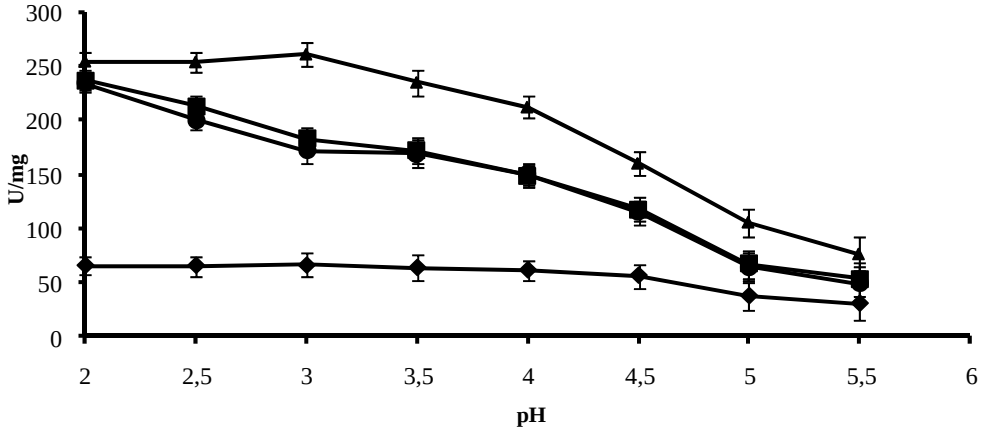
B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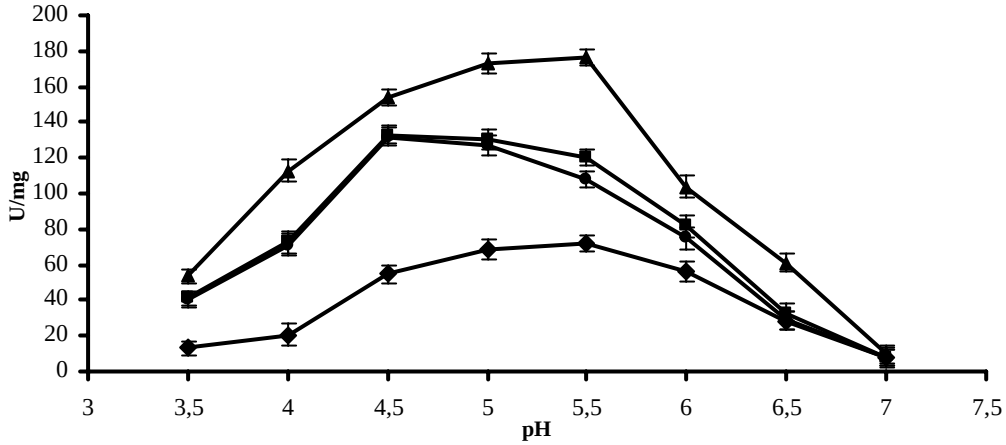
C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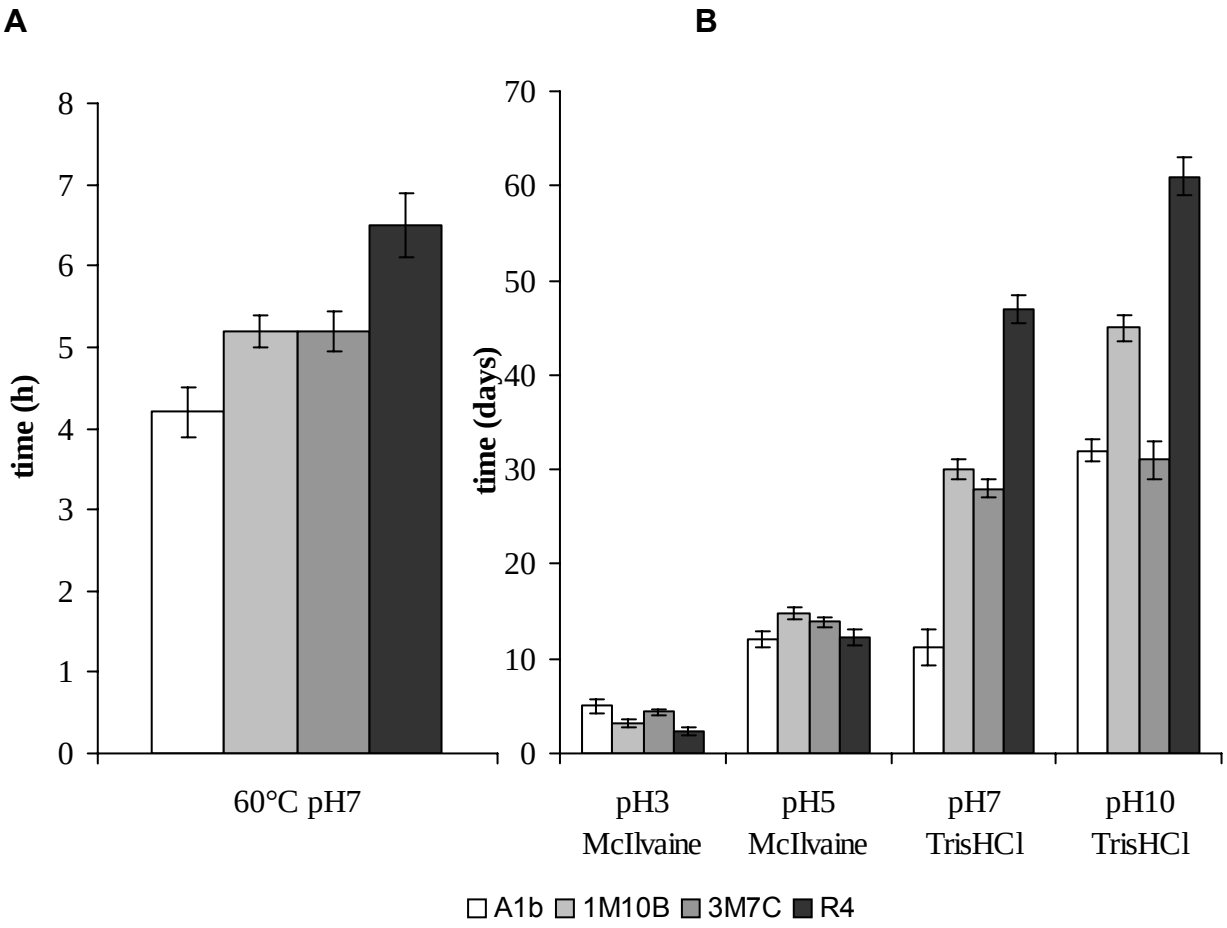
pH

Fig. 1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B
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60
70
60°C pH7
pH3 McIlvaine
pH5 McIlvaine
pH7 TrisHCl
pH10 TrisHCl
A1b
1M10B
3M7C
R4

Fig. 2

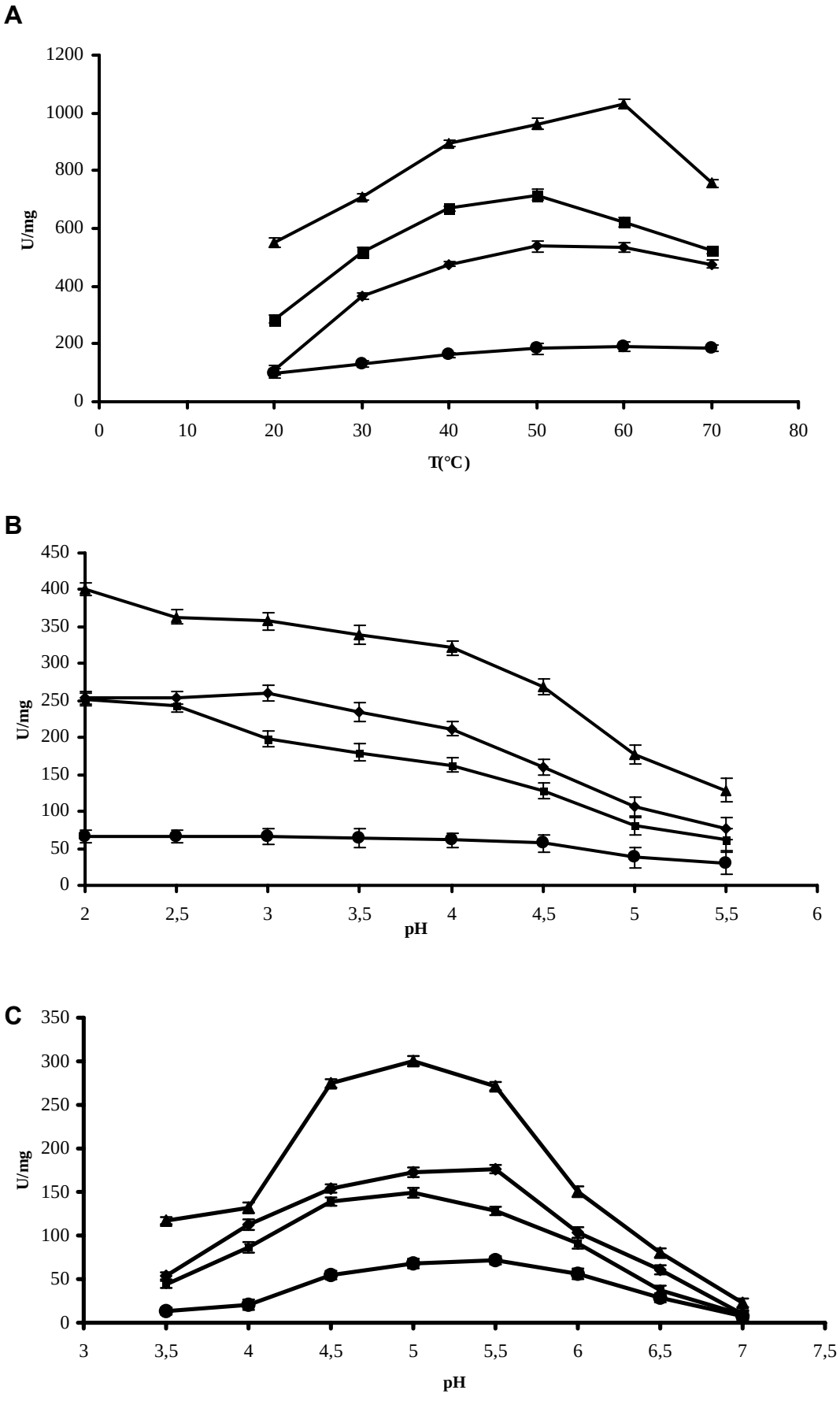
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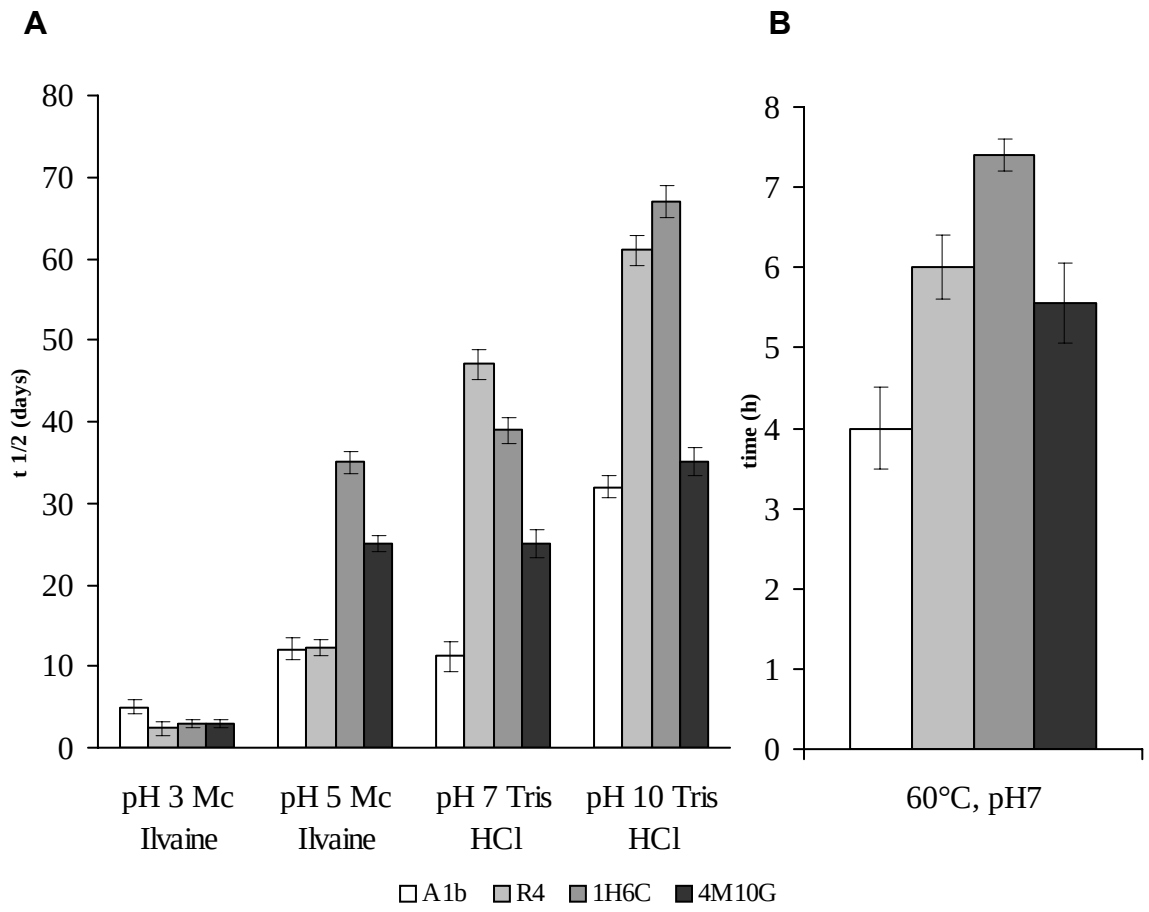
Fig. 3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8
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2
1
0
pH 3 Mc Ilvaine
pH 5 Mc Ilvaine
pH 7 Tris HCl
pH 10 Tris HCl
60°C, pH7
A1b
R4
1H6C
4M10G

Fig.4

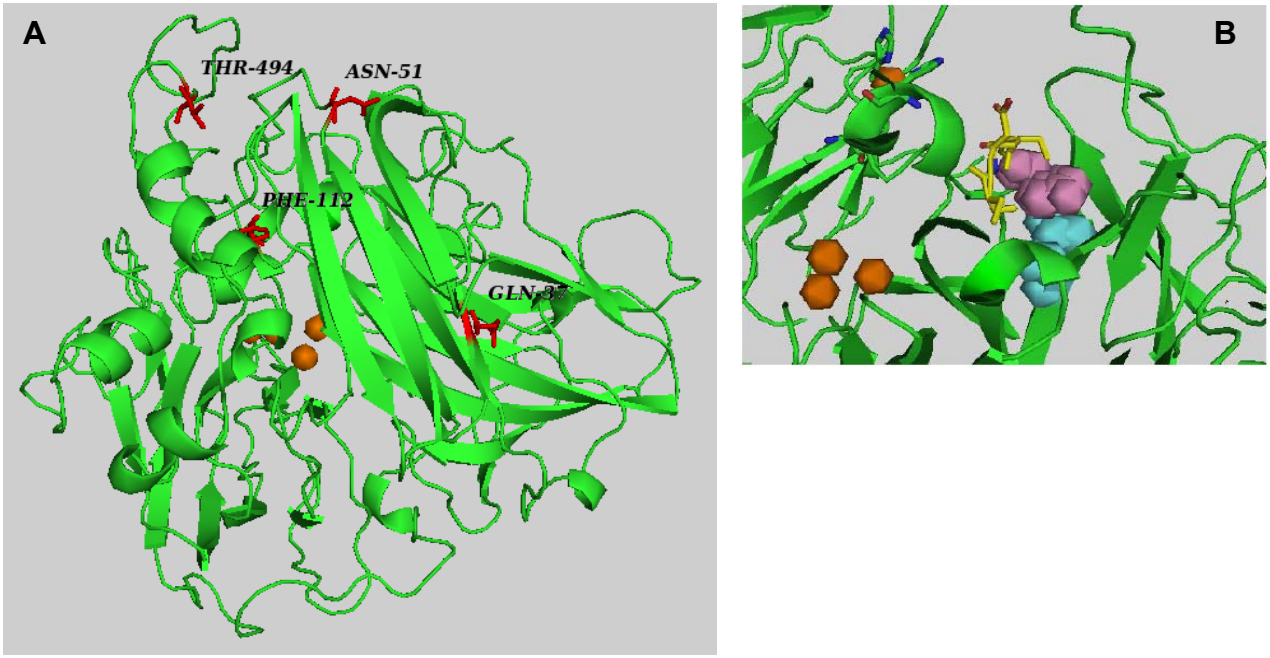

Fig. 5

3. Conclusions

In the chapter 2 the employ of a new molecular evolution technique and the improvement of random library of POXA1b laccase were described.

The R4 chimeric laccase contains the mutations of the two parental enzymes (1M10B and 3M7C), previously selected for their increased specific activity as well as improved stability. As the main improvement following the chimera construction, an increased stability was revealed at 60 °C , and even more at neutral and alkaline pH values, with a half-life of 1 month and half at pH 7 and two months at pH 10. Improvement of stability properties observed in R4 makes it a more appropriate scaffold to carry out directed evolution, taking into account the direct relationship between stability, mutational robustness, and evolutionary capacity.

A new collection of 1100 mutants was obtained using R4 as template. When this collection was screened by assaying activity towards ABTS, the 4M10G and 1H6C mutants showed 2- and 4-fold higher laccase activity specific production than that of R4 and of the POXA1b wild-type, respectively. These new variants were characterized showing a significant increase in stability at pH5 (~3 fold increase of stability). Moreover, the mutant 1H6C stores the stability of parental enzyme at alkaline pHs. At 60°C the mutants show similar stability compared with that of the wt protein.

In order to investigate structural determinants of the improved properties of the new mutant 1H6C with respect to the parental enzyme R4, structural and computational analyses were performed mapping mutations on the 3D model previously obtained for POXA1b. The substitution of V with L in position 148 could influence the oxidation rate of the reducing substrate but also the interaction between the domains 2 and 3.

In conclusion, a new strategy of enzymes mutagenesis allowed to develop a more efficient bio-catalyst that was proved to be both more versatile and more durable, exhibiting activity in wider temperature and higher stability at acidic (t1/2 at pH 5=35 days) neutral (t1/2 at pH 7=38 days) and alkaline (t1/2 at pH 10=62 days) pH values.

Chapter 3

1. Introduction

Laccases have attracted a lot of research interest in the last decades. As a widespread and heterogeneous class of oxidative enzymes, laccases have been the object of deep biochemical investigations. At the same time, interest in laccases is ever increasing due to their potential use in several biotechnological and environmental applications.

Large amounts of laccase are required for fundamental studies on laccase properties and, especially, for their practical use. Nevertheless, laccases secreted from wild type plant or fungal organisms may not be suitable for commercial purposes mainly because of low yields or high cost of preparation procedures. Heterologous expression should be better suited for large-scale production, because of the potential of expressing different laccases in one selected optimised host. At least 29 fungal laccase genes, including five from *Trametes villosa* and *Trametes sanguinea*, four form *Rhizoctonia solani*, three from Badiomycetes I-62 (CECT 20917) and *Pleurotus ostreatus*, two from *Agaricus bisporus*, *Pycnoporus cinnabarinus*, and one each from *Neurospora crassa*, *Coriolus hirsutus*, *Phlebia radiata*, *Coprinus congragatus*, *Mauginiella* sp. and *Volvariella volvacea*, have been cloned and expressed in heterologous hosts [1]. Eukaryotic hosts such as *Aspergillus oryzae*, *Aspergillus niger*, *Pichia pastoris*, *Saccharomyces cerevisiae* and *Trichoderma reesei* proved to be the most suitable microorganisms for laccase expression since they can provide efficient post-translational modifications required by laccases, such as glycosylation and disulphide bond formation.

Previous studies suggest that yeasts such as *Pichia pastoris* and *Saccharomyces cerevisiae* are convenient systems for rapid expression of laccase genes. However, production levels in yeast have been quite low (up to approx 5 mg/L), whereas filamentous fungi in general have given 2-30 times higher levels (10-135 mg/L) [2]. Filamentous fungi, such as *A. niger*, have the ability to produce and secrete exceptionally large amounts of properly folded proteins with the correct cofactors incorporated and can produce proteins that contain O- and N-linked glycans without extensive hyperglycosylation [3-5]. Filamentous fungi are, at least so far, the best producers of recombinant laccases, with protein yields from 70 mg/L [6] to 230 mg/L [7]. Even more than a century ago, fungi were know to produce and secrete enzymes in large quantities, which has resulted in an increasing interest in studing and using filamentous fungi in industrial processes as well as in the laboratory. Over the years, classical genetic studies have resulted in an accumulation of knowledge about fungal genetics and fungal biochemical pathways [8].

The development of molecular-biological techniques has opened new ways to use filamentous fungi for the production of homologous and heterologous proteins. In particular, species of the genera *Aspergillus* and *Trichoderma* are being used extensively for large-scale production of proteins. Initially, work on heterologous protein production in *Aspergilli* was started with *Aspergillus nidulans*, the species that has been studied since the early 1940s [9].

During the last decade other species, mainly *Aspergillus niger*, *Aspergillus awamori* and *Aspergillus oryzae*, have been used more frequently, since higher levels of recombinant proteins can be obtained with these strains.

Members of the genus *Aspergillus* occur in a wide variety of habitats. Some are common as saprophytes in soil, while other are found on stored food and in decaying vegetation. Raper and Fennel [10] identified 18 distinct grups, later classified into

subgenera and sections by Samson [11]. Some of these groups consist of toxigenic fungi (e.g. *A.flavus*), but some members are ideal organisms for industrial applications (e.g., *A. oryzea* and *A. niger*). In particular, they are able to produce a wide range af enzymes for the degradation of plant cell wall polysaccharides (e.g., pectinases and xylanases), of major importance for food and feed industries [12,13].

Filamentous fungi, such as *A. niger*, have the capability to secrete large amounts of proteins into their growth medium. Because several products produced with *A. niger* have the GRAS (Generally Recognized As Safe) status, this filamentous fungus is an attractive host for production of homologous and heterologous proteins [3,8,14-16].

1.1 Heterologus expression in *Aspergillus niger*

A. niger is included in *Aspergillus* subgenus *Circumdati*, section *Nigri.* The section *Nigri* includes 15 related black-spored species that may be confused with *A. niger,* including *A. tubingensis, A. foetidus, A. carbonarius, and A. awamori* [17,18]. A number of morphologically similar species was recently described by Samson et al. [18]. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food.

A. niger is cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574) and have been assessed as acceptable for daily intake by the World Health Organisation [19]. Many useful enzymes are produced using industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification. α-galactosidase, an enzyme that breaks down certain complex sugars, is a component of Beano and other medications which the manufacturers claim can decrease flatulence. Another use for *A. niger* within the biotechnology industry is in the production of isotope-containing variants of biological macromolecules for NMR analysis. In 2006 it was reported that a secreted RNase produced by *A. niger* called actibind has antiangiogenic and anticarcinogenic characteristics. *A. niger* is also cultured for the extraction of the enzymes glucose oxidase (GO) and Alpha-galactosidase (AGS). Glucose oxidase is used in the design of glucose biosensors, due to its high affinity for β-D-glucose [20]. Alpha-galactosidase can be produced by *A. niger* fermentation; it is used to hydrolyze alpha 1-6 bonds found in melibiose, raffinose, and stachyose. In the heydey of the opium trade, chandoo opium, which was meant to be smoked, was made by long term fermentation of *A. niger* and other molds on raw opium [21]. *A. niger* is the main agent in the fermentation of Pu-erh tea [22].

Record et al. (2002) published the first report of the production of a white-rot laccase in *A.niger*. *Pycnopourus cinnabarinus* laccase *lac*1 gene was overespressed in *A.niger* [6]. The laccase gene, lac1, was overexpressed successfully in *Pichia pastoris* as an active protein but with an hyperglycosylation increasing the molecular mass to 110 kDa as compared to the 70-kDa wild-type protein [14]. The production level of the recombinant protein in *Pichia* was high enough to allow the first structure function studies, but too low to consider industrial approaches. In order to produce large-scale level of *P. cinnabarinus* laccase, Record's group expressed the corresponding cDNA in *A. niger*. The corresponding cDNA was placed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter as a strong and

constitutive promoter. The laccase signal peptide or the glucoamylase preprosequence of *A.niger* was used to target the secretion. Both the signal peptides directed the secretion of laccase into the culture medium as an active protein, but the *A.niger* preprosequence allowed an 80-fold increase in laccase production. After Western blot analysis and N-terminal sequencing, the recombinant protein was identified and his molecular mass was 70 kDa, similar to that of the native form, suggesting a weak hyperglycosylation [2].

1.2 Recombinant expression of *P. ostreatus* laccases

Laccase cDNAs from *P. ostreatus* have been used to develop expression systems in the thee yeasts *K. lactis, S. cerevisiae* [23] and *P. pastoris*. In the table 1 the activity productions are shown.

	S. cerevisiae	***K. lactis***	***P. pastoris***
rPOXA1b	0.2 U ml^{-1} (7 days)	4.2 U ml^{-1} (3 days)	-
rPOXC	$8.4*10^{-4}$ U ml^{-1} (4 days)	0.09 U ml^{-1} (4 days)	-
rPOXA3	-	0.08 U ml^{-1} (14 days)	-
rPOX3	0.03 U ml^{-1} (2 days)	0.012 U ml^{-1} (3 days)	4 U ml^{-1} (8 days)
rPOX4	nd	nd	0.015 U ml^{-1} (8 days)

Table 1 Activity production of recombinant laccases from *P. ostreatus* in different hosts

For the heterologous expression of *P. ostreatus* laccases in *S. cerevisiae* three expression vectors were constructed for each protein, differing from each other for replication origins, promoter regions and secretion leader sequences. The plasmid with 2μ as replication origin, copper inducible CUP1 promoter region, and homologous laccase signal peptides gave the best results for both laccases. However, the native signal peptide always proved to be more effective in extracellular laccase production.

Recombinant expression in *K. lactis* was performed using both laccase and killer toxin signal peptide, and intra- and extracellular laccase productions were analysed. No recombinant enzyme was detected by immunoblot analysis in both compartments when killer toxin signal peptide was used. On the other hand, the laccase signal peptide allowed production and secretion of active laccase in the medium. Therefore, the signal peptide not only affects secretion but also production of recombinant enzymes in yeasts.

The apparent molecular mass of the recombinant proteins expressed in *K. lactis* is similar to that of the native ones, whereas *S. cerevisiae* extracellular rPOXA1b shows higher molecular mass. These data confirm the tendency of *K. lactis* with respect to *S. cerevisiae* to not hyperglycosylate recombinant proteins.

Performances of the *K. lactis* expression system (efficient secretion ability, weaker tendency to hypermannosilate proteins, and ease of manipulations) make this yeast a better producer than *S. cerevisiae*, at least for *P. ostreatus* laccases, and a promising host for deeper investigations. Unfortunately, in contrast to *S. cerevisiae*, the budding yeast *K. lactis* shows, as also described by Zeeman and Steensma, 2003 [24], variable, but in general low, gene targeting efficiency. The introduction of the cDNAs in the appropriate locus of the expression vector by homologous

recombination in *K. lactis* always led to the production of several (70%) mutants able to grow on selective medium but unable to express any laccase activity, probably because of low targeting efficiency.

For this reason, the results obtained with recombinant expression of *P. ostreatus* laccases in *S. cerevisiae* encouraged to project new oxidative catalysts using molecular evolution techniques. Therefore, the *S. cerevisiae* laccase expression systems were further used to set up directed evolution experiments, despite the fact that the amount of secreted laccase was not very high.

Another expression system for the laccase from *P. ostreatus* was *P.pastoris*. In particular, it was used to express the new laccase rPOX2and rPOX4 [25]. After eight days of culture, up to 4U/ml laccase levels were achieved for the best producing rPOX3 clone, whereas rPOX4 activity levels reached up to 0.015U/ml in the clone showing the highest laccase production. These results indicate a significant improvement of rPOX3 laccase production compared to the levels reached in *S. cerevisiae* (around 0.03 U/ml at the 2nd day) and *K. lactis* (around 0.012 mU/ml at the 3rd day) (table 1). Moreover, it is worth to note that rPOX4 laccase activity was not detected in neither of these two hosts, while it could be revealed, even if at low level, in *P. pastoris*.

These preliminary results indicate that *P. pastoris* could be a promising expression system for *P. ostreatus* laccases if compared to *S. cerevisiae* and *K. lactis*. However, literature data report for an higher level of glycosylation of the enzyme expressed by *P. pastoris*.

This appendix deals with the experimental work carried out in the Laboratory of Biotechnology at University of Science of Luminy, Marseille during my two months of foreign experience. The challenge was to design strategies based on heterologous expression in *A. niger* to maximize the yield of recombinant laccases, in the attempts to raise production levels sufficient to enable deeper understanding of the structure/function relationships and, consequently, industrial applications of *P. ostreatus* laccases POXA1b and its mutant 1H6C.

2. Results and Discussion

Cloning and expression of POX3 and POX4 laccases

The expression of *P. ostreatus* POXA1b and 1H6C coding cDNAs was carried out in the heterologous host *A. niger*. To this aim, both sequences, were cloned in the pAN52.4 expression vector. Laccase secretion in the extracellular medium is driven by the signal peptide of the 24 amino-acid glucoamylase (GLA) preprosequence from A*. niger*. Previously studies have shown that using GLA peptide signal the laccase activity has an increase of 80-fold compared to other construction as the laccase signal peptide [6].

A. niger D15#26 strain is deficient in oritidine-5'- phosphate decarboxilase (pyrG), the terminal enzyme in uridine-5'-phosphate biosynthesis. In a cotransformation experiment, *A. niger* D15#26 was transformed with a mixture of plasmid pAB4-1, containing the *pyrG* gene as a selection marker, and the expression vectors containing the laccases cDNA from *P. ostreatus* and its variant. Transformants were selected for their abilities to grow on a minimum medium plate without uridine. For

each construct, approximately 100 uridine prototrophic trasformants were obtained per microgram of expression vector. Both POXA1b and 1H6C expression vectors (pAN52.4-A1b and pAN52.4-1H6C) were successfully transformed into the filamentous fungi. Cotransformants containing the laccases cDNA were tested for laccase expression by growing on minimum medium plates supplemented with ABTS. Recombinants expressing laccase were identified by appearance of a green zone around the colonies after 7-10 days at 30°C. Colorated zone on the plate were not observed in the case of control transformants lacking the laccase cDNA (Fig.1). Seventeen and twenty-two clones were cultured in liquid medium for POXA1b and 1H6C, respectively, and then assayed at optimal day production. Results for laccase activity were ranging from 800-38500 IU/L (day 8) and 5000-55000 IU/L (day8), respectively, for *A. niger* transformant by pAN52.4-A1b and pAN52.4-1H6C. The best clone was selected for each construction in order to study the time course of the laccase activity.

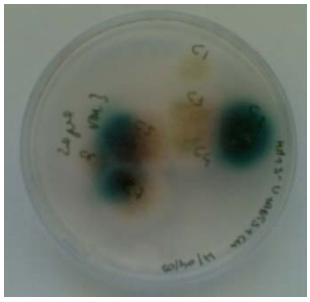

Fig.1 Activity laccases test on *Aspergillus niger* transformants

Analysis of laccase production in liquid culture

Preliminary results on laccase production in liquid medium were obtained performing *A.niger* cultures in shake-flasks. For both the expression vector, the laccase activity was found in the culture medium, indicating that laccase was secreted from *A. niger*. Activity was not found in the control culture (transformation with pAB4-1, without pAN52.4-A1b or pAN52.4-1H6C). In both cultures, mycelia dry weight increased until day 10. In additional the pH was maintained by supplementation with citric acid around pH5.0. A time-course analysis of rPOXA1b and r1H6C culture supernatants was initially performed on the recombinant clones selected by the first screening on plate (Fig.2).

After eight days of culture, up to 400U/ml laccase levels were achieved for the best producing rPOXA1b clone, whereas r1H6C activity levels reached up to 550U/ml in the clone showing the highest laccase production.

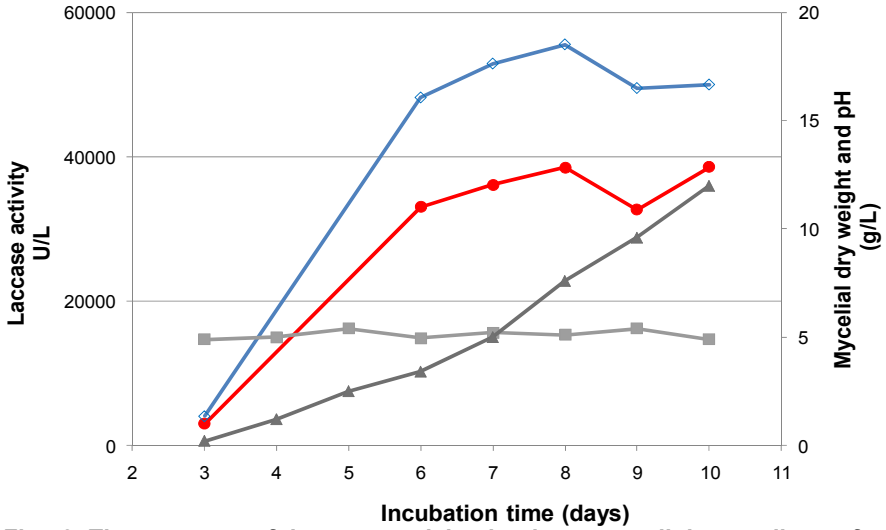

Fig. 2 Time course of laccase activity in the extracellular medium of *A.niger* cultures producing wild-type and mutated enzyme. Activity (◊ 1H6C, ● POXA1b), mycelia dry weight (▲) and pH (■).

These results indicate a significant improvement of rPOXA1b laccase production compared to the levels reached in *S. cerevisiae* (around 0.2 U/ml at the 7rd day), *K. lactis* (around 4.2 U/ml at the 3rd day) shown in table 1.

Production of the recombinant laccases was checked by elettroforesis on an SDS/polyacrylamide gel (Fig.3). A clear band of around ~80KDa was observed corresponding to the wild-type laccase from *P. ostreatus*. Immune detection of laccase was performed using antibodies raised against the *P. ostreatus* laccase. The Western blot analysis (data not shown) showed a unique band corresponding to ~ 80KDa protein demonstrating that this protein is the recombinant laccase.

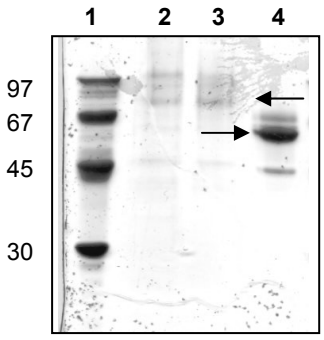

Fig. 3 SDS-PAGE analyses of rPOXA1b. Lane 1, molecular mass standards lane 2, rPOXA1b; lane 3, r1H6C; lane 4, native protein (POXA1b)

Previously, the *P.ostreatus* laccase POXA1b produced in *K. lactis* has a slightly higher apparent molecular mass where in *S. cerevisiae* was demonstrated to have a molecular mass higher than the native laccase, suggesting that an heterologous protein with hyperglycosylation was produced [23]. This phenomenon was also described for other laccases as for exempla the *Trametes villosa* laccase produced in *A. oryzae* [26]. Glycosylation was 0.5% of the molecular mass of the native laccase and 10% for the recombinant laccase. In the heterologous production of the *P. ostreatus* laccase in *K.lactis* and in *S. cerevisiae* [23] or the *T. villosa* laccase in *A. oryzae* [26], additional carbohydrates were added to the recombinant laccase, but had apparently no effect on their enzymatic activity [23,26]. In the experiment described before, the recombinant laccase produced by *A. niger* has the same molecular mass than the native laccase, suggesting weak hyperglycosylation. For this reason, *A. niger* seems to be the most adapted host for fungal laccase overproduction.

3. Material and methods

Strains, culture media

The *Escherichia coli* strain Top 10 (*F-mcr*A D (*mrrhsd*RMS-*mcr*BC) *f80lac*ZDM15 D*lac*X74 *deo*R *rec*A1*ara*D139 D (*ara–leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) was used in all DNA manipulations. *E.coli* was grown in Luria–Bertani (LB) medium (in g L^{-1}: 10 bacto tryptone, 10 NaCl, 5 yeast extract), supplemented, when required, with 100 µg mL^{-1} of ampicillin.

A.niger strain D15#26 ($pyrg^-$) [27] was used for heterologous expression. After cotransformation with vectors containing, respectively, the *pyrG* gene and the laccase cDNA, *A. niger* was grown on selective solid minimum medium (without uridine) containing 70 mM $NaNO_3$, 7 mM KCl, 11 mM KH_2HPO_4, 2 mM $MgSO_4$, glucose 1% (w/v), and trace elements (1000x stock solution consists of: 76 mM $ZnSO_4$, 178 mM H_3BO_3, 25mM $MnCl_2$, 18mM $FeSO_4$,7.1 mM $CoCl_2$, 6.4 mM $CuSO_4$, 6.2 mM Na_2MoO_4, 174 mM EDTA).

Chemicals

Restriction enzymes and Takara polymerase were, respectively, purchased from Promega. [a-32P]dCTP and Lonza was purchased from Amersham Pharmacia Biotech.

DNA sequencing

Sequencing by dideoxy chain-termination method was performed by Primm Sequencing Service (Naples, Italy) using specific oligonucleotide primers.

Expression vectors

The expression vectors was constructed using a PCR cloning approach, and the cloned PCR products were checked by sequencing. Table 2 shows the primers, vectors, and restriction sites used in the cloning strategy, and Table 3 lists the primer sequences. The construct pUC18 contained the laccases cDNA corresponding to the laccases gene, *poxa1b* from *P. ostreatus* (GenBank accession no AJ005018) and his mutant *1H6C* (Fig.3). The laccase signal peptide was replaced by the 24 amino-acid glucoamylase (GLA) preprosequence from A*. niger*. The *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (gpdA) promoter, the 5' untranslated region of the gpdA mRNA, and the *A. nidulans trp*C terminator were used to drive the expression of the laccase encoding sequence.

Expression vectors	**Primers**		**Cloning vectors**	**Cloning site restriction fragments**	**Cloning site vectors**
	Forward	Reverse			
pUC18	(POXA1b-1H6C)/Eco	(POXA1b-1H6C)/Hind	**pAN52-4(a)**	*Bss*HII±*Hind*III	*Bss*HII±*Hind*III

(a) EMBL accession number Z32750.

Table 2. Cloning strategy. For each expression vector are indicated the name of the primers used for amplification of the laccase DNA and addition of cloning sites, recipient *Aspergillus* expression vector and restriction sites used in the final cloning procedure.

Oligonucleotides	**Sequences**	**Restriction sites**
(POXA1b-1H6C)/Eco	TTG AAT TCG CGC GCT AGC ATT GGG CC R A S I G	*Eco*RI
(POXA1b-1H6C)/Hind	TCC GGC AAG CTT TCA TGC TTT CAA TGG St A K L P	*Hind*III

Table 3. Oligonucleotides used for cDNA amplification and cloning. St, stop codon. Restriction sites are underlined.

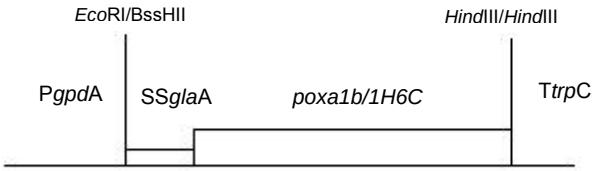

Fig. 3 Laccases gene expression vector. For an explanation, see Experimental procedures and Table 1.

Aspergillus transformation and laccase production

Fungal cotransformation was basically carried out as described by Punt & van den Hondel [28] using each of the laccase expression vectors and pAB4-1 [29] containing the *pyrG* selection marker, in a 10 : 1 ratio. Transformants were selected for uridine prototrophy. Cotransformants containing expression vectors were selected as

described in the following section. In order to screen the laccase production in liquid medium, 50 mL of culture medium containing 70 mM $NaNO_3$, 7mM KCl, 200 mM Na_2HPO_4, 2mM $MgSO_4$, glucose 10% (w/v), trace elements and adjusted to pH 5 with a 1 M citric acid solution were inoculated by 1 x 106 spore·mL^{-1} in a 300 mL flask. The culture was monitored for 12 days at 30 °C in a shaker incubator (200 r.p.m.). pH was adjusted to 5.0 daily with 1-M citric acid.

Screening of the laccase activity and laccase assay

Agar plate assay on selective medium (minimum medium without uridine) with 200 µM ABTS were used for the selection of transformants secreting laccase. Plates were incubated for 10 days at 30 °C and checked for development of a green color. From liquid culture medium, aliquots (1 mL) were collected daily and cells were removed by filtration (0.45 µm). Laccase activity in the culture supernatant was assayed by monitoring the oxidation of 2 mM ABTS at 420 nm to the respective radical (ε_{420} = 36 $mM^{-1}\cdot cm^{-1}$) [30], in the presence of 0.1 M sodium citrate buffer pH 3.0. Activity is indicated in international units (IU) which are the amount of laccase that oxidizes 1 µmol of substrate per min.

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5. Conclusions

Aim of the work described in this chapter has been to overexpress the POXA1b laccase and its variant 1H6C using the filamentous fungi *A. niger* as host. The necessity to produce large amounts of enzymes for biotechnological applications and the low yields of laccases secreted from wild-type fungal organisms claims for an heterologous expression of the enzymes of interest.

Three different hosts have been used for recombinant expression of laccases from *P. ostreatus:*

1. *K. lactis*: good level of production, no hyperglycosylation but variable, and in general low, gene targeting efficiency;
2. *S. cerevisiae*: amount of secreted laccase not very high, high glycosylation, no problem related to low gene targeting efficiency;
3. *P. pastoris*: high level of enzyme production but extensive hyperglycosylation.

On the basis of these data *A. niger* has been chosen as new expression system for its higher level of enzyme production and its lower glycosylation respect to the other hosts used before.

The cDNAs of *poxa1b* and *1h6c* were placed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter as a strong and constitutive promoter. The glucoamylase preprosequence of *A. niger* was used to target the secretion. The best clones selected show an increase of 100-fold of the laccase activity respect to the other expression systems. In fact, after eight days up to 400 and 500 U/ml laccase levels were achieved for the best clones producing respectively rPOXA1b and r1H6C.

The identity of the recombinant proteins was further confirmed by immunodetection using Western blot analysis. The molecular mass of the mature laccase was ~ 80 kDa as expected, similar to that of the native form (65 KDa), suggesting no hyperglycosylation.

These preliminary results indicate that *A. niger* could be a promising expression system for *P. ostreatus* laccase if compared to other expression systems. However, the laccase production reported in these scouting experiments, could probably be enhanced by further optimization of medium and cultivation conditions.

Conclusions

Conclusions

In this work six new mutants of POXA1b laccase from *P. ostreatus* were selected for their improved stability properties and activity against different substrates (phenolic and non-phenolic) and in different operative conditions (different pHs).

Three new mutants were selected from the already available collection of 2300 mutants of POXA1b laccase. Two variants (2L4A, 3L7H) derived from the screening of the library against DMP and one (1L9A) from the screening of the collection against ABTS at pH5, were purified, fully characterized and compared with the wild type protein. These new variants show a significant increase in stability at acidic pH (pH5). One of the mutants (2L4A) was also proved to mantain high stability of wild type at alkaline pHs (pH10). This variant also represents a more versatile enzyme with respect to the variety of xenobiotics degraded and the operative conditions. 2L4A and 3L7H mutants were shown to be able to decolourize industrial dyes with complex trisazo-, polyazo- and stilbene-type structures, in the absence of mediators.

At the same time, a new semi rational mutagenesis approach was carried out to improve POXA1b properties and enlarge collection of its random variants. The R4 chimeric laccase was rationally "designed" and "constructed" by joining the mutations of previously selected POXA1b random variants (1M10B and 3M7C). The chimera construction provided an enhancement of stability features ($t_{1/2}$ at pH10= ~ 60 days), making the novel enzyme R4 more appropriate as a scaffold for directed evolution. A library of 1000 randomly mutated variants of R4 was prepared and screened for the ability of oxidizing ABTS selecting the new mutants 4M10G and 1H6C. These new variants showed 2- and 4-fold higher laccase activity specific production than that of R4 and of the POXA1b wild-type, respectively. Characterization of 4M10G and 1H6C showed that they display a significant increase in stability at pH5 (~3 fold increase of stability). Moreover, the mutant 1H6C maintains the stability of parental enzyme at alkaline pHs.

To investigate the causes of the improved activity and stability properties shown by the mutants selected, structural and computational analyses were performed. The identified mutations, although not located in active sites, were proved to indirectly influence the efficiency - affecting the conformation of catalytic site - or improve enzyme stability - allowing additional interactions.

In particular, inspection of the protein model suggested that the Q272H mutation, characteristic of the 2L4A mutant, stabilizes the protein structure at pH 5, because it allows additional interactions - hydrogen bonds and electrostatic interactions - between the positively charged imidazolic ring of the His272 and the side chain of Asp287. This interaction could also cause a movement of the loop where it is located in position 272, involved in the formation of the reducing substrate-binding pocket, thus affecting enzymatic activity.

The S285N mutation, characteristic of the 3L7H mutant, is located very close to the Asp287 residue, both aminoacids belonging to the Gly282-Thr289 loop that seems to play an important role for protein stability. On the other hand, the N328S mutation, found in 3L7H, could have effect on the enzymatic activity, being located in a loop potentially interacting with the reducing substrate.

The 1L9A mutant presents one substitution (R284H) besides L112F. This substitution is also located very close to the Asp287 residue, both aminoacids

belonging to the Gly282-Thr289 loop that seems to have an important role for the protein stability.

The R4 chimeric laccase contains the mutations K37Q, K51N, L112F (from 1M10B) and P494T (from 3M7C). Aminoacids in position 37and 51 are generally conserved in so far analysed laccases. Therefore, the substitutions K37Q, K51N restore conserved residues in the protein sequence, suggesting that the relatively fast directed evolution mimics the same result of the slower natural evolution. Position 37 is located near the N-terminus loop (Ser-Ile-Gly-Pro-Arg) with which a Glutamine residue can establish different interactions compared to Lysine residue, stabilizing the protein. Molecular dynamic simulations on 3D model structure of 3M7C had shown the latter mutation to affect flexibility of the protein, increasing the accessibility of the T1 copper site and thus leading to an improved activity of the enzyme.

Sequence analyses of the selected mutants led to the identification of the nucleotide substitutions producing the mutations V126I for the 4M10G variant and V148L for the 1H6C mutant (besides the mutations of the parental R4 enzyme). In particular, position 148 is located in a closely packed region of the domain 2 adjacent to the reducing substrate binding site, that is located in a cleft between the domains 2 and 3. The substitution of V with L, increasing the size of the side chain, could further improve the packing of this region. Moreover, the close contact of the leucine side-chain with the aromatic ring of Y208 could change the conformation of the loop 204-208 forming the bottom of the reducing substrate binding site where the Asp 205, involved in the interaction with the aromatic substrate, is located. This change could, in turn, influence the oxidation rate of the reducing substrate but also the interaction between the domains 2 and 3.

To maximize the yield of recombinant laccases, in order to deepen structure/function relationships and improve industrial applications of *P. ostreatus* laccases, POXA1b and its best variant 1H6C were expressed in the filamentous fungi *A. niger*. The best clones selected show an increase of 100-fold of the laccase activity production with respect to other expression systems. The identity of the recombinant proteins was confirmed by immunodetection using Western blot analysis. The molecular mass of the mature laccase was ~ 80 kDa as expected, similar to that of the native form (65 KDa), suggesting no hyperglycosylation.

In conclusion, this work allowed widening of a library up to 3300 mutated laccases and the identification and characterization of six "new" laccases more active and more stable than the protein used as template. The identified mutations have been proved, although not directly involved in catalysis, to influence the efficiency and the stability of the enzyme, increasing our knowledge of structure-function relationships in this class of enzymes. Moreover *A. niger* was demonstrated to be a promising expression system for *P. ostreatus* laccases.

Publications and Communications

PUBLICATIONS

- Faraco V, C. Pezzella, A Miele, P Giardina,G Sannia. Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. 2008 Biodegradation 2009 Apr;20(2):209-20. Epub 2008 Aug 30.

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EXPERIENCES IN FOREIGN LABORATORIES

October - December 2008: *Department of Molecular Biology and Genetic, Istanbul Technical University (ITU), Istanbul (Turkey)* under the supervision of Prof. Dr. C. Tamerler.

April - June 2009: *Institut National de la Recherche Agronomique (INRA), Università della Provenza a del Mediterraneo – Ecole Superieure d'Ingénieurs de Luminy (ESIL), University of Science of Luminy, Marsiglia (France)* under the supervision of Dr. E. Record.

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Other Publications

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ORIGINAL PAPER

Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes

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Abstract The effect of *Phanerochaete chrysosporium* and *Pleurotus ostreatus* whole cells and their lignino-lytic enzymes on models of colored industrial wastewaters was evaluated. Models of acid, direct and reactive dye wastewaters from textile industry have been defined on the basis of discharged amounts, economic relevance and representativeness of chemical structures of the contained dyes. *Phanerochaete chrysosporium* provided an effective decolourization of direct dye wastewater model, reaching about 45% decolourization in only 1 day of treatment, and about 90% decolourization within 7 days, whilst *P. ostreatus* was able to decolorize and detoxify acid dye wastewater model providing 40% decolourization in only 1 day, and 60% in 7 days. *P. ostreatus* growth conditions that induce laccase production (up to 130,000 U/l) were identified, and extra-cellular enzyme mixtures, with known laccase isoenzyme composition, were produced and used in wastewater models decolourization. The mixtures decolorized and detoxified the acid dye wastewater model, suggesting laccases as the main agents of wastewater decolourization by *P. ostreatus*. A laccase mixture was immobilized by entrapment in Cu-alginate beads, and the immobilized enzymes were shown to be effective in batch decolourization, even after 15 stepwise additions of dye for a total exposure of about 1 month.

Keywords Textile dyes decolourization · Industrial effluent treatment · Ligninolytic fungi · Laccase · Manganese-peroxidase · Color industry

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Introduction

Dyes are widely used within the food, pharmaceutical, cosmetic, textile and leather industries. During industrial processing, up to 40% of the used dyestuff are released into the process water (Vaidya and Datye 1982), producing highly colored wastewaters that affect aesthetics, water transparency, and gas solubility in water bodies. Moreover and most importantly, there is a general concern regarding toxicity of some of these dyes. Because of both the high discharged volumes and the effluent composition, wastewaters from the textile industry can be considered as the most polluting among all industrial sectors, thus greatly requiring appropriate treatment technologies (O'Neill et al. 1999). All the dyes used in the textile industry are designed to resist fading even upon exposure to many chemicals including oxidizing agents. Although some a-biotic methods

for the reduction of several dyes exist, these require highly expensive catalysts and reagents (Robinson et al. 2001b). Biotechnological approaches were proven to be potentially effective in treatment of this pollution source in an eco-efficient manner (Willmott et al. 1998; McMullan et al. 2001; Robinson et al. 2001b; Borchert and Libra 2001; Beydilli et al. 1998; Zissi and Lyberatos 2001). The white rot fungi (WRF) are, so far, the microorganisms most efficient in degrading synthetic dyes, with basidiomycetous fungi that are able to depolymerize and mineralize lignin. This WRF's property is due to the production of extracellular lignin-modifying enzymes (LMEs), which, because of their low substrate specificity, are also able of degrading a wide range of xenobiotic compounds (Barr and Aust 1994; Pointing 2001; Scheibner et al. 1997) including dyes (Glenn and Gold 1983; Pasti-Grigsby et al. 1992; Paszczynski et al. 1992; Spadaro et al. 1992). The main LMEs are manganese peroxidases (MnP), E.C. 1.11.1.13, (Glenn et al. 1986), lignin peroxidases (LiP), E.C. 1.11.1.14 and laccases (Lac), E.C. 1.10.3.2, (Edens et al. 1999). LiP, MnP, and laccase play significant roles in dye metabolism by WRF (McMullan et al. 2001), due to the structural similarity of the most commercially relevant dyes to lignin (sub)structures amenable to be transformed by LMEs. However, the profiles of LME production during dye decolourization can be different in different fungi. For instance, Lac is the main enzyme involved in dye decolourization by cultures of *Phlebia tremellosa* (Kirby et al. 2000; Robinson et al. 2001a), *Pleurotus sajor-caju* (Chagas and Durrant 2001), and *Pleurotus ostreatus* (Palmieri et al. 2005a). Whereas, MnP, with or without LiP cooperation, was reported as the main enzyme involved in dye decolourization by *Phanerochaete chrysosporium* (Chagas and Durrant 2001; Kirby et al. 1995), and LiP was considered as the principal decolorizing enzyme of *Bjerkandera adusta* (Robinson et al. 2001b).

There is a gap in current knowledge of decolourization and, even more, of mineralization mechanisms, that limits our capacity to evaluate the true technical potential of WRF and their LMEs. Moreover, these difficulties are even greater if one considers that industrial effluents show a complex composition that is extremely variable even within the same factory, as the case of textile industry well demonstrates. Thus, decolourization of real effluents requires an appropriate choice of fungal strains as well as of operative conditions. Real textile dye effluents contain not only dyes but also salts, sometimes at very high ionic strength and extreme pH values, chelating agents, precursors, by-products, surfactants, etc. . Thus, in spite of the high efficiency in dye decolourization by some strains, decolorizing a real industrial effluent is quite troublesome.

In this report, models of acid, direct and reactive dye wastewaters from textile industry -defined on the basis of discharged amounts, economic relevance and representativeness of chemical structures of the contained dyes- were used to evaluate bioremediation capabilities of the WRF *Pleurotus ostreatus* and *Phanerochaete chrysosporium* and their LMEs.

Materials and methods

Microorganisms

Strains of the WRF *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) and *P. chrysosporium* Burdsall M1 (DSM 13583), were maintained through periodic transfer at 4°C on agar (1.5% w/v) plates containing undiluted and 10-fold diluted PDY medium [24 g/l potato dextrose (Difco, Detroit, Michigan, USA) and 5 g/l yeast extract (Difco)], respectively. The fungus *P. chrysosporium* shows a higher growth rate than *P. ostreatus* in PDY medium, and a comparable growth of both the fungal strains was obtained using a 10-fold diluted PDY medium for *P. chrysosporium*.

Dye containing wastewater models

Composition of the model wastewaters containing dyes that were used in this work is reported in Table 1. The wastewaters were sterilized by tindallyzation, consisting of three 1 h cycles of incubation at 60°C with 24 h interval between two cycles.

Treatment of dye containing wastewater models by *P. chrysosporium* and *P. ostreatus*

The inocula for liquid cultures were prepared by preinoculating 300 ml of ME broth [20 g/l malt extract (Difco)] in 1 l Erlenmeyer flask with 6 agar plugs of *P. ostreatus* (11 mm diameter) or *P. chrysosporium* (14 mm diameter) mycelia, from the edge of a

Table 1 Composition of colored wastewater models

Wastewater model	Dye				[Dye] (g/l)	Salt	[Salt] (g/l)	pH	Base or Acid	Concentration of base or acid (g/l)
	Name	Abbreviation	Chemical structure class	λmax						
Direct	Direct Blu 71	DrBu 71	Trisazo	582	1.00	NaCl	5	9	Na_2CO_3	2
	Direct Red 80	DrR 80	Polyazo	542						
	Direct Yellow 106	DrY 106	Stilbene	419						
Reactive	Reactive Blue 222	RBu 222	Disazo	280, 609	1.25	Na_2SO_4	70	10	NaOH	2
	Reactive Red 195	RR 195	Monoazo	291, 543						
	Reactive Yellow 145	RY 145	Monoazo	292, 419				12	Na_2CO_3	20
	Reactive Black 5	RBk 5	Disazo	593						
Acid	Acid Blue 62	Abu 62	Anthraquinonic	595, 637	0.10	Na_2SO_4	2	5	Acetic Acid	2
	Acid Yellow 49	AY 49	Monoazo	402						
	Acid Red 266	AR 266	Monoazo	307, 500						

7-days-old agar culture, in a temperature-controlled incubator at 28°C on rotary shaker (at 125 rpm). Fifty milliliters of a 5-day-old pre-culture were transferred in 1 l flasks containing 450 ml of model wastewater in the presence or in the absence of 2% malt extract. Cultures were incubated in the dark at 28°C on a rotary shaker at 125 rpm.

Performances of fungal cells in model wastewater decolourization were evaluated by recording light absorption spectra between 280 and 800 nm at different times (every 24 h), compared with the corresponding spectra of un-inoculated controls. Decolourization was measured as the extent of decrease of spectrum area recorded between 380 and 740 nm with respect to a control sample. All spectra were recorded after 1:100 dilution of the sample in water.

Samples of fungal cultures of wastewater treatment were daily withdrawn and used to perform enzyme activity assays.

Enzyme assays

Laccase activity was assayed using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate (Giardina et al. 1996). The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$).

Dye-decolourizing peroxidase (DYP) activity was assayed using Remazol Brilliant Blue R (RBBR) as substrate (Shin et al. 1997). The assay mixture contained 50 μM RBBR and 0.1 mM H_2O_2 in 20 mM sodium acetate buffer, pH 4.0. RBBR degradation was followed by absorbance decrease at 592 nm ($\varepsilon = 9{,}000\ M^{-1}\ cm^{-1}$).

Aryl alcohol oxidase (AAO) activity was determined using veratryl alcohol as substrate (Sannia et al. 1991). The reaction mixture contained 4 mM veratryl alcohol in 50 mM sodium phosphate buffer, pH 6.0. Oxidation of veratryl alcohol to veratrylaldehyde was followed by absorbance increase at 310 nm ($\varepsilon = 9{,}300\ M^{-1}\ cm^{-1}$).

Manganese peroxidase (MnP) activity was determined using manganese sulfate as substrate (Giardina et al. 2000). The reaction mixture contained 0.5 mM manganese sulfate and 0.1 mM H_2O_2 in 50 mM sodium malonate buffer, pH 4.5. Oxidation of Mn^{2+} to Mn^{3+} was followed by absorbance increase at 270 nm ($\varepsilon_{270} = 11{,}590\ M^{-1}\ cm^{-1}$) due to the formation of malonate—Mn^{3+} complex.

Lignin peroxidase (LiP) activity was determined using veratryl alcohol as substrate (Tien and Kirk 1984). The reaction mixture contained 2 mM veratryl alcohol and 0.5 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5. Oxidation of veratryl alcohol was followed by measuring the absorbance increase at 310 nm ($\varepsilon_{310} = 9{,}300\ M^{-1}\ cm^{-1}$).

All the enzyme activities were measured at 25°C and expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes one μmole of substrate in 1 min.

Production and characterization of laccase mixtures from *P. ostreatus*

A total of 50 ml of a pre-culture of *P. ostreatus* were inoculated in 1 l Erlenmeyer flasks containing 450 ml of PDY [24 g/l potato dextrose (Difco), 5 g/l yeast extract (Difco)], containing 0.15 mM $CuSO_4$ (Sigma) and supplemented with 2 mM ferulic acid (Sigma) after 2 days culture. After 8 days of fungal growth, the broth was filtered, and proteins were precipitated by the addition of 80% $(NH_4)_2SO_4$ at 4°C and centrifuged at 10,000*g* for 30 min. Protein precipitate was resuspended in 50 mM sodium phosphate buffer pH 6.5 and extensively dialyzed against the same buffer. The sample was centrifuged, and the supernatant was concentrated on an Amicon PM-10 membrane (Millipore). Enzyme samples were treated with 1 mM PMSF (Sigma) and stored at −80°C to be used in the treatment of dye containing wastewater models or for analyses of laccase isoenzyme composition.

Laccase isoenzyme fractionation

Ultra-filtrated protein samples were loaded on a Resource Q (GE Healthcare) column equilibrated with 50 mM Tris–HCl buffer pH 7. The column was washed at a flow rate of 1 ml/min with 3 ml of buffer, and a 0–0.5 M NaCl linear gradient (20 ml) was applied. Fractions containing laccase activity were pooled and concentrated.

Non-denaturing polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed at alkaline pH under nondenaturing conditions. The separating and stacking gels were, respectively, at 9 and 4% acrylamide, buffer solutions were 50 mM Tris–HCl (pH 9.5) for separating gel and 18 mM Tris–HCl (pH 7.5) for stacking gel, and the electrode reservoir solution was 25 mM Tris, 190 mM glycine, pH 8.4. Gels were stained for laccase activity using ABTS as substrate.

Treatment of dye containing wastewater models by laccase mixtures from *P. ostreatus*

Batch decolourization experiments using laccase mixtures as free enzymes were performed incubating crude preparation of laccase mixture containing different enzyme amounts (0.1, 1, 10 or 100 U/ml) with wastewater models, at room temperatures. Decolourization was evaluated recording UV-VIS absorption spectra at different times (10 min, 30 min, 1 h, 4 h, 24 h) of enzyme incubation and calculated as the extent of decrease of spectrum area recorded between 380 and 740 nm in comparison with the corresponding area of the spectrum of the untreated wastewater model.

Immobilization of laccase mixtures from *P. ostreatus*

The immobilization was carried out according to the procedure reported by Palmieri et al. (2005b). 2000 U of crude laccase preparation of laccase mixture were mixed with 35 ml of 3% sodium alginate solution (low viscosity, Sigma, St. Louis, MO), centrifuged at 4,000 rev min^{-1} for 5 min to remove air bubbles, and extruded drop by drop through a needle (0.4 mm internal diameter) into a 0.15 M $CuSO_4$ aqueous solution (pH 4.0) under continuous stirring. The resulting spherical blue beads were left to solidify for at least 30 min in the copper solution and then washed exhaustively with distilled water until pH 5.0–5.5 was reached. The total wet weight of beads obtained from 1 ml of sodium alginate solution was about 0.7 g. The beads were stored wet at 4°C.

Batch-mode decolourization by immobilized *P. ostreatus* laccase mixtures

In a typical experiment, 1 g of beads (with a mean diameter of 2.7 mm) containing about 50 U laccase activity was added to 2 ml of acid dye wastewater model solution. The reaction was incubated at room temperature under vigorous stirring. Each experiment was performed in a repeated sequencing batch decolourization. Each cycle consisted of the addition of the acid dye wastewater model solution to the laccase alginate beads. A new cycle was initiated when no further change in the dye (Abu 62) concentration was observed by monitoring the absorbance decrease at 637 nm. Control samples, consisting of alginate beads without any entrapped enzyme, were run in parallel under identical conditions. Each experiment has been repeated at least three times, and standard deviations were determined.

Production of manganese peroxidase and lignin peroxidase containing mixtures from *P. chrysosporium*

Different *P. chrysosporium* growth conditions in liquid culture have been tested for MnP and LiP activity production. 50 ml of a pre-culture of *P. chrysosporium* (6 agar plugs with 14 mm diameter from the edge of 7-days-old agar culture in 1 l flask containing 300 ml of 2% malt extract) were inoculated in 1 l Erlenmeyer flasks containing 450 ml of 10-fold diluted [2.4 g/l potato dextrose (Difco), 0.5 g/l yeast extract (Difco)] PDY broth or 2% malt extract broth supplemented with 0.1 or 1 mM $MnSO_4$. Culture broth was concentrated by ultra-filtration by using a 30 kDa cartridge in the Quix Stand Benchtop system (GE Healthcare).

Analysis of detoxification abilities

Toxicity of wastewater models before and after fungal treatment or incubation with enzyme mixture was measured with Lumistox 300 system (HACH LANGE, S.r.l. Milan—Italy), using freeze-dried *Vibrio fischeri* bioluminescent bacteria as test micro-organism. The inhibition of the natural light emission of these microorganisms, caused by toxic substances, was measured.

All the samples to be tested were prepared according to the manufacture's instructions. Conductivity, pH and optical density (OD) were measured. The pH was set to 7.0 ± 0.2 with HCl or NaOH and their salt content was adjusted to 2% NaCl when the measured conductivity was lower than 35 mS/cm.

Both acid and direct dye model wastewaters (with and without malt extract addition) showed O.D. values >1,800 mE, that required preparation of wastewaters dilutions to have final O.D. values <1,800 mE. All the measures were performed in color correction mode in order to reduce the interferences caused by physical absorption or light scattering. Toxicity values (defined as percent of inhibition of bacterial bioluminescence) of these wastewaters at the new defined concentrations were measured.

Analysis of COD

Determination of COD (Chemical Oxygen Demand) with the dichromate method was performed using HACH (COD High range vials) apparatus (HACH LANGE, S.r.l. Milan—Italy) according to the manufacture's instructions. A calibration curve was obtained using HACH COD standard solution (800 mg O_2/l). Appropriate dilutions of each sample were assayed.

Results and discussion

Treatment of dye containing wastewater models by *P. chrysosporium* and *P. ostreatus* cultures

The effect of *P. chrysosporium* and *P. ostreatus* liquid cultures on colored acid, direct and reactive dye industrial wastewater models was evaluated both in the presence and in the absence of nutrients. The wastewater models were defined in the frame of European Union Sixth Framework Program funded project SOPHIED (contract NMP2-CT2004-505899) on the basis of discharged amounts by textile industry, economic relevance and representativeness of chemical structures of the contained dyes (Table 1). Values of pH and salt concentration were chosen as those reproducing the characteristics of the real industrial effluents.

P. chrysosporium caused an effective decolourization of direct dye wastewater models when supplemented with nutrients, reaching 46% decolourization in only 1 day of treatment, and 87% decolourization within 7 days. Spectra analysis revealed that fungal treatment is effective on all the dyes present in the wastewater model, giving a complete depletion of the peaks at 582, 541 and 419 nm corresponding to the dyes Direct Blue 71 (Trisazo), Direct Red 80 (Polyazo) and Direct Yellow 106 (Stilbene), respectively (Fig. 1a). However, a new absorbance peak centered at 357 nm was observed in the spectrum of the wastewater model after fungal treatment, thus suggesting the formation of a new product inducing a significant shift of wastewater colour from dark to yellow. The complete removal of the major visible light absorbance peaks and the significant spectral change observed after *P. chrysosporium* treatment of direct wastewater suggest a degradation of the dyes by the fungus (Knapp et al. 1995). Fungal treatment of the wastewater also determined a pH shift of the growth medium from 8.4 to 4.5 within 7 days. Spectra of samples of direct

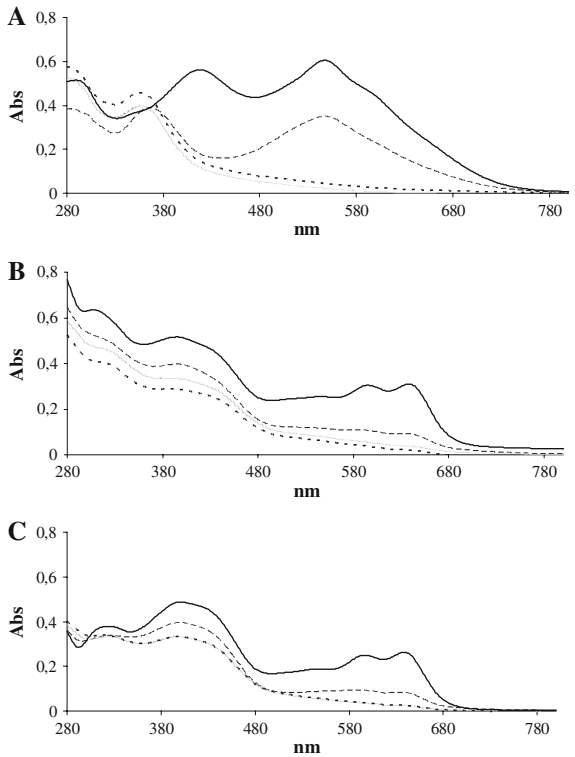

Fig. 1 Light absorption spectra between 280 and 800 nm of: direct dye wastewater treated by *P. chrysosporium* (**a**), acid dye wastewater treated by *P. ostreatus* in the presence (**b**) and in the absence (**c**) of malt extract, after 0 (——), 1 (– – –), 7 (·····) and 14 days (·····) of treatment

dye wastewater model at different pH values in the range 8.4–4.5 were recorded allowing us to rule out that the variations observed in the spectrum after fungal treatment were due to a pH change. We could not directly correlate the decolourization of the wastewater by *P. chrysosporium* to the production of oxidative enzyme activities potentially involved in the process, since residual color interfered with assays of MnP and LiP activities, the enzymes reported as the mainly responsible for decolourization by *P. chrysosporium* (Champagne 2005).

P. ostreatus proved to be capable to decolorize acid dye wastewater model with and without addition of malt extract, providing 40% decolourization after only 1 day of treatment in the absence of nutrient, and reaching up to 60 and 66% of decolourization after 7 and 14 days, respectively, in the presence of nutrients. Spectra analysis revealed that fungal treatment is more effective on the dyes Acid Blue 62 (anthraquinonic dye) and Acid Red 266 (azo dye), with a dramatic reduction of the maximum absorbance peaks at 637 and 595 nm, due to Abu62, and of maximum absorbance peak at 500 nm due to AR266. On the other hand, Acid Yellow 149 (azo dye) seems to be more recalcitrant to biodegradation, its maximum absorbance peak at 402 nm being only slightly decreased (Fig. 1b). These results indicated a different efficiency of *P. ostreatus* in degrading the tested acid dyes.

Oxidative enzyme activities [laccase, Mn Peroxidase (Mnp), Lignin peroxidase (LiP) dye decolourizing peroxidase (DyP, Johjima et al. 2003), Veratryl Alchol Oxidase (VAO)] were assayed in decolorized samples treated by *P. ostreatus*. Only laccase activity was revealed since the first day of incubation, and a constant level of enzyme production (10^{-3} U/ml) during fungal treatment of the wastewater model was observed. Therefore, laccase activity seems to be the oxidative activity mainly involved in dye decolourization by *P. ostreatus*.

No decolourization of reactive wastewater model was obtained, neither by *P. chrysosporium* nor by *P. ostreatus*, both in the presence and in the absence of added nutrients. This can be ascribed to the loss of fungi vitality, due to the extreme pH (10–12) and salt concentration (70 g/l) conditions of the waste. As a matter of fact, it has been demonstrated that both the fungi loose their vitality after treatment of reactive dye wastewater, whilst vitality was retained after treatment of the acid and direct dye wastewater models. The observation that *P. chrysosporium* was able to decolourize the direct wastewater, characterized by a high pH value (9), suggested that the loss of the fungus vitality during the treatment of reactive dye wastewater can be ascribed to the higher salt concentration (70 g/l) of this wastewater in comparison with that of direct wastewater (5 g/l).

Hence the fungal strains *P. ostreatus* and *P. chrysosporium* showed different specificities in decolorizing the dye wastewater models. *Phanerochaete chrysosporium* proved to be able to decolourize direct dyes having complex -Trisazo, Polyazo and Stilbene-structures, whilst *P. ostreatus* did not decolorize these dyes but was showed to be able to decolourize anthraquinonic and aniline azo dyes. That is consistent with some previously reported studies (Knapp et al. 1995; Swamy and Ramsay 1999; Balan and Monteiro 2001; Martins et al. 2003; Eichlerova et al. 2005; Nozaki et al. 2008), showing different specificities of WRF in dye decolorization.

The different decolourization specificities of *P. ostreatus* and *P. chrysosporium* can be due to the different profiles of produced LMEs, mainly laccases in *P. ostreatus* and MnP in *P. chrysosporium*.

Treatment of dye containing wastewater by *P. ostreatus* laccases

In order to test the ability of *P. ostreatus* laccases to decolorize dye wastewater models and to verify the hypothesis that laccase activity is the oxidative activity mainly involved in decolourization of acid dye wastewater by *P. ostreatus*, an extracellular enzyme mixture endowed with high laccase activity levels was produced from *P. ostreatus* and incubations of the wastewater models with the laccase mixture were performed.

High levels of laccase activity production (up to 130×10^3 U/l) were obtained in a potato-dextrose (24 g/l) broth supplemented with yeast extract (5 g/l), 0.15 mM $CuSO_4$ and 2 mM ferulic acid, a low cost growth medium (4.4 euro/l). In this growth condition a quite constant production level of laccase activity was also detected throughout the 8–11 days period. Laccase isoenzyme composition was determined in culture supernatants, withdrawn after 8 days of fungal growth, and fractionated by ammonium sulphate precipitation and anionic exchange chromatography fractioning. Two laccase activity peaks were obtained. The corresponding fractions were pooled, concentrated, and analyzed by electrophoretic separation on native PAGE gels stained for laccase activity. The identity of the laccase isoenzymes in the laccase activity peaks was ascertained on the basis of the different electophoretic mobility of the laccase isoenzymes. The extracellular enzyme mixture was showed to contain POXA3 (Palmieri et al. 2003) and POXC (Giardina et al. 1996) isoenzymes, the latter one being the most abundant (99%).

When the wastewater models (Table 1) were prepared for incubations with the enzyme mixture, they were not tindallysed, since antiseptic conditions are not strictly required. Reactive dye wastewater model was used at pH 10.

The laccase mixture decolorized acid dye wastewater model up to 35% decolourization after incubation with only 0.1 U/ml of laccase in 24 h, and up to 30% after only 1 h incubation with 1 U/ml. An increase in enzyme concentration does not correspond to a proportional increase in the extent of decolourization (Fig. 2). The modifications of the absorption spectra following the enzymatic treatment of the wastewater model are similar to those produced by *P. ostreatus*, thus suggesting that laccases are the main agents of the wastewater model decolourization by the fungus. A quick depletion of the maximum absorbance peaks at 637 and 595 nm—due to Acid Blue 62—was observed just after an incubation of 10 min. This behaviour was confirmed by treating the single dyes with the laccase mixtures, that provided a fast decolourization of Acid Blue 62 (anthraquinone dye), a slower decolourization—taking 24 h—of Acid Red 266 (azo dye), whilst Acid Yellow 49 (azo dye) was resistant to laccase decolourization. The differences in decolourization efficiency can be ascribed to structural differences of the dyes and to substrate specificity of the laccase isoenzymes. As reported in literature, anthraquinonic dyes are good substrates for laccases, while degradability of azo dyes depends on their different aromatic substitution patterns (Chivukula and Renganathan 1995), and among them phenol and aniline azo dyes can be degraded by laccases (Kandelbauer et al. 2004). This could allow us to explain the more efficient degradation of the anthraquinone-type dye Acid Blue 62, compared with that of the aniline azo dye Acid Red 266. Lack of a hydroxyl or an amine group on the aromatic ring of the azo dye Acid Yellow 49 should explain the inability of *P. ostreatus* laccases to degrade this dye.

The laccase mixture proved to be poorly active in the decolourization of direct dye wastewater, even when up to 100 U/ml of enzyme were used. The extreme conditions of pH and salt concentration of this wastewater model could prevent efficient

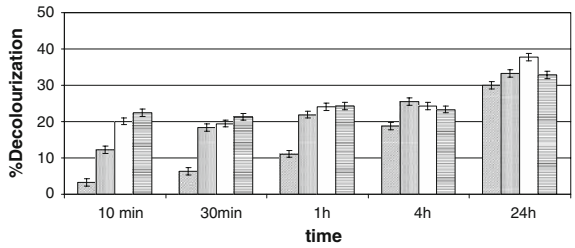

Fig. 2 Percent of decolourization of acid dye wastewater model after incubation with various amounts (0.1 U/ml, ▨; 1 U/ml, ▩; 2U/ml, □; 10 U/ml, ▤) of laccase mixture, at room temperature, and recording UV-VIS absorption spectra at different times (10 min, 30 min, 1 h, 4 h, 24 h)

functioning of laccases in decolourization. In fact a slight increase in decolourization is achieved by lowering the pH of direct dye wastewater from 9 to 5. Spectra analysis revealed that the enzymatic treatment is effective only on Direct Blue 71 (Trisazo), as indicated by the decrease of absorbance intensity at the wavelength of maximum absorption of this dye (582 nm), while the other two dyes Direct Red 80 (Polyazo) and Direct Yellow 106 (Stilbene) showed to be resistant to the enzymatic degradation.

No decolourization was observed for reactive dye wastewater after incubation with laccase mixture, even if the enzyme concentration was increased up to 100 U/ml. In order to verify that the absence of any decolourization effect is due to the extreme pH value (10) of the reactive wastewater, decolourization experiments were performed lowering the pH of the wastewater model to 6. No decolourization was observed for reactive dye wastewater although the pH lowering, thus ruling out the hypothesis that the extreme pH value of this wastewater model had inhibited enzyme decolourization activity.

Treatment of wastewater models by laccase mixtures from *P. ostreatus* entrapped in Cu-alginate beads

Effectiveness of *P. ostreatus* laccase mixture entrapped in Cu-alginate beads in decolourizing RBBR (Remazol Brilliant Blue R) had been previously established (Palmieri et al. 2005a, b). Although the reported data suggested the possibility to use this system in wastewater treatment, it was necessary to verify the stability of the support in the wastewater models. No alteration of beads consistency was observed after 24 h incubation in acid dye wastewater model, whereas a big stress was given by direct dye wastewater, and a complete dissolution of beads was produced by reactive dye wastewater model.

The enzymatic extracellular mixture was immobilized by entrapment in Cu-alginate beads and the immobilized enzymes used in batch decolourization experiments on acid dye wastewater model.

The dye decolourization process was efficient even after 15 stepwise dye additions taking about 1 month, providing up to 80% decrease of absorbance at 637 nm (Fig. 3a). Even if the time needed to achieve 50% decolourization exponentially increased (Fig. 3b), the $t_{1/2}$ of the 15th cycle was as low as 7 h.

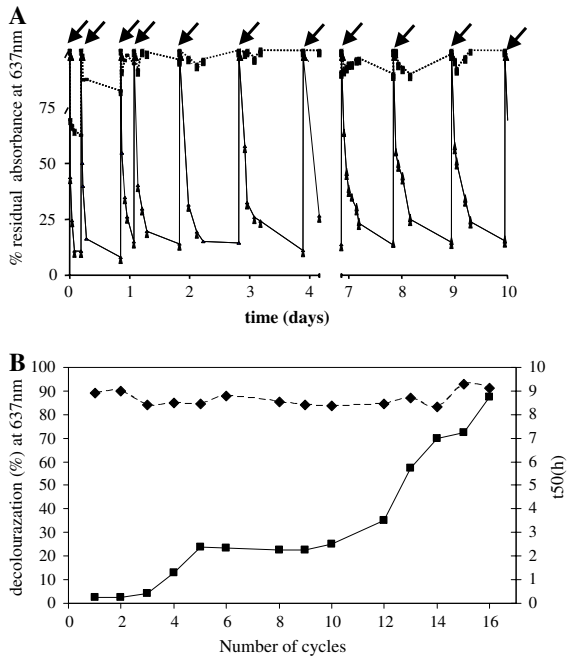

Fig. 3 Decolourization of acid dye wastewater model obtained by crude laccase mixture entrapped in copper alginate beads. (**a**) Decolourization percentage (residual absorbance at 637 nm) during 10 cycles (▲) compared to that obtained in the presence of alginate beads with no enzyme entrapped (■). Arrows indicate new dye additions. (**b**) Decolourization percentage (◆) during 16 cycles and time needed to obtain 50% decolourization (t_{50}) for each cycle (■)

Production of manganese peroxidase mixtures from *P. chrysosporium* for treatment of dye containing wastewater models

To test the performance of *P. chrysosporium* extracellular enzymes in the decolourization process, conditions to optimize enzyme (MnP and LiP) production and to prepare enriched enzyme mixtures have been investigated. Among the different *P. chrysosporium* growth conditions tested, the best selected growth medium for MnP production contained potato-dextrose broth (2.4 g/l) added with yeast extract (0.5 g/l) and 0.1 mM $MnSO_4$. Further increase of $MnSO_4$ concentration resulted in a decrease of MnP activity production. However, LiP activity was not detected in any tested condition.

The enriched enzyme mixture containing MnP activity was used in batch decolourization experiments performed incubating 0.1 or 0.01 U/ml enzyme with direct dye wastewater model and recording UV-VIS absorption spectra at different times (1, 24 and

48 h). Incubations were carried out in H_2O or 50 mM Sodium Malonate pH 4.5 with or without addition of 0.5 mM $MnSO_4$ and of 0.1 mM H_2O_2, by using the wastewater at pH 9 or lowering pH to 4.5. The best performance of the enzyme mixture was observed in decolorizing the 10-fold diluted wastewater model, in 50 mM Sodium Malonate pH 4.5 in the presence of $MnSO_4$. The mixture was able to decolorize undiluted wastewater giving about 40% decrease of absorbance at 600 nm after only 1 h, whilst to observe a complete depletion of the 600 nm absorbance peak, a 10-fold dilution of the wastewater was required. When decolourization was evaluated as decrease of spectrum area between 380 and 740 nm, only 15% decolourization was revealed after 48 h, whilst about 50% of decolourization after 24 h was observed on 10-fold diluted direct dye wastewater model. The addition of 0.1 mM H_2O_2 did not affect the decolourization efficiency. When tested on each of the dyes present in the direct dye wastewater model, the MnP containing samples were shown to be more effective in decolorizing Direct Blue 71, reaching 50% of decolourization, evaluated as the decrease of spectrum area between 380 and 740 nm of the undiluted dye, after 24 h.

Only some of the spectral modifications produced by *P. chrysosporium* treatment were observed after enzyme incubations, thus suggesting involvement of other fungal metabolites or enzymes during in vivo decolourization process.

Variability of MnP production by fungus and low stability of the enzymes hindered further scale up of the system. The low stability is reported as a common property of MnP enzymes (Sutherland and Aust 1996; Timofeevski and Aust 1997).

Analyses of detoxification and COD reduction abilities

Toxicity of the acid dye wastewater model after 24 h treatment with 0.1U/ml of the laccase mixture and at the end of treatment with *P. ostreatus* was evaluated by using Lumistox 300. EC50 could be only measured for acid dye wastewaters with malt extract added, and the values determined for untreated and fungal treated were 43.3 and 10.4% respectively. For the acid dye wastewater without malt extract, toxicity of a 50% dilution with a final O.D. values <1,800 mE was tested. Reduction of the toxicity of the wastewater was provided by fungal treatment both in the presence and in the absence of added nutrients, whilst a less extent of toxicity reduction was obtained by the enzyme incubation (Table 2), thus suggesting the involvement of other fungal components in detoxification during in vivo process.

Toxicity of direct dye wastewater model treated by *P. chrysosporium* could not be evaluated because of the low reproducibility of toxicity measurements obtained with these samples, due to too low sample concentration (required by the color correction mode).

Reduction of COD of acid and direct dye wastewaters after fungal treatment was not detectable in the presence of added malt extract because of the high contribute of the nutrient to overall COD value. On the other hand, 33% COD reduction of acid dye

Table 2 Detoxification abilities of *P. ostreatus* and its laccases evaluated by using Lumistox 300 (reduction of % Inhibition of *Vibrio fisheri* growth by dye after fungal or laccase treatment)

Wastewater	Bioremediation system	Toxicity
Acid with malt extract added	Control (tyndalized)	EC50:10.4%
	P. ostreatus	EC50: 43.3%
Acid	Control (tyndalized)	% Inhibition (at 50% diluition): 37.8% ± 1.1%
	P. ostreatus	% Inhibition (at 50%diluition): 6.1%
Acid	Control (Not tyndalized)	% Inhibition (at 50%diluition) = 28.6 ± 6.0%
	Laccase mixture	% Inhibition (at 50%diluition) = 11.5 ± 3.2%
Direct with malt extract added	Control (tyndalized)	Not detectable
	P. chrysosporium	Not detectable

wastewater without malt extract was produced by *P. ostreatus* treatment. 31% COD reduction of the wastewater was measured after enzyme treatment, whilst no COD reduction of model wastewater was provided by *P. chrysosporium*.

Conclusions

In conclusion, in this report decolourization capabilities of the fungi *P. chrysosporium* and *P. ostreatus* and of free and immobilized laccase mixtures from *P. ostreatus* on industrial dye wastewaters have been demonstrated. A different decolourization specificity was exhibited by the fungi, *P. chrysosporium* being active in decolorizing the direct wastewater model containing dyes with complex—Trisazo, Polyazo, Stilbene- structures, whilst *P. ostreatus* provided decolourization of the acid wastewater model. Laccases were identified as the main agents of wastewater decolourization by *P. ostreatus* and their preference in decolourizing anthraquinonic type dye and aniline mono-azo dye allowed us to explain ability of *P. ostreatus* to decolourize acid wastewater model. On the other hand, *P. ostreatus* is unable to decolourize the direct wastewater model, possibly because of the presence of complex poly-azo and stilbene structures, not degraded by laccases. A system based on immobilized laccase mixtures was also shown active in decolurization and can be further improved for large scale applications. On the other hand, manganese peroxidases are involved in direct wastewater model decolourization by *P. chrysosporium*, and the different profiles of LMEs produced by the fungi can explain their different decolorization specificities.

It's worth noting the ability of *P. chrysosporium* to decolourize the direct wastewater that is characterized by a high (9) pH value, whilst for majority of the fungi the optimum pH for dye decolourization lies in the acidic range (Asgher et al. 2008; Kapdan et al. 2000; Parshetti et al. 2007). However, such low pHs are not suitable for the wastewater treatment and hence, fungal strains able to decolourize dyes efficiently at wider pH ranges are desirable for industrial applications.

While many studies were devoted to biodecolourisation of the textile dyes, few manuscripts have been reported on decolourization of dye effluents in which the presence of salts and high dye concentration may be inhibitory to biological agents. Decolorization of reactive dye industry effluents was demonstrated by the fungi *Aspergillus fumigatus* (Jin et al. 2007) and *Phanerochaete sordida* (Harazono and Nakamura 2005), and by fungal oxidative enzymes (Zille et al. 2003; Mohorcic et al. 2006). Knapp and Newby (1999) reported decolourization of a diluted chemical industry effluent containing a diazo-linked chromophore by white-rot fungi. Taking also into account the high dye concentrations and the presence of salts in the tested wastewaters, *P. chrysosporium* and *P. ostreatus* exhibited good decolourization performances.

The potential of *P. ostreatus* to be used as an effective detoxifying microorganism for acid dye wastewaters have also been demonstrated. To the best of our knowledge, this is the first report on this detoxifying potential.

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