DEVELOPMENT OF OXIDATIVE BIOSYSTEMS FOR THE TREATMENT OF INDUSTRIAL EFFLUENTS

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Alla mia famiglia

SUMMARY

Laccases are multicopper oxidative enzymes, useful for biotransformation of environmental organic pollutants and exploited industrially in various oxidative processes. These copper-containing enzymes couple the oxidation of various substrates, polyphenols, aminophenols, methoxyphenols and aryl amines, with the simultaneous reduction of molecular oxygen to water. The white-rot fungus *Pleurotus ostreatus* expresses multiple laccase genes encoding isoenzymes with different physico-chemical and catalytic characteristics: the typical POXC (Palmieri et al. 1993), the white POXA1w (Palmieri et al. 1997), the most stable POXA1b (Giardina et al. 1999) and the two heterogeneously heterodimeric POXA3a and POXA3b (Palmieri et al. 2003). Moreover heterologous expression of *Pleurotus ostreatus* POXC and POXA1b laccases in two yeasts, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, was performed (Piscitelli et al. 2005). The development of new bio-based processes using laccases requires deeper understanding of the structure/function relationships of native enzymes and the "invention" of better performing molecules, more suited to industrial applications, as treatment of industrial effluents. In this context research has been aimed to characterize role and interaction of the subunits of heterodimeric laccases POXA3 from *P. ostreatus*, and to develop and characterize "new" better performing laccases subjecting *P. ostreatus* isoenzymes to directed evolution.

As for POXA3 isoenzymes, two genes encoding POXA3a and POXA3b small subunits have been identified: the corresponding amino acid sequences show only two amino acid substitutions. The heterogenity of small subunits POXA3 isoenzymes was associated with a different N-glycosylation at Asn150. *In vitro* experiments aimed at separating the POXA3 large and small subunits, using different denaturing agents and conditions, have demonstrated very strong interactions between them. Two unfolding intermediates can be singled out: a partially denatured form, with bound subunits, or an irreversibly unfolded form in which separation of the subunits had occurred. Heterologous expression of the large POXA3 subunit alone and co-expression of both large and small subunits in *Kluyveromyces lactis* have been performed. Reported data indicate a significant increase of laccase activity in the case of subunit co-expression, and a role of the small subunit in the stabilization of the POXA3 complex.

Library of new phenol-oxidases was constructed introducing mutations at different frequency in *poxa1b* and poxc cDNAs and shuffling *poxa1b* and *poxc* cDNAs. Mutated cDNAs were expressed in *S. cerevisiae*. A rapid, reliable and sensible screening method to isolate more active mutants was set up. More than 1100 clones were screened and one mutant exhibiting activity higher than that of wild type was isolated. This mutant was used as parent to produce a second generation of mutants. 1200 new mutants were screened and three "more active" clones were identified. All four mutants isolated were parctilally purified and fully characterized. Some mutants exhibit not only higher specific activity but also higher stability respect to that of wild type protein. 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.

RIASSUNTO

Le biotecnologie rappresentano uno sforzo continuo di ricerche e applicazioni per migliorare la qualità della vita. Il rapporto McKinsey citato sulla rivista "*The Economist*" del 2004 stimava che circa il 5% dei prodotti chimici derivavano dall'industria biotecnologica e prevedeva un valore più che raddoppiato a partire dal 2010. L'utilizzo di enzimi a livello industriale è oggi il risultato del rapido sviluppo avutosi negli ultimi decenni, anche se il loro impiego per la produzione di cibi e bevande, trova radici ben più lontane: Sumeri e Babilonesi producevano vino e birra sin dal 6000 a.C.

I vantaggi offerti dagli enzimi rispetto ai convenzionali catalizzatori chimici vanno dalla loro produzione, a partire da fonti rinnovabili, alla loro biodegradabilità e all' estrema selettività. Non poche sono le applicazioni in cui gli enzimi hanno brillantemente sostituito i reattivi classici: proteasi e cellulasi al posto dei fosfati nei detersivi, amilasi e pectinasi al posto dell'idrossido di sodio nell'industria tessile, sono solo alcuni esempi. Se da un lato l'impiego di enzimi può rendere meno tossici alcuni processi, dall'altro si può anche pensare ad un loro impiego nel campo del risanamento ambientale. I problemi connessi all'utilizzo dei metodi convenzionali, come l'incenerimento, per la rimozione di sostanze tossiche dall'ambiente provocano il crescente interesse verso lo sviluppo di trattamenti alternativi economici e ecocompatibili. In questo contesto i funghi white-rot rappresentano una possibile promessa. Essi sono infatti in grado di secernere un sistema enzimatico ossidativo capace di degradare una varietà di sostanze xenobiotiche. Negli ultimi anni la ricerca intorno alle ossidasi e perossidasi prodotte da tali organismi è stata molto intensa, legittimata soprattutto dal fatto che risultano molto promettenti per una serie di applicazioni biotecnologiche. 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. Il fungo basidiomicete white rot *Pleurotus ostreatus,* come la maggior parte delle specie fungine, produce più di un enzima ad attività fenolo ossidasica, cinque isoenzimi (POX-: Phenol OXidase-) sono stati 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, così come di altri, enzimi, il loro reale utilizzo a livello industriale richiede da un lato la loro sempre più completa caratterizzazione, dall'altro lo sviluppo di nuove fenolo ossidasi più adatte alla specifiche richieste delle applicazioni industriali.

Questo lavoro di ricerca ha avuto come scopi 1) la caratterizzazione di una famiglia di laccasi naturali, gli isoenzimi POXA3, secrete dal fungo basidiomicete *P. ostreatus,* e 2) lo sviluppo di nuove ossidasi, più attive, mediante l'evoluzione guidata di alcune delle laccasi da *P. ostreatus*.

Isoenzimi POXA3 *da P. ostreatus*

I due isoenzimi POXA3a e POXA3b, separabili mediante cromatografia a scambio anionico, mostrano una inusuale struttura eterodimerica, costituita da una subunità maggiore, di 67 kDa, omologa alle altre laccasi note, e da una subunità minore,

eterogenea perché presente in una forma di 16 o di 18 kDa. Un unico gene era stato isolato, codificante per le subunità maggiori di POXA3a e POXa3b.

In primo luogo si è effettuata un'analisi su frammenti di cDNA codificanti la subunità maggiore degli isoenzimi POXA3; tale analisi ha rivelato la presenza di due varianti, differenti per dodici nucleotidi, derivanti da un fenomeno di *spilicing* alternativo. Mappe peptidiche indicano la presenza di entrambe le sequenze amminoacidiche, derivanti dalle due varianti di *splicing*, sia in POXA3a che in POXA3b, fatto che amplia ulteriormente la già nota eterogeneità degli enzimi POXA3.

Si è successivamente proceduto alla caratterizzazione delle subunità minori. Mediante una selezione di una collezione di frammenti di DNA genomici, sono state isolate due sequenze geniche, codificanti la subunità minore, differenti per soli sei nucleotidi. I cDNA, relativi alle due sequenze, codificano sequenze peptidiche differenti per soli due residui; tali sequenze non mostrano alcuna omologia con proteine presenti in banca dati, non consentendo dunque di formulare ipotesi sul ruolo della subunità minore sulla base della sola struttura primaria. L'analisi delle sequenze amminoacidiche dedotte, unita alle mappe peptidiche ottenute mediante spettrometria di massa MALDI ha consentito 1) l'assegnazione univoca di una delle due sequenze alla subunità minore di POXA3a e dell'altra alla subunità minore di POXA3b; 2) la comprensione della ragione molecolare dell'eterogeneità caratterizzante le subunità minori degli isoenzimi POXA3, presenti nella forma di 18 o 16 kDa se soggette o meno una modifica post-traduzionale; 3) l'individuazione dei processi che determinano la formazione della forma matura delle subunità minori degli enzimi POXA3: oltre alla rimozione del peptide segnale, un ulteriore evento proteolitico si verifica all'estremità N-terminale, con successiva formazione di un piroglutammico per ciclizzazione di un residuo di glutammina, evento che blocca l'estremità amminoterminale.

Allo scopo di comprendere il ruolo della subunità minore nei complessi POXA3, sono state esplorate molte condizioni per dissociare reversibilmente il complesso, utilizzando due agenti denaturanti: il cloruro di guanidinio (Gnd-HCl) e il sodio dodecil solfato (SDS). Incubazioni con Gnd-HCl determinano una perdita di attività proporzionale alla concentrazione di denaturante utilizzata; il processo di denaturazione sembra essere irreversibile, non c'è infatti recupero di attività da parte dei campioni denaturati se sottoposti a diluizione o dialisi. Analisi condotte mediante cromatografia ad esclusione molecolare mostrano una chiara separazione delle subnità costituenti il complesso quando il campione viene incubato in Gnd-HCl 6M, mentre, a concentrazione più basse, il cromatogramma mostra sempre un unico picco, contenente entrambe le subunità.

Diverso è il comportamento osservato quando si è utilizzato l'SDS : la perdita di attività è completa ed immediata anche a basse concentrazioni di denaturante (0,1%). Il processo di denaturazione risulta però essere reversibile, l'attività può infatti essere recuperata del tutto per diluizione del campione. Analisi mediante cromatografia ad esclusione molecolare ha dimostrato la mancata separazione del complesso in questa condizione così come quando il processo di denaturazione viene favorito da tempi più lunghi di incubazione e da concentrazioni più alte di agente denaturante (fino a 4%). Solo quando il campione viene denaturato termicamente (100°C) si osservano due picchi separati sul cromatogramma, testimonianti l'avvenuta dissociazione del complesso. Nel tentativo di separare le due subunità in condizioni più blande è stato valutato l'effetto del pH, unitamente alla

presenza dell'SDS, sulla stabilità del complesso. Soltanto a pH 4 si ha separazione delle subunità, ma la denaturazione non è reversibile, come testimoniato dal mancato recupero di attività per diluizione o dialisi della subunità maggiore da sola o unita alla minore.

In definitiva, anche se non legate covalentemente, le due subnità costituenti il complesso POXA3 godono di un'interazione tutt'altro che debole. E' possibile inoltre individuare almeno due intermedi denaturati: uno, in cui le subunità sono ancora legate, capace di recuperare quasi del tutto l'attività originaria, e un altro, corrispondente al complesso dissociato, incapace di recuperare l'attività.

Non essendo riusciti a dissociare il complesso nativo in maniera reversibile, per ottenere la subunità maggiore, in forma monomerica e strutturata, e confrontarne le caratteristiche con quelle del complesso, si è adottata la strategia dell'espressione ricombinante. Si è proceduti infatti all'espressione della subunità grande da sola e alla coespressione di subunità maggiore e minore per via eterologa nel lievito *Kluyveromyces lactis*. La subunità grande mostra un peso molecolare poco più alto della proteina nativa da *P. ostreatus*, confermando la bassa tendenza di *K. lactis* ad iperglicosilare; la subunità minore viene secreta come due isoforme, differentemente glicosilate, similmente a quanto osservato per la proteina nativa da *P. ostreatus*. Attività fenolo ossidasica è stata osservata sia nel caso dell'espressione della sola subunità grande che nel caso della coespressione delle due subunità, con alcune differenze:1) l'attività nelle colture del lievito che esprime la subunità grande da sola è sempre minore rispetto a quella misurata nelle colture del lievito che esprime entrambe le subunità; 2) la frazione di subunità maggiore che non riesce ad essere secreta è maggiore quando la subunità maggiore viene espressa da sola rispetto a quando viene coespressa assieme alla subunità minore. Questa ultima differenza potrebbe essere imputabile ad una difficoltà nella secrezione a causa di una non corretta strutturazione, fenomeno che sembrerebbe meno significativo quando è presente anche la subunità minore ed è possibile la formazione del complesso.

Questi dati indicano un aumento dell'attività catalitica della subunità maggiore connesso alla presenza della subunità minore, suggerendo, per quest'ultima, un ruolo nell'attivazione del sistema enzimatico. Il fatto che la subunità maggiore, espressa in forma di monomero, non possa essere attivata mediante l'aggiunta della subunità minore, indica inoltre che è probabilmente necessario un *co-folding* delle due per la formazione del complesso al pieno della sua attività. Inoltre, la maggiore stabilità dell'attività fenolo ossidasica osservata in presenza della subunità minore, consente di ipotizzare un ulteriore ruolo di quest'ultima nella stabilizzazione della subunità maggiore.

Evoluzione guidata di laccasi da *P. ostreatus*

La disponibilità di un efficiente sistema di espressione eterologa pone le basi per lo sviluppo di nuove ossidasi mediante tecniche di evoluzione molecolari. Due sono i lieviti in cui erano state espresse con successo le laccasi da *P. ostreatus*: *Saccharomyces cerevisiae* e *Kluyveromyces lactis*, il secondo più efficiente del primo.

Entrambi i lieviti sono stati analizzati come potenziali ospiti per l'espressione di nuove fenolo ossidasi. Sfortunatamente *K. lactis*, pur se più efficiente nell'espressione, si è dimostrato meno efficiente, rispetto a *S. cerevisiae*, nella corretta ricombinazione omologa, necessaria invece per l'ottenimento di un numero elevato di cloni

ricombinanti, con un numero limitato di passaggi. Su queste basi si è preferito a *K. lactis* il convenzionale *S. cerevisiae*.

Una volta scelto l'ospite si è messo a punto un metodo di selezione che rispondesse alle seguenti caratteristiche: 1) rapidità, in modo da consentire l'analisi di un elevato numero di mutanti; 2) affidabilità, per minimizzare falsi positivi o negativi; 3) sensibilità, caratteristica essenziale perché la strategia scelta, di sviluppare nuove laccasi introducendo un numero piccolo di mutazioni per ogni gene, procede necessariamente attraverso miglioramenti di piccola entità e dunque difficili da apprezzare se non con un metodo sensibile.

Tra le tecniche disponibili per introdurre variazioni casuali in sequenze geniche, sono state scelte la *Error Prone PCR* (EP-PCR), la più frequentemente utilizzata tra le tecniche non ricombinative, e il *DNA shuffling*, il più tradizionale tra i metodi ricombinativi. Reazioni di polimerizzazione a catena (PCR) con frequenza di mutazione bassa, media e alta sono state condotte utilizzando come stampo il cDNA codificante la fenolo ossidasi POXA1b; analogamente PCR con bassa frequenza di mutazione sono state condotte utilizzando come stampo il cDNA codificante la fenolo ossidasi POXC. Un'ulteriore collezione di cDNA mutati è stata realizzata mediante il *DNA shuffling* dei cDNA codificanti le laccasi POXC e POXA1b. Le collezioni così ottenute sono state espresse in *S. cerevisiae*, dando luogo a una *library* di più di mille mutanti. L'intera collezione è stata sottoposta al metodo di selezione messo a punto. E' importante notare che per i mutanti derivanti dalla EP-PCR, aumentando la frequenza di mutazione, il numero di mutanti incapaci di produrre proteine attive cresce notevolmente. Questo dato è in accordo con la teoria generalmente accettata che utilizzando frequenze di mutazioni basse, aumenta la probabilità di individuare mutazioni vantaggiose, poiché, in generale, incrementando il numero di mutazioni per chilobasi aumenta il rischio di mutare residui essenziali per la funzionalità dell'enzima. Su queste basi, per accumulare mutazioni positive, il miglior mutante (1M9B) isolato dalla selezione di questa prima *library* è stato esso stesso sottoposto a nuovi cicli di EP-PCR, ottenendo anche questa volta più di mille mutanti. La selezione di questa seconda *library* ha consentito l'individuazione di altri tre cloni (1L2B, 1M10B e 3M7C) più attivi del clone 1M9B, e dunque più attivi della proteina wild type. Per quanto riguarda i cloni derivanti dal *DNA shuffling*, soltanto una bassa percentuale di essi è capace di produrre attività fenolo ossidasica, a testimonianza del fatto che, anche se i cambiamenti più significativi in un enzima possono essere ottenuti proprio con lo *shuffling,* la tecnica è accompagnata dal rischio di danneggiare determinanti strutturali essenziali, durante la "ricostruzione" del gene, producendo cDNA codificanti proteine inattive.

Le quattro proteine selezionate, e la proteina ricombinante wild type, dopo un opportuno processo di arricchimento, sono state caratterizzate da un punto di vista cinetico e catalitico, utilizzando tre substrati (ABTS, DMP, SGZ). Tutte le proteine hanno mostrato un attività specifica verso l'ABTS, il substrato utilizzato durante la selezione, maggiore rispetto a quella del wild type. Tre proteine si mostrano più attive anche verso il DMP, suggerendo un aumento di attività substrato-indipendente. Valori molto bassi si osservano invece per le attività specifiche verso la SGZ che resta dunque un "cattivo" substrato così come per le laccasi da *P. ostreatus.* I valori delle costanti di Michaelis delle proteine mutate per l'ABTS sono simili a quello della proteina wild-type; diversamente, verso il DMP, tutte le proteine analizzate mostrano un'affinità maggiore rispetto a quella della proteina wild type; verso la SGZ tutte le

proteine mostrano la stessa affinità, tranne il mutante 1L2B che mostra un valore di K_M più basso rispetto a quello della proteina wild type.

Un'ulteriore caratterizzazione delle proteine isolate è consistita nello studio dell'effetto della temperatura e del pH sull'attività fenolo ossidasica: tutte le proteine analizzate, inclusa la wild type, si mostrano discretamente attive nell'intervallo 40-70°C; il profilo di attività in funzione del pH è sostanzialmente lo stesso per tutte le proteine, l'unica differenza sta nel fatto che le proteine mutate sembrano risentire maggiormente dell'incremento di pH a valori superiori a 4.4.

E' stata inoltre valutata la stabilità delle nuove laccasi in funzione del pH e della temperatura: il miglior clone derivante dalla selezione della prima *library* (1M9B) mostra in tutte le condizioni analizzate una stabilità sensibilmente più bassa rispetto a quella della proteina wild type. Tra i cloni isolati dalla *library* derivante dal secondo ciclo di EP-PCR, il clone 3M7C è più stabile della proteina wild type a pH acido, mentre il clone 1M10B lo è a pH alcalino. Tutte le proteine mutate, tranne 1M9B, mostrano una termoresistenza paragonabile a quella della proteina wild type.

I mutanti isolati e caratterizzati, sono stati dunque sequenziati, l'analisi del *codon usage* di *S. cerevisiae*, ha permesso di escludere che le mutazioni osservate potessero essere causa di un aumento o una diminuzione del livello di espressione delle proteine mutate rispetto alla proteina wild type. Per localizzare i residui mutati nella struttura tridimensionale delle proteine, sono stati costruiti mutanti *in silico* sostituendo nel modello tridimensionale di POXA1b gli amminoacidi originali con quelli mutati. L'analisi di tali modelli ha consentito di formulare alcune ipotesi sulle relazioni struttura-funzione dei determinanti modificati a seguito dell'evoluzione guidata. Il mutante 1M9B, identificato mediante la selezione della prima *library*, presenta una singola mutazione, in posizione 112 di una Leucina con una Fenilalanina; questa sostituzione può essere considerata responsabile dell'aumento dell'attività specifica così come della diminuzione della stabilità. La posizione 112 è localizzata all'interno del canale che rende il sito T3 accessibile al solvente, ogni variazione in tale posizione potrebbe influenzare la stabilità della coordinazione a uno dei due ioni rame del sito T3, influenzando quindi anche la stabilità dell'attività laccasica. Nel caso del mutante 1M9B, la catena laterale della fenilalanina, più estesa rispetto a quella della leucina, potrebbe essere in grado di stabilire interazioni con residui in posizioni più distanti, per esempio la Leucina in posizione 457, localizzata sull'α-elica in cui è posizionata anche un residuo di istidina coordinante il rame del sito T1. Tale interazione potrebbe essere responsabile di un aumento di potenziale a seguito dell'allungamento del legame Cu1-N (His456), similmente a quanto ipotizzato per spiegare l'elevato potenziale redox della laccasi da *Trametes versicolor*.

I tre mutanti ottenuti dalla selezione della seconda *library,* esibiscono oltre che una maggiore attività specifica anche una maggiore stabilità. Il mutante 1L2B presenta tre mutazioni, oltre quella in posizione 112. Le tre mutazioni, in maniera indipendente o sinergica, sono in grado di compensare la destabilizzazione dell'attività fenolo ossidasica causata dalla sostituzione in posizione 112. Il mutante 1M10B presenta due mutazioni in posizione 37 e 51, posizioni occupate da residui ammidici in gran parte delle laccasi. Le due mutazioni sostituiscono due lisine con un residuo di glutammina e uno di asparagina, testimoniando come, nello sviluppo di enzimi migliori, la relativamente rapida evoluzione guidata possa dare gli stessi risultati della più lenta evoluzione naturale. L'ultimo mutante analizzato, 3M7C, presenta una

singola mutazione, oltre quella in posizione 112. La mutazione sostituisce un residuo di Prolina con un residuo di Tirosina in una zona piuttosto variabile, la regione C-teminale, capace di creare una sorta di tappo nella cavità che da al solvente l'accesso al sito T3. L'incremento di attività e di stabilità osservato per il mutante 3M7C sono in linea con i dati di letteratura che associano alla regione C-terminale un ruolo determinante nella funzionalità delle laccasi.

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 DNA *shuffling* e la successiva identificazione e caratterizzazione di quattro "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.

Il presente lavoro di ricerca ha, dunque, consentito una più approfondita conoscenza delle relazioni struttura-funzione delle laccasi, validi enzimi dal punto di vista biotecnologico. In particolare, lo studio è stato concentrato sulla caratterizzazione di un'atipica famiglia di fenolo ossidasi naturali, gli enzimi POXA3 da *P. ostreatus*, unico caso di laccasi eterodimeriche ed esempio emblematico dell'eterogeneità che può caratterizzare tale classe di enzimi per molteplicità di geni codificanti, *splicing* alternativo e modifiche post-traduzionali. E' stata inoltre sviluppata una collezione di 2300 nuove laccasi, per selezione della quale sono state identificati quattro enzimi più attivi del wild type, fatto che da un lato consente di ipotizzare lo sviluppo di fenolo ossidasi sempre più "*directedly evolved*" per una specifica applicazione industriale e dall'altro ha consentito di individuare alcuni residui, apparentemente marginali, coinvolti invece nella funzionalità e nella stabilità di tali enzimi.

1. Introduction

Biocatalysis in industrial processes

Although nowadays is often associated only with advances in medical therapeutics and production of pharmaceuticals, biotechnology is much more generally defined as 'the application of micro organisms, cells or components thereof (e.g., enzymes) for the production of useful goods and services'. As such it has a particularly important role to play in the development of sustainable industrial processes. For example, a McKinsey report cited by *The Economist* (2004) estimated that 5% of global chemical sales are derived in part currently from industrial biotechnology and it is projected that this will more than doubled from 2010. The report suggests that some of these new biobased processes will result from the emerging techniques of recombinant DNA technology, metabolic engineering, functional genomics and proteomics, bioinformatics, and so on, which are rapidly outpacing advances in the more traditional and catalytic-based chemical processes. Others are likely to be stimulated by the needs of improved pollution control and the potential for using renewable, agricultural-based raw materials.

The enzyme industry today is the result of a rapid development seen primarily over the past four decades, thanks to the evolution of modern biotechnology. Enzymes found in nature have been used since ancient times in the production of food products such as cheese, beer, wine (Fig.1) and vinegar, and in the manufacture of commodities such as leather, indigo and linen.

Figure 1 Viticulture scene in the tomb of Nakht on the West Bank at Luxor (ancient Thebes). Dynasty XVIII, (1.543-1.292 a.C.)

All of these processes relied on either enzymes produced by spontaneously growing micro organisms or enzymes present in added preparations such as calves' rumen or papaya fruit. The enzymes were, accordingly, not used in any pure or well-characterized form. The development of fermentation processes during the later part of the last century, aimed specifically at the production of enzymes by use of selected production strains, made it possible to manufacture enzymes as purified, well-characterized preparations even on a large scale. This development allowed the introduction of enzymes into true industrial products and processes, for example, within the detergent, textile and starch industries.

Enzymes offer substantial and increasingly important advantages over chemical catalysts in that they can be derived from renewable resources, are biodegradable, work under relatively mild conditions of temperature and pH, and tend to offer exquisite selectivity in both reactant and product stereochemistry (Zaks, 2001).

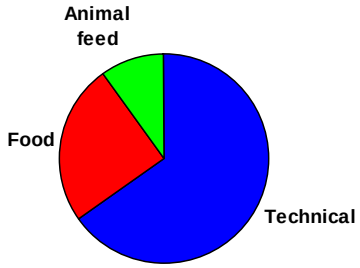


Figure 2 Segmentation of the industrial enzyme market.

The industrial enzyme market is traditionally divided into three segments (McCoy, 2000). The largest, at 65% of sales, is that of technical enzymes and includes enzymes used in the detergent, starch, textile, leather, pulp and paper, and personal care industries (Fig.2). Food enzymes, the second largest segment at 25% of the market, includes enzymes employed in the dairy, brewing, wine and juice, fats and oils, and baking industries. Finally, feed enzymes, comprising enzymes used in animal feeds, contributes approximately 10% of the market. Examples where enzymes make a significant difference can be found in numerous applications: in laundry detergents phosphates have been replaced with enzymes such as proteases (Gupta et al., 2002) and cellulases (Ito, 1997), in bread making chemical emulsifiers are replaced with lipases (Collar et al., 2000), and in textile factories sodium hydroxide is replaced with amylases (Pandey et al., 2000) and pectinases (Kashyap et al., 2001). To date, successful applications of biocatalysts have been largely confined to hydrolytic enzymes such as lipases, esterases, acylases and hydantoinases, but this situation is changing with the recent development of enzymes for a wider range of transformations, including asymmetric reduction, oxidation and carbon–carbon bond formation (Schoemaker et al. 2003). The list of enzyme applications grows, bringing with it a list of significant social and environmental benefits.

Global attention has focused in recent years on finding new ways to sustain and manage the environment. Biotechnology is an essential tool in this endeavour because it can provide new approaches for understanding, managing, preserving, and restoring the environment. Over the past few decades huge amounts of industrial pollutants have been released into the environment. A large number of them, i.e. those structurally related to natural compounds, are readily degraded or removed by microorganisms found in soil and water. However, superimposed on the wide variety of pollutants present in the environment is an increasing number of novel industrial compounds rarely found in nature. These xenobiotic compounds are usually removed slowly and tend to accumulate in the environment. Due to the high degree of toxicity, their accumulation can cause severe environmental problems. Because of the problems associated with pollutant treatment by conventional methods, such as incineration or landfills, increasing consideration has been placed on the development of alternative, economical and reliable biological treatments.

White-rot fungi produce various isoforms of extracellular oxidases including laccases, Mn peroxidases and lignin peroxidases, that are able to degrade and mineralize recalcitrant plant polymer lignin. This oxidative enzymatic system of white-rot fungi (WRF) is directly involved in the degradation of various xenobiotic compounds and dyes (Wesenberg et al., 2003). The detoxification potential of WRF can be harnessed thanks to emerging knowledge of the physiology of these organisms as well as of the biocatalysis and stability characteristics of their enzymes. Although natural microorganisms collectively exhibit remarkable evolutionary capabilities to adapt to a wide range of chemicals, natural evolution occurs at a relatively slow rate, particularly when the acquisition of multiple catalytic activities is necessary. In these cases, the

acceleration of these events via genetic engineering is helpful since the desirable traits can be carefully designed and controlled. The drive toward this goal represents the essence of environmental biotechnology.

Although highly attractive for both the treatment of industrial effluents and chemical synthesis, use of enzymes on an industrial scale often entails problems. Mother Nature has supplied a vast array of enzymes with diverse functions and properties. However, many man-made applications require activities never needed by living organisms in nature. In these cases, it is necessary to reengineer an appropriate natural enzyme to exhibit the necessary function.

Evolutionary design approaches have enjoyed considerable attention in modifying native proteins. The generation of stable enzymes with improved or novel catalytic activities is a fascinating topic of modern protein biochemistry. This goal is relevant as for basic research as for applications in biotechnology. In addition, the search for new biocatalysts can have a major impact on the applications of enzymes in industrial processes. Enzyme engineering can, for instance, force enzymatic reactions to proceed in a desired direction, enhance their selectivity, and their stability.

Two principally different strategies can be applied to change enzymatic properties at wish. One is rational design, which is the planned redesign of the protein sequence by site-directed mutagenesis.

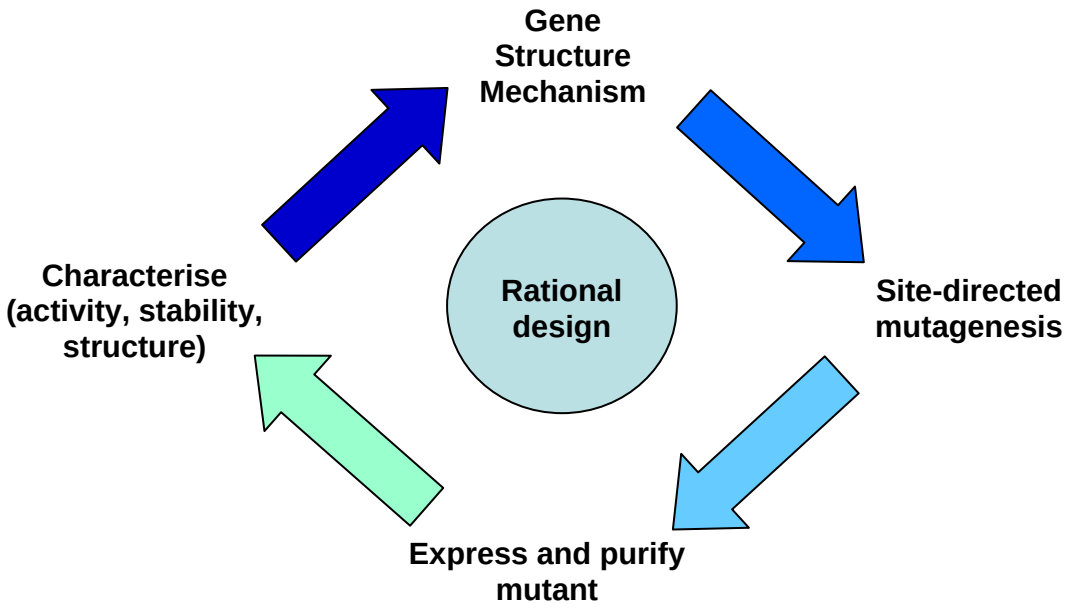


Figure 3 Schematic representation of the process of rational design

The rational redesign cycle (Fig.3) requires the appropriate gene and suitable knowledge of the structure and mechanism of action of the enzyme. Selected residues are targeted for site-directed mutagenesis and after expression and purification the properties of the new enzyme/s are assessed. Further residues may be targeted in further rounds of site-directed mutagenesis. This strategy, which has

been applied extensively with variable success, depends on detailed structural and mechanistic information on the parent enzyme. Site-directed mutagenesis has been successful, for example, in redesigning the substrate specificity of a large number of common classes of enzymes, such as oxidoreductases (dehydrogenases and reductases), hydrolases (acetylcholinesterases and proteases), transferases and restriction enzymes (Penning et al., 2001). A disadvantage of using crystal structures to identify residues thought to be responsible for sustrate specifity is that this approach may ignore residues relatively distant from the active site.

The second strategy is directed evolution, which requires the gene (or genes) of interest, but there is no requirement for a detailed knowledge of structure or function. Diversity may be introduced using a methods of random mutagenesis, and after expression variants with the desired property are selected or screened out of the mixture.

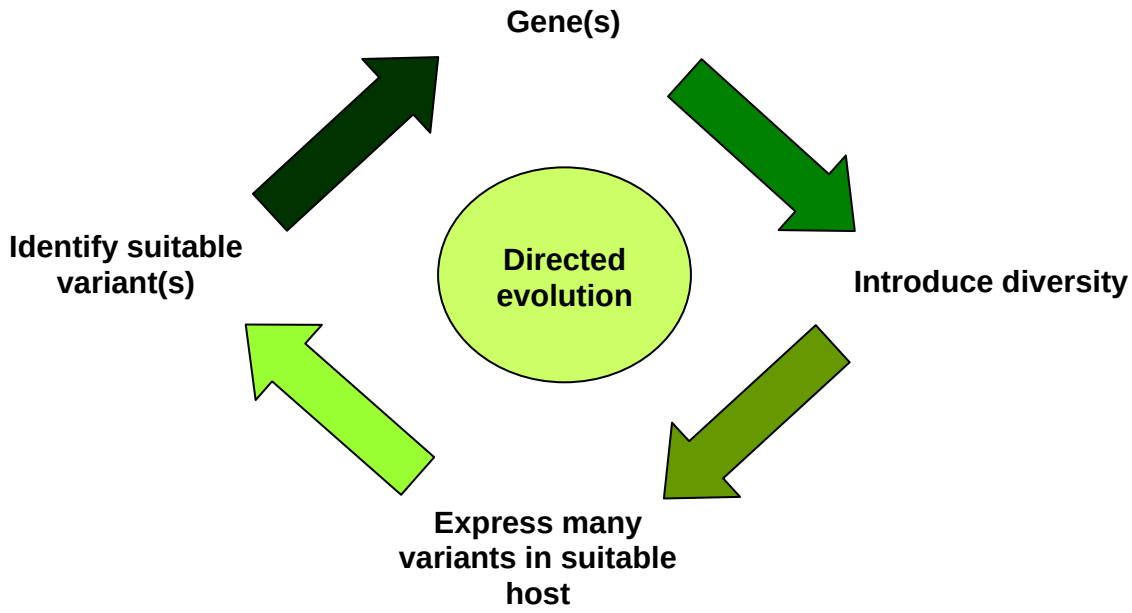


Figure 4 Schematic representation of the process of directed evolution

During the last few years, directed evolution has emerged as the method of choice for engineering functions and properties of enzymes. Rational redesign is beset by problems, most notably the amount of data that has first to be accumulated on each individual enzyme under study. By contrast with rational enzyme redesign, directed evolution does not rely on the detailed understanding of the relationship between enzyme structure and function. Instead, this technique relies on Darwinian principles of mutation and selection: enough diversity is created in the starting gene and then screening or selection methods are used to identify variants with the desired improvements.

Directed evolution: achieving enzymatic molecular diversity

Directed evolution is achieved by iterative cycles of random mutagenesis, recombination and functional screening of a library of variants from which mutants

with improved or new properties can be identified. A range of strategies for the introduction of diversity into the starting gene(s) are available, and these can be broadly divided into two classes: (i) non-recombinative methods and (ii) recombinative methods, and can vary from creating libraries with a few as 200 variants to many tens of thousands of variants. These two strategies are also named asexual and sexual evolution.

Non-recombinative methods

In an asexual evolution random mutagenesis is targeted preferentially towards the gene encoding one parent protein. An enzyme library is generated, then screened for improved properties. The best enzyme identified in the first generation can then be further optimized in subsequent cycles.

Non-recombinative methods generally create diversity via point mutation and include the directed substitution of single amino acids, the insertion or deletion of more than one amino acid, for example by cassette mutagenesis, and random mutagenesis across the whole gene. Combinatorial cassette mutagenesis is performed using synthetic oligonucleotide primers to mutagenize a relatively short DNA sequence (Derbyshire et al., 1986). This method is useful only when the targeted amino acids are in the same stretch of primary sequence.

Thus, a variety of methods are available depending on the extent of mutation required. In cases where a high-resolution structure of the target protein with bound substrate or inhibitor is available, residues which contact the substrate can be identified and can be hypothesized to be responsible in varying degrees for the natural reaction specificity. Mutation of these contacting residues to all other 19 amino acids by saturation mutagenesis (sm) can often lead to the identification of variants with significantly altered substrate specificity. For example, Wang and co-workers (2001) used saturation mutagenesis at five positions in the active site of the *Methanococcus jannaschii* tyrosyl transfer RNA (tRNA) synthetase to alter the amino acid specificity so that it accepts only an unnatural amino acid. Using several rounds of positive and negative growth selection, a mutant synthetase was obtained which had a k_{cat}/K_M for the target unnatural amino acid *O*-methyl-L-tyrosine, 100-fold higher than for the natural substrate tyrosine.

However, the simplest , and still a popular, method of choice for introducing diversity in gene(s) is Error-Prone PCR (EP-PCR). It was developed by Leung in 1989 who exploited the ability of thermostable DNA polymerases lacking proof-reading activity, for example *Taq* polymerase to introduce random mistakes during the extension of the new DNA strand. EP-PCR is able to produce large libraries of random mutants in a sequential fashion where deleterious mutations are accumulated in conjunction with beneficial ones. This can seriously limit the evolutionary process because of the high probability that the effect of the few beneficial mutations are lost due to the many deleterious ones. The mutation rate can be adjusted so that, usually, an average of 1–2 amino acid mutations is introduced per gene product (Moore et al. 1997; 1998).

Recombinative methods

Despite the important and growing use of non-recombinant methods for variant library production, the most significant changes in enzyme function have been created using recombinative methods, and DNA shuffling is still the most popular method of sexual evolution. Briefly, the original DNA shuffling technique (Stemmer,

1994) provides that homologous sequences from different templates hybridize and prime each other, and the resulting crossovers are locked in by polymerase extension. Multiple cycles of this fragment reassembly result in a library of chimeric DNA sequences. Parent genes can originate from an asexual evolution, from related natural sequences, or from enzyme variants generated by rational design.

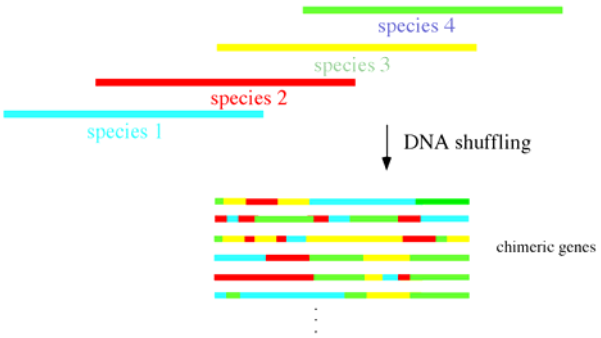


Figure 5 Schematic representation of the process of DNA shuffling

In an example of the use of DNA shuffling two highly homologous triazine hydrolases which differ at only nine amino acid positions were shuffled and variants with up to 150-fold higher activities were identified (Raillard et al., 2001)

The features of the DNA shuffling method have been subject of studies highlighting that the process is strongly affected by biases, resulting in limited possibilities for recombining point mutations in close regions, and generating libraries with an over-representation of parental sequences.

These observations led to modifications and improvements of the original basic protocols, in order to further explore the sequence mutation space and to limit the required number of screening tests to be performed to isolate the desired variants (Valetti and Gilardi, 2004).

Laccases: blue enzymes for white biotechnology

Laccase is one of the very few enzymes that have been studied since the end of 19th century. It was first identified in the exudates of *Rhus vernicifera*, the Japanese lacquer tree (Yoshida, 1883). A few years later its presence was also demonstrated in fungi (Bertrand, 1896); more recently, proteins with features typical of laccases have been identified in insects (Kramer et al., 2001) and prokaryotes (Claus, 2003). Although known from long time, laccases attracted considerable attention only after the beginning of studies on enzymatic degradation of wood by white-rot fungi and now fungal laccases are also being extensively exploited for many other industrial purposes.

Laccases belong to the group of blue multicopper oxidases that catalyze a one-electron oxidation concomitantly with the four-electron reduction of molecular oxygen to water (Solomon et al., 1996, 2001;Messerschmidt, 1997). The catalysis carried out by all members of this family is guaranteed by the presence of different copper centres in the enzyme molecule. In particular, all blue multicopper oxidases are

characterized by the presence of one type-1 (T1) copper, together with at least three additional copper ions: one type-2 (T2) and two type-3 (T3) copper ions, arranged in a trinuclear cluster. Two histidines and one cysteine serve as ligands for type-1 Cu at the T1 center. Usually, in type-1 centers a sulfur from a methionine functions as an additional axial ligand, in the case of TvL, there is a phenylalanine in this position. Eight histidines serve as ligands for binding of type-2 and type-3 Cu at the T2/T3 cluster (Messerschmidt 1997; Ducros et al., 1998; Hakulinen et al., 2002; Piontek et al. 2002). The histidines and the cysteine are spread over four highly conserved amino acid regions. These regions are considered fungal laccase signature sequences (L1-L4) and include not only residues involved in copper binding, but also residues responsible to maintain a local three-dimensional fold (Kumar et al., 2003). The different copper centres can be identified on the basis of their spectroscopic properties. The T1 copper is characterized by a strong absorption around 600 nm, whereas the T2 copper exhibits only weak absorption in the visible region. The T2 site is electron paramagnetic resonance (EPR)-active, whereas the two copper ions of the T3 site are EPR-silent due to an antiferromagnetic coupling mediated by a bridging ligand. Substrates (phenols and aromatic or aliphatic amines) are oxidized by 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 (Messerschmidt, 1997) (Fig. 10).

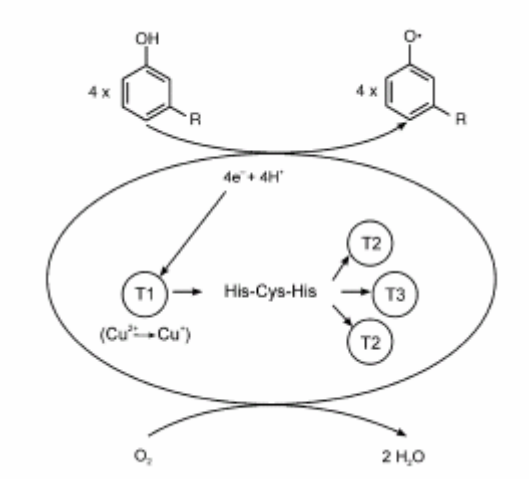


Figure 6 Catalytic cycle of laccase

The physiological function of these biocatalysts, which can be secreted or intracellular, is different in the various organisms but they all catalyse polymerization or depolymerization processes. It has been proposed that laccases are involved in cuticle sclerotization in insects and in the assembly of UV-resistant spores in *Bacillus* species. In plants they are involved in cell wall formation and, together with peroxidases, in lignification: there is no doubt that laccases are among the enzymes involved in delignification processes by white rot fungi (Mayer et al., 2002). Additionally, these enzymes can protect fungal pathogens from toxic phytoalexins and tannins, thus they are an important virulence factor in many fungal diseases (Mayer et al., 2002).

Structural properties

Current knowledge about the structure and physico-chemical properties of fungal laccase proteins is based on the study of purified proteins. Up to now, more than 100

laccases have been purified from fungi and have been characterized. Based on the published data some general conclusions about laccases had been drawn (Baldrian, 2006). Typical fungal laccase is a protein of approximately 60–70 kDa with acidic isoelectric point around pH 4.0 (Table 2).

Table 1 Properties of fungal laccases (*Baldrian, 2006*)

Property		n	Median	Min	Max
Molecular weight (Da)		**103**	**66000**	**43000**	**383000**
pI		**67**	**3.9**	**2.6**	**6.9**
Temperature optimum (°C)		**39**	**55**	**25**	**80**
pH optimum	**ABTS**	**49**	**3.0**	**2.0**	**5.0**
	2,6-Dimethoxyphenol	**36**	**4.0**	**3.0**	**8.0**
	Guaiacol	**24**	**4.5**	**3.0**	**7.0**
	Syringaldazine	**31**	**6.0**	**3.5**	**7.0**
K_M (µM)	**ABTS**	**36**	**39**	**4**	**770**
	2,6-Dimethoxyphenol	**30**	**405**	**26**	**14720**
	Guaiacol	**23**	**420**	**4**	**30000**
	Syringaldazine	**21**	**36**	**3**	**4703**
K_{cat} (s^{-1})	**ABTS**	**12**	**24050**	**198**	**350000**
	2,6-Dimethoxyphenol	**12**	**3680**	**100**	**360000**
	Guaiacol	**10**	**295**	**90**	**10800**
	Syringaldazine	**4**	**21500**	**16800**	**28000**

n, number of observations

It seems that there is considerable heterogeneity in the properties of laccases isolated from ascomycetes, especially with respect to molecular weight (Baldrian, 2006). Several species produce a wide variety of isoenzymes, the molecular basis for the production of different isoenzymes is the presence of multiple laccase genes in fungi (Chen et al., 2003). Fungal laccases are generally monomeric proteins. Only a few exception to the typical laccase homodimeric structure are reported. The wood-rotting species *Phellinus ribis* (Min et al., 2001), *Pleurotus pulmonarius* (Souza & Peralta, 2003) and *Trametes villosa* (Yaver et al., 1996), the mycorrhizal fungus *C. cibarius* (Ng & Wang, 2004) and the ascomycete *Rhizoctonia solani* (Wahleithner et al., 1996) produce laccases with homodimeric structure, being composed of two identical subunits with a molecular weight typical for monomeric laccases. The ascomycetes *G. graminis*, *M. indicum* and *P. anserina* also produce oligomeric laccases. In *M. indicum* a single band of 100 kDa after gel filtration resolved into three proteins (24, 56 and 72 kDa) on SDS-PAGE (Thakker et al., 1992): *G. graminis* produces a trimer of three 60-kDa subunits (Edens et al., 1999); *P. anserina* laccase is a heterooligomer (Molitoris & Esser, 1970); and one of the laccases purified from *A. mellea* has a heterodimeric structure (Curir et al., 1997). According to Wood (Wood, 1980), *A. bisporus* laccase consists of several polypeptides of 23–56 kDa. (Perry et al., 1993), on the basis of Western blot analyses, suggested that the native Lac2 of the same species is produced as a dimer of identical polypeptides, one of which is then partially proteolytically cleaved. SDS–PAGE and MALDI-MS analyses of purified POXA3a and POXA3b laccases from *P. ostreatus* reveal the presence of

three different polypeptides of 67, 18 and 16 kDa, whereas the native proteins behave homogeneously, as demonstrated by the presence of a single peak or band in gel filtration chromatography, isoelectric focusing and native-PAGE analysis (Palmieri et al., 2003).

Like many fungal extracellular enzymes, laccases are glycoproteins. The extent of glycosylation usually ranges between 10% and 25%, but laccases with a saccharide content higher than 30% were reported: e.g. *Coriolopsis fulvocinnerea*, 32% (Shleev et al., 2004), and *P. pulmonarius* , 44% (Souza & Peralta, 2003). Glycosylation of fungal laccases is one of the main problem for the heterologous production of the enzyme. It was proposed that in addition to the structural role, glycosylation can also participate in the protection of laccase from proteolytic degradation (Yoshitake et al., 1993).

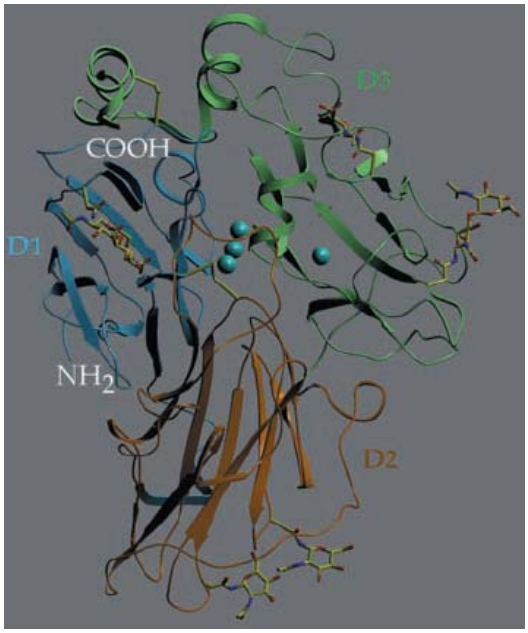


Figure 7 Ribbon diagram of *Trametes versicolor* laccase. The arrangement of the domain structure is depicted in different color coding (D1–D3). Copper ions are drawn as blue spheres.

The typical metal contents, featuring the four copper ions located in two copper centers, shows some exception: the enzyme from *P. ribis* with catalytic features typical for laccases lacks Cu1 and contains one Mn atom per molecule. The structural differences are probably also responsible for the relatively high pH optimum for ABTS oxidation (Min et al., 2001). The 'white' laccase POXA1w from *P. ostreatus* contains only one copper atom, together with two zinc and one iron atoms per molecule (Palmieri et al., 1997).

Until recently, the three-dimensional structure of five fungal laccases has been reported: *Coprinus cinereus* (in a copper type-2-depleted form) (Ducros et al., 1998), *T. versicolor* (Bertrand et al., 2002; Piontek et al., 2002), *P. cinnabarinus* (Antorini et al., 2002), *M. albomyces* (Hakulinen et al., 2002) and *R. lignosus* (Garavaglia et al., 2004), the latter four enzymes with a full complement of copper ions. Moreover, the three-dimensional structure of the CotA laccase from *Bacillus subtilis* endospore has also recently been published (Enguita et al., 2003, 2004). The *Trametes versicolor* laccase structure is a monomer, organized in three sequentially arranged domains (Fig.11), and has dimensions of about 65 X55 X 45 $Å^3$. Each of the three domains is structured in a similar β-barrel type architecture, related to that of small blue copper proteins such as azurin or plastocyanin. Domain 1 comprises two four-stranded β-sheets and four 3_{10}-helices. Three of the 3_{10}-helices are in connecting peptides between the β-strands, and one is in a segment between domain 1 and 2. The second domain has one six-stranded and one five-stranded β-sheet, and like in domain 1, there are three 3_{10}-helixes in peptides connecting individual β-strands and domains 1 and 3, respectively. A 3_{10}-helix between domains 2 and 3 forms part of a 40-residue-long extended loop region. Finally, domain 3 consists of a β-barrel formed by two five-stranded β-sheets and a two-stranded β-sheet that, together with an ά-helix and a β-turn, form the cavity in which the type-1 copper is located. The tri-nuclear copper cluster (T2/T3) is embedded between domains 1 and 3 with both

domains providing residues for the coordination of the coppers. The third domain has the highest helical content with one 3_{10}-helix and two ά-helices located in the connecting regions between the strands of the different β-sheets. Finally, at the C-terminal end of domain 3, three sequentially arranged ά-helices complete the fold. A 13-aminoacid-long ά-helix at the C-terminal portion 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.

Despite the amount of information on laccases as well as other blue multicopper oxidases, neither the precise electron transfer pathway nor the details of dioxygen reduction in blue multicopper oxidases are fully understood (Garavaglia et al., 2004). A detailed structural comparison between a low redox potential (E^0) *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 (Piontek et al., 2002). This was later confirmed by studies on *R. lignosus* laccase with a high redox potential (Garavaglia et al., 2004). However, more effort will be needed to elucidate the relation between the structure of the catalytic site and the substrate preference of different laccase enzymes.

Laccases application

Commercially, laccases have been used to delignify woody tissues, produce ethanol, and to distinguish between morphine and codeine. A very wide variety of bioremediation processes employ laccases in order to protect the environment from damage caused by industrial effluents. Research in recent years has been intense, much of it elicited by the wide diversity of laccases, their utility and their very interesting enzymology.

Textile industries

At present, the main technological application of laccases are in textile, dye and printing industries, in processes related to decolourization of dyes. For example, laccases from the lignin-degrading basidiomycetes *Trametes versicolor*, *Polyporus pinisitus* and the ascomycete *Myceliophthora thermophila* were found able to decolorize the azo dye Direct Red 28, the indigoid Acid Blue 74 and anthraquinonic dyes. Due to their higher oxidative capacities, the laccases from the two basidiomycetes decolorized dyes more efficiently than that of the ascomycete (Claus et al., 2002).

Pulp and paper industries

The removal of lignin from woody tissues is a process that has attracted a very great deal of research, especially due to its importance in the pulp and paper industry. One of the approaches to delignification of wood fibers for preparation of pulp has been the use of laccases for this purpose (Argyropoulos, 2001). It has also been shown that the ability of laccases to break down ligno-cellulose is increased by certain phenolic compounds acting as mediators (Bourbonnais et al., 1995; Eggert et al.,1996).

Ethanol production

To improve the production of fuel ethanol from renewable raw materials, laccase from the white rot fungus, *Trametes versicolor*, was expressed under control of the PGK1 promoter in *S. cerevisiae* to increase its resistance to phenolic inhibitors in

lignocellulose hydrolysates (Larsson et al., 2001). The results from a detailed study of the system showed that phenolic compounds are important as fermentation inhibitors, and that there is a definite advantage of using laccase expressing yeast strains for producing ethanol from lignocellulose.

Drug analysis

A new enzymatic method based on laccase has been developed to distinguish morphine from codeine simultaneously in drug samples injected into a flow detection system (Bauer et al., 1999). An enzyme sensor was constructed based on laccase and glucose dehydrogenase immobilized at a Clark oxygen electrode. Morphine is oxidized by laccase with consumption of oxygen and regenerated by glucose dehydrogenase. Laccase cannot oxidize codeine, so the sensor is selective for morphine. Morphine is detected between 32 nM and 100 mM. The rapid and technically simple method allows discrimination between morphine and codeine in less than 1 min after injection at a sampling rate for quantitative measurements of 20 per hour.

Wine clarification

Laccase immobilized on a copper-chelate carrier that can be regenerated was used successfully to remove phenols from white grape must (Servili et al., 2000). Phenols are partially removed by the enzymatic treatment, especially ferulic and o-coumaric acids. Laccase was shown to form two complex compounds with ferulic acid (Carunchio et al., 2001).

Bioremedation

Laccases exhibit broad substrate specificity and are thus able to oxidize a broad range of xenobiotic compounds including chlorinated phenolics (Royarcand & Archibald, 1991; Roper et al., 1995; Ullah et al., 2000; Schultz et al., 2001; Bollag et al., 2003), synthetic dyes (Chivukula & Renganathan, 1995; Rodriguez et al., 1999; Wong & Yu, 1999; Abadulla et al., 2000; Nagai et al., 2002; Claus et al., 2002; Soares et al., 2002; Peralta-Zamora et al., 2003; Wesenberg et al., 2003; Zille et al., 2003), pesticides (Nannipieri & Bollag, 1991; Jolivalt et al., 2000; Torres et al., 2003) and polycyclic aromatic hydrocarbons (Johannes et al., 1996; Collins et al., 1996; Majcherczyk et al., 1998; Majcherczyk & Johannes, 2000; Cho et al., 2002; Pozdnyakova et al., 2004). They can bleach Kraft pulp (Reid & Paice, 1994; Paice et al., 1995; Bourbonnais & Paice, 1996; Call &Mucke, 1997;Monteiro & de Carvalho, 1998; de Carvalho et al., 1999; Sealey et al., 1999; Balakshin et al., 2001; Lund et al., 2003; Sigoillot et al., 2004) or detoxify agricultural byproducts including olive mill wastes or coffee pulp (Martirani et al., 1996; D'Annibale et al., 2000; Kissi et al., 2001; Tsioulpas et al., 2002; Velazquez-Cedeno et al., 2002; Olivieri et al., 2006).

However, the low redox potential of laccases (450–800mV) compared to those of ligninolytic peroxidases (41 V) only allows the direct degradation of low-redoxpotential compounds and not the oxidation of more recalcitrant aromatic compounds, including some synthetic dyes or polycyclic aromatic hydrocarbons (PAH) (Xu et al., 1996). A possibility for the oxidation of compounds with high redox potentials is the use of redox mediators. From the description of the first laccase mediators, ABTS (Bourbonnais & Paice, 1990), to the more recent use of the -NOH-type, synthetic mediators such as 1-hydroxybenzotriazole, violuric acid and N-hydroxyacetanilide or TEMPO, a large number of studies have been performed on the mechanisms of oxidation of nonphenolic substrates (Bourbonnais et al., 1998; Xu

et al., 2000; Fabbrini et al., 2002; Baiocco et al., 2003), the search for new mediators (Bourbonnais et al., 1997; Fabbrini et al., 2002), and their applications in the degradation of aromatic xenobiotics (Bourbonnais et al., 1997; Johannes et al., 1998; Kang et al., 2002; Keum & Li, 2004). Nevertheless, the laccase-mediator system has yet to be applied on the process scale due to the cost of mediators and the lack of studies that guarantee the absence of toxic effects of these compounds or their derivatives. The use of naturally occurring laccase mediators would present environmental and economic advantages and increase the feasibility of the laccasemediator system for use in biotechnology. (Camarero et al., 2005).

In addition to substrate oxidation, laccase can also immobilize soil pollutants by coupling to soil humic substances – a process analogous to humic acid synthesis in soils (Bollag, 1991; Bollag & Myers, 1992). The xenobiotics that can be immobilized in this way include phenolic compounds and anilines such as 3,4-dichloroaniline, 2,4,6- trinitrotoluene or chlorinated phenols (Tatsumi et al., 1994; Dawel et al., 1997; Dec & Bollag, 2000; Ahn et al., 2002). The immobilization lowers the biological availability of the xenobiotics and thus their toxicity.

The current development in laccase catalysis research and the design of mediators along with the research on its heterologous expression opens a wide spectrum of possible applications in the near future. Last but not least, the considerable amount of data concerning the properties of fungal laccases accumulated in the past years could allow us to select a protein suitable for a specific application (e.g. temperature-resistant or pH-stable).

Laccase evolution: rational design and directed evolution

Laccases have attracted considerable interest in various fields of research, and a significant number of new and promising technological applications can be wished. To this end, detailed biochemical knowledge about these enzymes and their structure-function relationship have to be defined and “new” laccases, useful in unnatural industrial condition have to be found. Rational design and directed evolution are valid tools in this context.

Rational design

Not many experiments of site-directed mutagenesis have been performed on laccases. Based on comparative studies carried out against *Zucchini* ascorbate oxidase (zAO) and human serum ceruplasmin (hCP) (Messerschmidt, 1997; Messerschmidt et al., 1992; Zaitseva et al., 1996) and on the postulation that the co-ordination geometry and ligands of type-1 (T1) Cu might determine the redox potential (E^0) of this site (Malstom, 1997; Gray and Malstrom, 1983) Xu et al.(1998) performed site-directed mutations on *Rhizoctonia solani* laccase, enzyme endowed with high-T1 Cu E^0, and *Myceliophthora thermophila* laccase, enzyme endowed with low-T1 Cu E^0, targeting a pentapeptide segment located near the T1 site. Triple mutations resulted in significant changes in pH optimum, k_M and k_{cat}, although the redox potentials were not significantly altered. In contrast single mutations of the residue corresponding to axial methionine ligand in zAO (L513F for MtL and L470F for RsL) did not yield significant changes in the enzymatic properties, including redox potential. Nevertheless the F463M mutation in *Trametes villosa* laccase (Xu et al., 1999) resulted in an altered UV-visible spectrum and a decreased redox potential (by

0.1 V). In oxidizing phenolic substrate, this mutation led to a more basic optimal pH as well as an increase in k_{cat} and k_M. These effects are attributed to a significant perturbation of the T1 copper center caused by the coordination of the axial methionine (M463) ligand. Moreover site-directed mutagenesis has been used to replace Met502, a so-called weak axial ligand of the T1 site, in CotA laccase by the non-coordinating hydrophobic residues leucine and phenylalanine (Durao et al., 2006). These substitutions did not lead to major structural changes in the geometry of the centre or in the overall fold of the enzyme. Thus, an increase of the redox potential of both mutant enzymes by as much as 100 mV has been attributed to the weakening in the T1 Cu coordination. Furthermore, the mutations in the axial ligand have a profound impact on the thermodynamic stability of the enzyme.

As well as mutations of the axial ligand, other experiments of rational design have been performed. When the C-terminus of a laccase from *Trametes versicolor* expressed in *Pichia pastoris* is truncated, the barrier to heterogeneous electron transfer is reduced as indicated by electrochemical studies (Gelo-Pujic et al., 1999). When laccase gene from Ascomycete *Melanocarpus albomyces* was expressed in *Saccharomyces cerevisiae* C-terminus truncation enhanced six fold laccase production (Kiiskinen and Saloheimo, 2003).

Moreover site-directed mutagenesis of a laccase produced by *Trametes versicolor* was performed, providing that the highly conserved aspartic residue in position 206 plays a leading role in catalysis: the D206N mutation led to a significant shift of the pH (ΔpH=1.4) for optimal activity against 2,6-dimethoxyphenol (Madzak et al., 2006).

Directed evolution

To the best of our knowledge only one laccase was subjected to experiment of directed evolution: a thermophilic laccase from *Myceliophthora thermophila* (MtL) was improved by the use of directed evolution both *in vivo* and *in vitro* using a yeast expression system (Bulter et al., 2003). The MtL gene was subjected to random error-prone PCR, expressed in yeast, and screened through nine rounds of *in vivo* shuffling, leading to a 170-fold enhancement in expression level. The highly expressing MtL laccase T2, evolved in yeast, contains 13 mutations. Three of them are located at the three processing sites of the protein, namely the signal sequence, the prosequence and the C-terminal sequence. Presumably, these mutations adjust the MtL sequence to the different specificities of the *S. cerevisiae* proteases compared to those of the natural fungal organism. Whereas N-terminal processing and glycosylation are probably required for secretion, C-terminal processing may play a role in activation of the enzyme. Indeed, the authors have shown that a dramatic loss in activity was observed for the C-truncated mutant, suggesting that the C terminus is essential for early post-translational processing steps. The *Myceliophthora thermophila* laccase variant T2 was used to create a library by error-prone PCR with 1.1 to 3.5 mutations per kb. After only 1 generation of directed evolution, one mutant displayed about 3.5-fold higher activities than parent type in the presence of 20% acetonitrile or 30% ethanol (Alcade et al., 2005).

The thesis: Development of oxidative biosystems for the treatment of industrial effluents

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 research has been aimed to develop new laccases for the treatment of industrial effluents. Work description has been organised in two parts:

1) In the first part we analyze the heterogeneity of POXA3 laccases isoenzymes from *P. ostreatus.* These proteins are laccases atypical for their heterodimeric structure. Moreover they are emblematic of the complexity that these enzymes can undergo by the different post-transcriptional and post-translational modifications. Gene cloning of POXA3 large and small subunits and detailed analysis of the deduced protein sequences are reported. Furthermore a deep investigation on the oligomeric nature of POXA3 isoenzymes, performed by means of heterologous expression and study of effect of denaturant agents on activity and quaternary structure, is reported.

2) In the second part we describe the directed evolution of *Pleurotus ostreatus* laccases. Two cDNAs encoding POXC and POXA1b, have been selected as "parent molecules" to guide the evolution of laccases with higher specific activity and different substrate specificities. The creation of genetics variants by errore-prone PCR and DNA shuffling is described. The chararacterization, from structural, catalytic and thermodynamic points of view, of the four selected mutants is reported. Furthermore analysis of 3-D model of mutants has also been performed.

2. Subunit role and interaction in heterodimeric laccases from *Pleurotus ostreatus*

Introduction

Laccases are multicopper oxidative enzymes, useful for biotransformation of environmental organic pollutants and exploited industrially in various oxidative processes. Multiple isoforms of laccases are usually secreted by each fungus depending on species and environmental conditions. Up to seventeen laccase encoding genes have been found in the genome of *Coprinopsis cinerea* (Kilaru et al., 2006). Laccases are the major extracellular components of the lignin-degrading system of the white-rot fungi belonging to the *Pleurotus genus* (Baldrian et al., 2005). The isoenzymes produced by Pleurotus ostreatus have been extensively studied. POXC laccase is the most abundantly produced under all the growth conditions examined (Palmieri et al., 1993); other isoenzymes secreted by the mycelium have also been purified and characterised (POXA1w, POXA1b and POXA2) (Palmieri et al., 1997; Giardina et al., 1999).

Two closely related laccase isoenzymes (POXA3a and POXA3b) (Palmieri et al., 2003), produced by *P. ostreatus* in copper supplemented cultures, were purified. They exhibit unusual structural features. Unlikely most of the known laccases that are monomeric proteins, both native isoenzymes are heterodimers constituted of a large (67 kDa) and a small subunit (18 or 16 kDa),. Laccases endowed with quaternary structure have been already found in *Phellinus ribis* (Min et al., 2001), *Trametes villosa* (Yaver et al., 1996) and *Rhizoctonia solani* (Wahleithner et al., 1996), but all of them are homodimeric proteins.

Despite their remarkably different chromatographic behaviour, the POXA3a and POXA3b isoenzymes show similar catalytic properties (Palmieri et al., 2003). Their large subunits share the same MALDI-MS peptide maps, corresponding to the amino-acid sequence encoded by the unique isolated poxa3 gene. This protein sequence is clearly homologous to other known laccase sequences, and contains all the putative copper-binding residues, as well as the five Cys residues found in all other laccases. Each small subunit -either from POXA3a or POXA3b- is produced as two isoforms of 18 and 16 kDa molecular mass. The aminoacid sequence of some tryptic peptides from the two isoforms did not show significant homology with other known proteins, leaving the nature and the role of this subunits still obscure.

The singular properties of POXA3a and POXA3b isoenzymes led us to deeply investigate their structural features, taking also into account their biotechnological applicative potential. Among *P. ostreatus* laccases, POXA3 isoenzymes are able to efficiently decolorize the anthraquinonic dye Remazol Brilliant Blue R, showing a catalytic efficiency six-fold higher than POXC (Palmieri et al., 2005).

With the aim of analyzing the heterodimeric nature of the POXA3 isoenzymes, in vitro experiments have been performed in order to separate the subunits and to analyze quaternary structure stability. Moreover, the availability of a laccase recombinant expression system in the yeast *Kluyveromyces lactis* (Piscitelli et al., 2005), provided us with a tool for in vivo analyses of POXA3. The large and small subunits were both individually expressed and co-expressed in the yeast system and their properties characterized and compared.

Materials and Methods

Organism and culture conditions. White-rot fungus, *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) was maintained through periodic transfer at 4°C on potato dextrose agar plates in the presence of 0.5 % yeast extract (Difco). Mycelia were grown in shaking flasks containing potato dextrose (24 g/l) broth with 0.5% yeast extract. 50 ml of a 5-day old culture were transferred in 1 litre flasks containing 450 ml broth supplemented with 150 μM $CuSO_4$. Enzyme purification was performed from 10 days culture broth.

Enzyme purification. Secreted proteins were precipitated from the filtered medium by addition of $(NH_4)_2SO_4$ up to 80% saturation and, after extensive dialysis, loaded onto a DEAE Sepharose Fast Flow (Pharmacia Biotech Inc.) column. Two fractions containing laccase activity, recovered with the equilibrating buffer, were separately pooled, concentrated on an Amicon PM-10 membrane and equilibrated in Tris-HCl 50 mM pH 8.0. Each pool was loaded onto an anion exchange Mono Q HR5/5 (Pharmacia) column in a fast protein liquid chromatography system (FPLC, Pharmacia) equilibrated with the same buffer. The enzyme was eluted with a linear gradient (0-0.3 M NaCl). Ammonium sulphate at 1M final concentration was added to the active fractions pooled and desalted. These fractions were loaded onto a Phenyl Superose PC 1.6/5 at 0.05ml/min. Elution was performed by saline linear gradient (1-0 M ammonium sulphate).

Protein deglycosylation was performed incubating laccase samples with 1 mU of endoglycosidase H in 20 mM sodium acetate, pH 5.2, 0.01% SDS, 5 mM DTT over night at 37 °C.

Isolation and sequencing of POXA3 small subunit gene and cDNA. Amplification experiments of *P. ostreatus* genomic DNA were performed at 44 °C annealing temperature, using the following oligonucleotide couples: 5'-CARATYCAYGTBAAYATYCC -3' and 5'-CATCATNGTNGTRTANGT-3' (Y=T/C, R=G/A;V=G/C/A, B=G/T/C, D=G/A/T, n=G/A/T/C). A 400 bp fragment was obtained, cloned in pGEM T Easy vector and sequenced. This fragment, labelled by random priming method, was used as probe to screen a *P. ostreatus* genomic library. Colony hybridisation experiments were carried out in 5xSSC at 65 °C (where 1xSSC is 0.15 M NaCl, 0.015 M sodium citrate).

Total RNA was extracted from lyophilised mycelia, harvested from three days culture as already described (Lucas et al., 1977). Reverse transcription reaction was performed using Superscript II (Gibco BRL) and following the manufacturer's instructions. Amplification experiments of specific cDNA were performed at 58 °C annealing temperature using the following oligonucleotide couple: 5'-GGAGCTAAATACGCTACAAGC-3' and 5'-AATTCGCGGCCGCTTTTTTTTTTTTTTT-3'. The amplified fragment was cloned in the pGEM T Easy vector and sequenced.

In situ digestion. Mass spectrometric analyses were peformed on the Coomassie blue-stained proteins excised from a preparative SDS electrophoresis on a 12% polyacrylamide gel (Laemli, 1970). Excised bands were washed with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubation in 10 mM dithiothreitol for 45 min at 56 °C, and carboxamidomethylated by using 55 mM iodoacetamide in 0.1 M NH_4HCO_3 for 30 min, in the dark, under nitrogen atmosphere at room temperature. The gel particles were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestions were carried out with

trypsin (15 mg/ml) in 50 mM ammonium bicarbonate pH 8.5 at 4 °C for 4 hr. The buffer solution was then removed and a new aliquot of the enzyme/ buffer solution was added for 18 hours at 37 °C. A minimum reaction volume, sufficient for complete rehydratation of the gel was used. Peptides were then extracted, washing the gel particles with 20 mM ammonium bicarbonate and 0.1% trifluoroacetic acid in 50% acetonitrile at room temperature and then lyophilized. Aliquots of the digests were directly analysed by MALDI-MS (Matrix Assisted Laser Desorption Ionisations Mass Spectrometry) or separated on a narrow bore Vydac C_{18} column (25x0.21 cm, 5 μm) (The Separation Group, Hesperia, CA) using 0.1% trifluoroacetic acid (Sigma) as solvent A and 0.07% trifluoroacetic acid in 95% acetonitrile (Baker) as solvent B. A linear gradient of solvent B from 5% to 65% in 60 min at flow rate of 0.2 ml/min was employed. The UV absorbance of the eluent was monitored at 220 nm.

Mass spectrometry analysis. MALDI mass spectra were recorded using a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystem). A mixture of analyte solution and α-ciano-4-hydroxy-cinnamic acid as matrix, was applied to the sample plate and air-dried. Mass calibration was obtained using the quasi-molecular ions (MH^+) from an external calibration mixture provided by the manufacturer. Raw data were analysed by using computer software provided by the manufacturer and are reported as monoisotopic masses.

Size exclusion chromatography Samples were loaded onto a gel filtration Superdex 75 PC 3.2/30 column using a SMART System (Pharmacia). Alternatively Superdex 75 HR 10/30 (24ml) and AKTA FPLC system (GE Healthcare) was used. After GndHCl incubation at different concentration, elution was performed with 50 mM sodium phosphate buffer pH 7.0, containing the corresponding concentration of GndHCl (flow rate 0.05 ml/min). After SDS incubation, elution was performed with 50 mM Na Citrate buffer (pH 4 or 5) or with 50 mM sodium phosphate buffer pH 7.0 containing 150 mM NaCl and the desired concentration of SDS.

Heterologous POXA3 expression. The *Kluyveromyces lactis* strain used for heterologous expression was CMK5 (*a thr lys pgi1 adh3 adh1::URA3 adh2::URA3*) (Saliola et al., 1999). The plasmid pYG132 was engineered from pKD1, a natural plasmid originally isolated from *Kluyveromyces drosophilarum* (Falcone et al., 1986) that can stably replicate in *K. lactis*. Expression of inserts is controlled by the ethanol-inducible *KlADH4* promoter (Mazzoni et al., 1992) and the *S. cerevisiae* phosphoglycerate kinase (*PGK*) terminator. In order to insert a Hind III site upstream and downstream of the cDNA, the 5' and 3' regions of large subunit *poxa3* cDNA were amplified by means of *Phusion* polymerase (Stratagene) with fwPOXA3 HindIII/ revPOXA3BamHI and fwPOXA3BamHI/ revPOXA3 HindIII primers (Table 1) respectively, using pGEM containing *poxa3* cDNA as template and cloned into pYG132 giving A3L vector. cDNA encoding for POXA3 small subunit was amplified with sspoxa3 5'SalI/sspoxa3 3' SalI primers, in order to insert a Sal I site upstream and downstream of the cDNA. The SalI DNA fragment containing the POXA3 small subunit encoding sequence, including its signal peptide, was ligated into A3L vector, giving A3LS vector.

K. lactis transformation was performed by electroporation with a Bio-Rad Micro-Pulser apparatus, as specified by the manufacturer. The cells were spread on YPPG (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} galactose) medium containing 100 μg ml^{-1} of geneticin G418, after an over night incubation at 28°C in YPPG. Agar plate assays on YPPG supplemented with 100 μg ml^{-1} of geneticin G418, 0.5% ethanol, 1 mM $CuSO_4$ and 0.2 mM ABTS were used for transformant selection. Plates were incubated inverted at 28°C and checked for the development of colour.

Primer	Nucleotide Sequences	Annealing T (°C)
fwPOXA3 *Hind*III	AAAC*AAGCTT*GAGCTC*ATGGTGCTCTCTACTAAG*	52
revPOXA3*BamH*I	GCGCACGGATCCAGTAG	56
fwPOXA3*Ba*mHI	CTACTGGATCCGTGCGC	56
revPOXA3 *Hind*III	AAATGCAT*AAGCTT*ACTGGAACTCGGGAGC	56
sspoxa3 5' *Sal*I	CCACGC*GTCGAC*ATGTTCTTCCGTCCTTCACTC	62
sspoxa3 3' *Sal*I	CCACGC*GTCGAC*TCATGCAAATCCGAGAGAAGC	62

Table 1. List of the primers used in this study. Nucleotides in bold are complementary to the laccase cDNA sequences. Nucleotides in italics are recognized by restriction enzymes.

100 μl of ethanol was added each day to the lid of the plate to compensate its evaporation. Some transformed clones for each expression vector were screened for laccase production by growing them in 10 ml selective medium (YPPG containing geneticin) supplemented with 1 mM $CuSO_4$ and 0.5% ethanol, at 28°C on a rotary shaker (150 r.p.m.). 0.5% ethanol was daily added and samples were taken at intervals for optical density and laccase activity determination. Better producing clones were chosen to study laccase production in 250 ml flasks containing 50 ml of selective YPPD (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} glucose) or YPPG medium, containing 0.5 or 1% ethanol, and 0.5 or 1mM $CuSO_4$, starting from 0.06 OD_{600}. Cultures were grown at 28°C or 20 °C on a rotary shaker. Preparative culture was performed in 1l flasks containing 200 ml of medium.

Western blots. Protein samples were resolved by using 12.5% SDS-PAGE gel (Laemmli, 1970) and transferred to a PVDF membrane using an electroblotting transfer apparatus (Trans-Blot Semi-Dry Transfer Cell, Bio-Rad, USA). POXA3 large subunit was detected by using anti-POXA1b [5] antibodies (cross-reacting with POXA3) at a 1:2,000 ratio, and peroxidase-conjugated anti-rabbit secondary antisera (1:40,000) (A9169, Sigma, MO, USA). For POXA3 small subunit detection, polyclonal antibodies were obtained by rabbit immunization with the recombinant protein produced in *Escherichia coli* using pET-22b(+) (Novagen) expression system (data not shown). 1:1,000 dilution of these antibodies and 1:40,000 dilution of peroxidase-conjugated anti-rabbit secondary antisera were used for small subunit detection. The membranes were developed by using SuperSignal West Femto Maximum Sensitivity Substrate detection kit (Pierce).

Results

Gene cloning

In a previous study, the amino acid sequence of some peptides from the tryptic digest of POXA3a small subunit had been determined (Palmieri et al., 2003). On the basis of these sequence data, degenerate oligonucleotide-primers were designed and used in amplification experiments on *P. ostreatus* genomic DNA. A *P. ostreatus* genomic library (Giardina et al., 1995) was screened using the amplified fragment. Two different genes (1 and 2) encoding the small subunits were identified, whose sequences only differed for 6 bases. The corresponding cDNAs were amplified, using specific oligonucleotides. The encoded amino acid sequences are reported in Fig. 1 and resulted to be different for 2 residues at positions 68 and 86. The deduced protein sequences did not share significant homology with any protein sequence in data banks.

Figure 1 Amino acidic sequence of POXA3a and POXA3b small subunits, the latter containing the indicated amino-acid substitutions. The putative signal peptide is underlined, and the six amino-acid residues cleaved at the N-termini are doubly underlined. N-glycosylation site is indicated by arrow.

As far as POXA3 large subunit, some cDNA fragments with sequences differing from that already published [6] were found by analysing several clones. An insertion of 12 bp (5'-CTTGTTCACTAG-3') at the 3' terminus of intron XII was detected, thus indicating that alternative splicing could occur at the junction between exons XII and XIII, with canonical splicing sequences conserved in both cases. The encoded amino acid sequence LeuPheThrArg is inserted between Pro287 and Leu288 of the large subunit sequence. Tryptic peptide maps of the large subunits showed the presence of both forms, with and without the four amino acid insertion, in all the isoenzymes.

Small subunit characterization

Analyses of MALDI-MS peptide maps of POXA3a and POXA3b small subunits (18 and 16kDa) allowed the two different amino acid sequences to be assigned (Tab. 2). As a fact, mass signals at m/z 2546.6 and 2546.8 corresponding to the tryptic peptide 45-68 are only present in POXA3a 18 and 16kDa subunits respectively, indicating that gene 1, encoding the protein containing Arg at position 68, corresponds to the POXA3a small subunits.

Table 2 Comparison of experimental and theoretical tryptic peptides of POXA3a and POXA3b small subunits (18 and 16 kDa forms)

Peptides (sequence 2)	MH^+ theoretical	POXA3a 16 MH^+ experimental	POXA3a 18 MH^+ experimental	Peptides (sequence 1)	MH^+ theoretical	POXA3b 16 MH^+ experimental	POXA3b 18 MH^+ experimental
21-26	706.4			21-26	706.4		
27-44	1949.0	1932.2	1931.0	27-44	1949.0	1932.5	1932.0
45-68	2547.3	2546.8	2546.6	45-123	8015.1		
69-123	5615.8						
124-151	3046.5	3045.4		124-151	3046.5	3047.0	
N-glyc.124-151			4261				
152-161	1126.7	1126.6	1126.5	152-161	1126.7	1126.8	1126.0
162-180	2138.1	2138.0	2136.9	162-180	2138.1	2138.3	2138.3
181-186	565.3			181-186	565.3		

It has been previously reported that none of the small subunits could be directly sequenced, since they have blocked N-termini. The cDNA deduced amino acid sequences were then submitted to SignalIP software (http://us.expasy.org) and a putative signal peptide, corresponding to the fragment 1-20, was recognized. Accordingly, no peptides generated from the N-terminal end could be detected in the tryptic maps of the small subunits. The mass signals at about m/z 1932 present in all the tryptic maps (Tab. 2), occurred 17 Da lower than expected for the peptide 27-44 suggesting that Gln27 had been converted into a pyrrolidone carboxyl residue. As a fact, these peptides did not provide any sequence when purified and submitted to Edman degradation. Therefore, further six aminoacid residues at the N-termini of each small subunit (from Ser21 to Arg26) should have been cleaved and Gln27 cyclization occurred, giving rise to blocked N-termini.

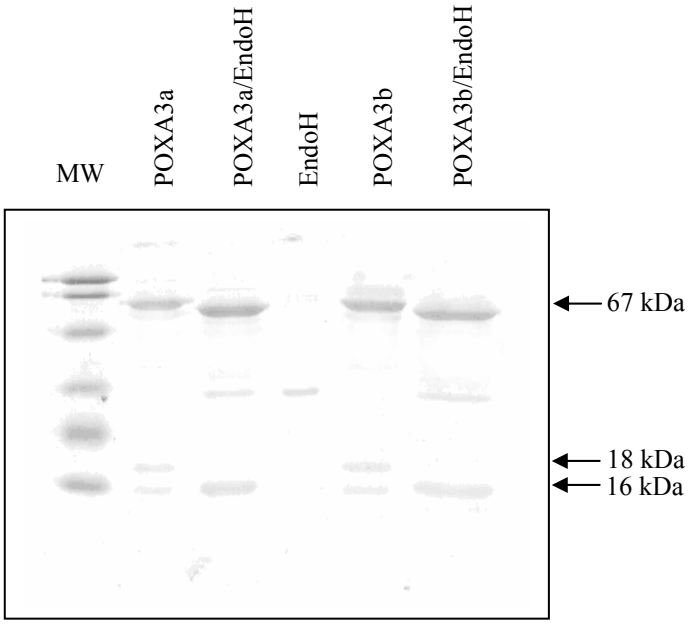


Figure 2 SDS PAGE of POXA3a and POXA3b before and after (POXA3a/EndoH and POXA3b/EndoH) treatment with endoglycosidase H

A single putative N-glycosylation site was inferred at Asn150 in the small subunit amino acid sequences. The signal at m/z 4261 found in the POXA3a 18 kDa small subunit (Tab. 2) was assigned to the peptide 124-151 containing Asn150 modified by high-mannose-type glycans with 2 mannose residues linked to the pentasaccharide core. On the other hand the signals at 3046 m/z observed in the mass spectra of POXA3a and POXA3b 16 kDa small subunits (Tab. 2) was assigned to the unmodified peptide 124-151. Therefore the mass differences between the 16 and 18 kDa subunits of both POXA3a and POXA3b are due to the presence of a glycosidic moiety attached to Asn150 in the 18 kDa subunits (Fig. 1).

These results were confirmed by incubation of POXA3a and POXA3b isoenzymes with endoglycosidase H followed by SDS PAGE analysis (Fig. 2). After deglycosylation treatment, the 18 kDa small subunit signals disappeared and only bands with mobility corresponding to the 16 kDa subunits were observed.

Taking into account the identified post-translational modifications, the expected molecular mass values of the small subunits are in agreement with those experimentally determined.

Isoenzyme heterogeneity

When POXA3a or POXA3b were loaded on Phenyl Superose, two active peaks from each isoenzyme have been eluted with the salt gradient, corresponding to the complexes formed by the large subunit and either the 18 or the 16 kDa small subunits, respectively. Therefore, four heterodimeric POXA3 isoenzymes have been separated (POXA3a16, POXA3a18, POXA3b16 and POXA3b18), as demonstrated by SDS-PAGE analysis. When kinetic parameters or stabilities at different temperatures or pH values of the four complexes were analysed, no significant differences could be observed. Moreover, two further peaks were eluted at the end of the gradient and demonstrated by mass spectral analysis to correspond to the free 18 and 16 kDa small subunits, accounting for about 10% (mol/mol) of the complexes.

Effect of denaturant agents on enzymatic activity and quaternary structure

In order to understand the role played by small subunits in POXA3, several conditions to reversibly dissociate the complexes were investigated, using two denaturant agents, GndHCl and SDS. POXA3b16 (0.15 mg/ml final concentration) was incubated with 1, 2, 3, 4, and 6M GndHCl at room temperature and the enzymatic activity was evaluated. After one hour incubation at 1M GndHCl the activity was unchanged, while at 2, 3, and 4 M GndHCl the activity decreased to 70, 38, and 15% respectively. At 6M GndHCl no residual laccase activity could be detected. However, following sample renaturation, no recover of activity was obtained after 1:10 dilution or dialysis of the samples in the refolding buffer (50 mM TrisHCl pH7, 0.5 mM $CuSO_4$, 50 mM NaCl). The irreversible effect of the denaturation process was observed even when a ten fold higher concentration of the protein was used, or the concentration of the denaturing agent was lowered (0.5- 1.5 M GndHCl) and the incubation times increased (up to 24 hours).

Enzyme samples incubated in 2 and 6M GndHCl for 1 hr were analysed by SEC under denaturing conditions. A clear separation of the large and small subunits could only be observed in the presence of the 6M GndHCl (peak I and peak II respectively, Fig. 3A). At lower GndHCl concentration a single peak (peak III, Fig. 3A) was eluted containing both subunits as demonstrated by SDS-PAGE (data not shown). These findings suggest that loss of enzymatic activity in the presence of 6M GndHCl might essentially be due to dissociation of the complex.

When incubations were performed in the presence of SDS, a different behaviour was observed, with noticeable loss of activity (95%) even at SDS concentrations as low as 0.1%. However, in this condition, a 100% of activity could be recovered after 1:20 sample dilution in the refolding buffer. The SDS denatured sample was then analysed by SEC using 0.1% SDS in the elution buffer. A single peak was eluted by the column and shown to contain both the large and small subunit. Likewise increasing SDS concentrations in the incubation mixture (from 0.1 to 4%), incubation times (from 30’ to 24 hr) and SDS concentrations in the eluting buffer (from 0.1 to

2%) did not affect subunit separation. As a fact an unique peak from SEC (peak III, Fig 3B) was always obtained. Only when the samples were incubated at 100 °C for 5 minutes in 4% SDS, two separate peaks on SEC (peak I and peak II, Fig. 3B) were observed.

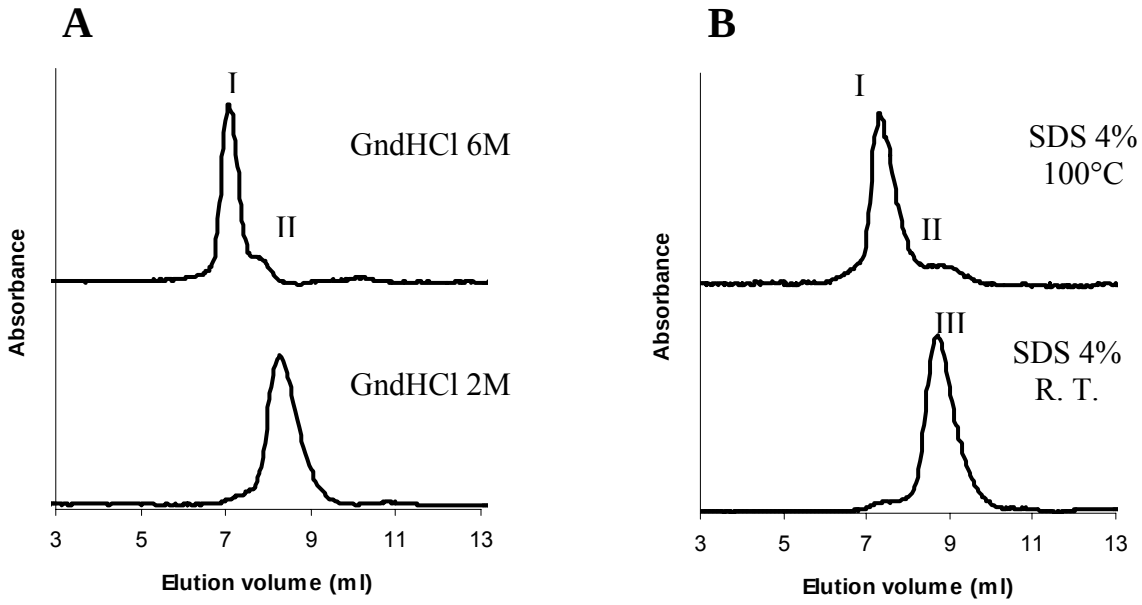


Figure 3 Size exclusion chromatography analyses of POXA3b in the presence of 6 or 2 M Guanidine Hydrochloride (panel A) or 0.1% SDS (panel B).
Samples were previously incubated in 6 or 2 M GndHCl for 1 hr (A) or in 4% SDS at 100 °C for 5 min or for 30min at room temperature (B).

Stability of POXA3a and POXA3b complexes

The effect of pH on POXA3a and POXA3b complexes stability in the presence of SDS was evaluated and compared. Enzyme samples were incubated in 4% SDS at different pH values for 30 minutes at room temperature. Dissociation of the complexes was evaluated by SEC analyses (Fig 4A) carried out in appropriate buffer solution containing 0.1% SDS and the peak identity was assigned by SDS PAGE analysis (Fig. 4B). At pH 7 both complexes showed the occurrence of a single chromatographic peak containing both the large and the small subunits. The complexes exhibited different retention time in these conditions, with POXA3a eluted before POXA3b, whereas under native conditions both isoenzymes shared the same elution volume. When the incubation was performed at pH 5, POXA3a showed the occurrence of two distinct peaks. The first eluted peak, marked α in Fig.4A, contained only the large subunit whereas both subunits occurred in peak β. In the same conditions, POXA3b was still eluted as a single component, suggesting a higher stability of this complex. Finally, at pH 4, both complexes dissociated and the large and the small subunits were eluted separately, as assessed by SDS PAGE (Fig. 4B).

Renaturation experiments were carried out on POXA3 isoenzymes incubated at pH 5 and pH 4 by dialysis against refolding buffer. About 80% of enzymatic activity was recovered from the samples at higher pH value while no activity could be detected for the enzymes incubated at pH 4.

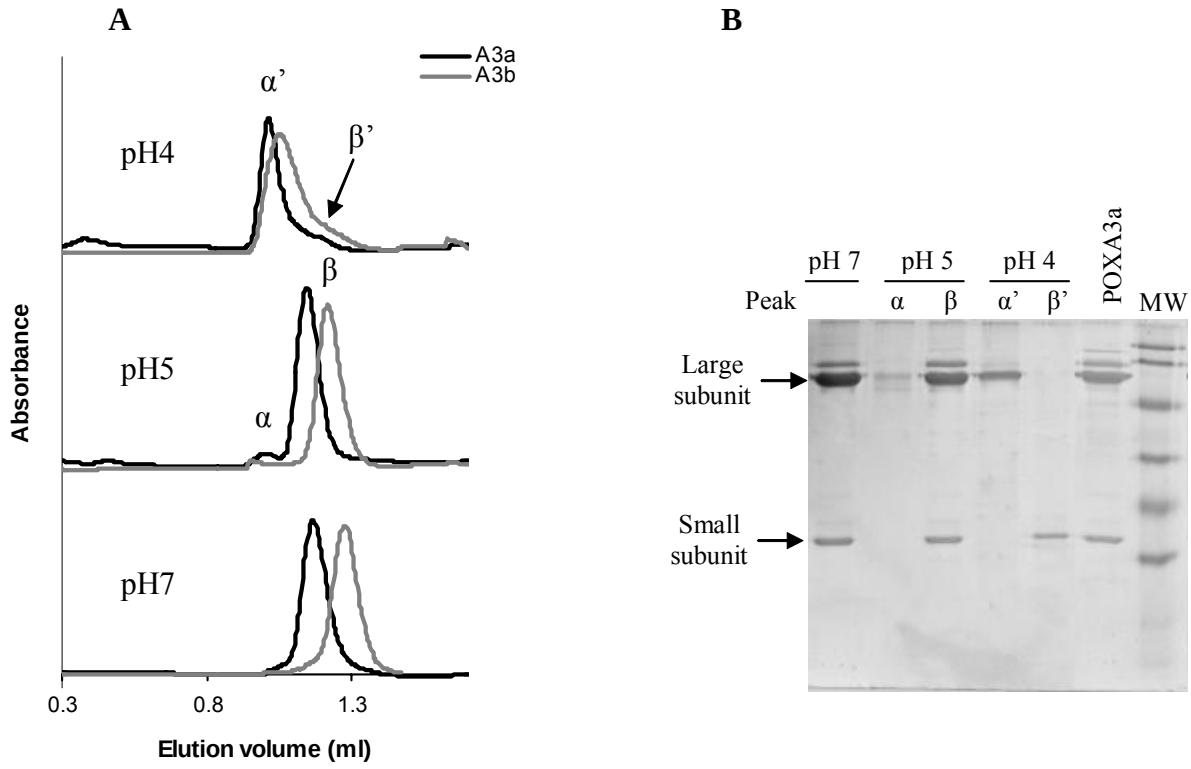


Figure 4 (A) Size exclusion chromatography analyses of POXA3a and POXA3b in the presence of 0.1% SDS at different pH values (4, 5 and 7). Samples were previously incubated in 4% SDS for 30 min at the corresponding pH. (B) SDS PAGE analysis of POXA3a samples from SEC at different pH values.

Attempts to renature fractions eluted from SEC at pH5 were then performed. No activity was recovered from peak α (Fig 4A) containing the large subunit alone, whereas renaturation of peak β containing both subunits yielded about 50% of recovered activity. Refolding of peak α was also performed in the presence of a molar excess of the free small subunit, resulting in no recovery of enzymatic activity.

POXA3 heterologous expression

The POXA3 large subunit was heterologously expressed in *K. lactis* (A3L vector), a yeast recombinant expression system already successfully set up to express POXC and POXA1b laccase isoenzymes from *P. ostreatus*. The co-expression of the POXA3 large and small subunits was also performed using the same expression system by exploiting the bidirectional functioning of the *Kl*ADH4 promoter (C. Mazzoni personal communication). The cDNA encoding the POXA3b small subunit was inserted upstream the *Kl*ADH4 promoter and the cDNA coding for the large subunit downstream the promoter sequence (A3LS vector)

Production of recombinant laccase activity was assayed on plates containing ABTS as substrate. All the transformants carrying A3LS vector were active, whereas no activity was secreted by any transformant carrying the A3L vector. Some

recombinant clones from each transformation were grown in liquid culture broth at 28 °C. After 10 days, extracellular laccase activity produced by the A3LS transformants ranged from 0 to 10 mU ml^{-1}, whilst no laccase activity was detectable in culture supernatants of any A3L transformant.

Cultures of the best producing A3LS clone were carried out in the presence of different carbon sources (glucose or galactose), ethanol (0.5 or 1%), and copper concentrations (0.5 or 1 mM $CuSO_4$), and at different incubation temperatures (20 or 28 °C) to improve laccase production. The presence of glucose, 0.5 % ethanol and 1 mM $CuSO_4$ allowed higher production of laccase activity. Moreover, decreasing the growth temperature from 28°C to 20 °C led to a further increase of laccase activity production up to four fold (Fig. 5). In the same growth conditions optimized for A3LS clone, low levels of extra-cellular laccase activity was detectable also in A3L transformants. The best producing A3L clone exhibited laccase activity, at the 14^{th} day, eight fold lower then that of A3LS clone (Fig. 5), while the protein concentration in the culture broths for both clones was comparable (0.16 mg ml^{-1}).

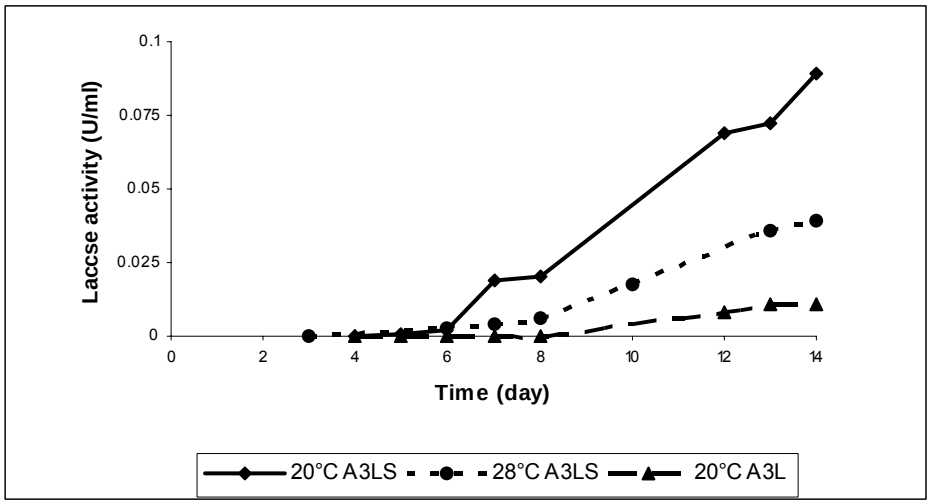


Figure 5 Time courses of laccase activity production by A3LS and A3L clones in the optimized culture conditions. In these conditions, no laccase activity was detectable in culture supernatants of A3L clones at 28 °C.

Western blot analyses of cellular extracts and culture supernatants withdrawn at the 4^{th}, 7^{th} and 11^{th} growth days of A3L and A3LS clones were performed. As shown in Fig. 6A and B, similar amounts of large subunit were secreted by both clones, while a higher intracellular amount of the protein was observed in A3L with respect to A3LS. Therefore, a higher total amount of recombinant large subunit was produces by the A3L clone, but the protein was poorly secreted. The secreted recombinant large subunit exhibited an apparent molecular mass (Mr) slightly higher than that of the native protein in both A3LS and A3L clones possibly due to different glycosylation patterns, whilst the intracellular forms have similar Mr values.

The expression and secretion of the small subunit in A3LS clone was evaluated by Western blot analyses, thus demonstrating the effective bidirectional functioning of the KlADH4 promoter (Fig. 6C). Two isoforms were detected, differing for their glycosylation moiety, as demonstrated by endo-glycosidase treatment of the protein

(lane marked with LSd). The recombinant small subunit was totally secreted, being not detectable in cellular extracts (data not shown).

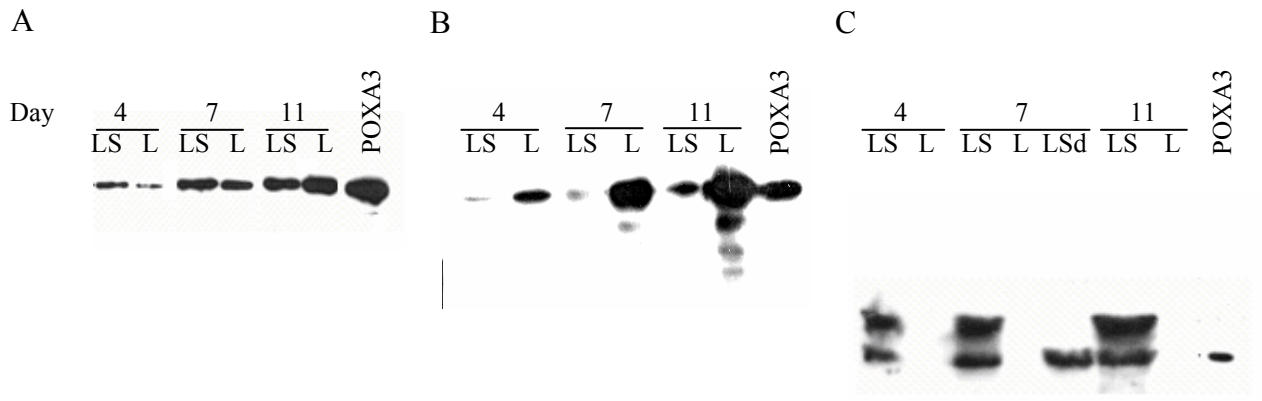


Figure 6 Western blot analyses of cellular extracts (A) and culture supernatants (B, C) withdrawn at different growth days of A3L (lanes marked with L) and A3LS (lanes marked with LS) clones, using antibodies cross-reacting with POXA3 large subunit (A, B) or POXA3 small subunit (C). Lane marked with LSd: culture supernatant of A3LS clone after endo-glycosidase treatment.

In an attempt to increase recombinant laccase isoenzyme activity, the POXA3b small subunit (16kDa) from *P. ostreatus* was added to A3L culture broth. However, no increase of laccase activity was observed even when enzyme samples were previously denatured in mild condition (0.1% SDS for 30min).

Furthermore, measurements of laccase activities in A3L and A3LS culture broth samples incubated at room temperature, revealed a significant higher stability for A3LS with respect to A3L samples, i.e. only 5% residual activity was measured after 5 hr for A3L, while 60% residual activity was held by A3LS.

Discussion

Pleurotus ostreatus produces a wide variety of laccase isoenzymes. This heterogeneity is certainly due to the multiplicity of the corresponding encoding genes, but the isoenzymatic pattern is made more complex by the different post-translational modifications (proteolytic processing, glycosylation, etc.) that each isoenzyme can undergo. Moreover alternative splicing can occur, further increasing the isoform number. The subfamily of POXA3 isoenzyme is emblematic of this complexity, being the enzyme a heterodimer showing further heterogeneity on both subunits. In this paper a deep analysis of all POXA3 forms is reported as an example of complexity of fungal isoenzyme pattern.

An unique gene codes for both POXA3a and POXA3b large subunits. Among the large subunit transcripts, the presence of two splice variants has been observed. In both cases, the splicing occurs at consensus splice junction sequences. A variant, 12 nt longer than the previously published nucleotide sequence, encodes a protein with an insertion of four amino acids between Pro287 and Leu288. Nowadays several authors have reported alternative splicing in fungi (Lodato et al., 2003; Larrondo et al., 2004; Stuardo et al., 2005), but, at the best of our knowledge, no information is available on the encoded protein variants. We have demonstrated that both splicing variants are translated and the two corresponding proteins were found in the purified POXA3a and POXA3b.

Two genes encoding POXA3 small subunits have been identified. The structural characteristics of POXA3 small subunits and the differences among them were analysed by comparison of the sequences deduced from the encoding genes and the tryptic maps of the proteins. These analyses allowed us to ascertain that: i) POXA3a and POXA3b small subunits differ for two amino acid substitutions; ii) differences between 18 and 16 kDa subunits of both POXA3a and POXA3b are only due to post-translational modification of the 16 kDa subunit by N-glycosylation at Asn150; iii) all the small subunits undergo a proteolytic cleavage at their N-termini followed by Gln27 cyclization. Therefore the unique difference between POXA3a and POXA3b enzymes seems to be related to the occurrence of two amino acid substitutions in their small subunits. Moreover, each isoenzyme can be further split into two forms differing for the presence of the glycosydic moiety in the small subunits. All of these isoenzymes contain both variants of the large subunit originating from the alternative splicing.

POXA3 large subunit is clearly homologous to fungal laccases and all known consensus sequences involved in copper binding are found (Palmieri et al., 2003). The reason why only this protein, among several other laccase isoenzymes produced in the same culture, is found associated with another polypeptide chain is not clear. The sequence of the small subunit does not show significant homology with any sequence in data banks, therefore no indication on the function of this subunit can be inferred from its primary structure. To investigate on the unusual structure of POXA3, we intended to reversibly denature the protein, take apart the two subunits and refold them to compare the characteristics of the complex with respect to those of the large subunit alone. Several attempts were performed to this aim, using different denaturant agents in various conditions. Unfortunately the complexes were shown to dissociate only in conditions that did not allow protein refolding. As a fact at least two different denatured intermediates can be singled out: i) a partially denatured, not active form with bound subunits whose activity can be recovered by renaturation, ii)

an irreversibly unfolded form in which separation of the subunits had occurred. Therefore POXA3 large and small subunits, although not covalently bound, interact very strongly. However a different behaviour of POXA3a and POXA3b in denaturing conditions was observed. Experiments at different pH values suggested a stronger interaction between the two POXA3b subunits, in agreement with previous data obtained using urea as denaturing agent. According to our structural data, this difference in stability might be ascribed to the only two amino acid substitutions found between POXA3a and POXA3b small subunit sequences. These amino acids might possibly be involved in subunit interaction, thus affecting the stability of complexes.

Heterologous expression of the large subunit alone and co-expression of both subunits in *Kluyveromyces lactis* were chosen as alternative strategy to obtain a folded separated large subunit to compare with the complex. Preliminary data from C. Mazzoni and co-workers suggested a possible bidirectional functioning of the KlADH4 promoter. Both subunits were successfully co-expressed under the control of this promoter by using a single vector, thus demostrating the bidirectional transcriptional activity of this promoter. This yeast expression system might be very useful in the production of recombinant heterodimeric proteins.

The recombinant secreted large subunit shows a slightly higher Mr respect to the native protein, confirming the low tendency of *K. lactis* to hyperglycosylate with respect to other yeast hosts ref). The small subunit is secreted by *K. lactis* as two isoforms differing for their glycosylation moiety, showing the same type of heterogeneity of the native proteins from *P. ostreatus*.

In optimized growth conditions laccase activity can be detected in both transformed yeast cultures, but the clone expressing the large subunit (A3L) exhibited always notably lower activity than that expressing both subunits (A3LS). Notably higher intracellular amount of the protein was observed in the yeast expressing the large subunit alone, while protein concentration in the culture broths was the same for both A3L and A3LS clones. The higher production of the large subunit by A3L could be due to a better functioning of the promoter working mono-directionally with respect to that promoting expression in both directions. On the other hand the higher expression of the large subunit does not correspond to its higher secretion level. This finding can be due to differences in the intracellular protein folding processes, or in the secretion mechanisms involving the single large subunit or the complex.

Reported data indicate a significant increase of laccase activity connected to the presence of the small subunit. Nevertheless the heterologously expressed POXA3 large subunit is unable to be activated by addition of the small subunit, suggesting that only a co-folding process can produce the fully active enzyme. Moreover in culture broth samples of A3LS a significant higher stability of laccase activity with respect to that of A3L was observed, allowing to hypothesize a role of small subunit in the stabilization of POXA3. This hypothesis is strengthened by the in vitro experimental results showing that separation of subunits implies the complete denaturation of the proteins.

3. Directed evolution of *Pleuortus ostreatus* laccases

Introduction

Laccases (E.C.1.10.3.2, p-benzenediol:oxygen oxidoreductase) belong to the blue oxidase family of proteins, along with ascorbic oxidases and ceruloplasmins. These copper-containing enzymes (multicopper oxidases) couple the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water (Yaropolov et al. 1994). Laccases are widely distributed in higher plants and fungi, and laccase-like activity has been found in some insects (Dittmer et al. 2004; Kramer et al. 2001) and bacteria (Claus 2003). These enzymes have a very broad substrate range and can oxidize polyphenols, aminophenols, methoxyphenols and aryl amines and are being increasingly evaluated for use in a variety of applications including the pulp and paper industry, bioremediation, and for analytical purposes (Mayer et al. 2002). The white-rot fungus *Pleurotus ostreatus* expresses multiple laccase genes encoding isoenzymes with different physico-chemical and catalytic characteristics. The amount of the various enzymes produced depends on culture conditions. So far, five isoenzymes secreted by this fungus have been purified and characterised: POXC (Palmieri et al. 1993), POXA1w (Palmieri et al. 1997), POXA1b (Giardina et al. 1999) and the two closely related isoenzymes POXA3a and POXA3b (Palmieri et al. 2003). POXC is the most abundantly produced in all growth conditions tested so far; POXA1w has unusual metal ion content; POXA1b is the most stable at alkaline pH; and POXA3a and POXA3b are atypical because of their heterodimeric structure. Four genes encoding laccases are known, *poxc* (previously named pox2) (Giardina et al. 1996), *pox1* (which codes for a laccase isoenzyme not identified yet) (Giardina et al. 1995), *poxa1b* and *poxa3* (Giardina et al. 1999; Palmieri et al. 2003). Heterologous expression of *Pleurotus ostreatus* POXC and POXA1b laccases in two yeasts, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, was performed (Piscitelli et al. 2005). 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. Directed evolution by DNA shuffling, random mutagenesis and recombination followed by screening or selection is a valuable tool for the engineering of enzymes. We report optimization of the catalytic properties of *Pleurotus ostreatus* laccases expressed in yeast, by directed evolution, and describe the positive variants found by screening of large variant libraries.

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	SOB
Bacto tryptone (Difco) 1%	Bacto tryptone (Difco) 2%
Yeast extract (Difco) 0.5%	Yeast extract (Difco) 0.5%
NaCl 1%	NaCl 10 mM
	$MgSO_4$ 20 mM
	$MgCl_2$ 10 mM

Selective medium was supplemented with 100 µg ml^{-1} of ampicillin; solid medium contained 15% of agar (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 r. p. m.). 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 Kluyveromyces lactis. The *Kluyveromyces lactis* strain used for heterologous expression was CMK5 (a thr lys pgi1 adh3 adh1::URA3 adh2::URA3). *K. lactis* was grown in YPPG medium at 28°C. Cultures were conducted at 28°C. Selective medium was YPPG supplemented with 100 µg ml^{-1} of geneticin, solid medium contained 15% of agar (Difco). Plate assay was performed on solid selective medium supplemented with enzyme substrate: 0.2 mM ABTS (2,2'–azinobis (3ethylbenzothiazoline-6-sulfonate)) or 0.1 mM DMP (2,6–dimethoxy-phenol). EtOH as inducer and copper (as $CuSO_4$) as cofactor were used.

Component	YPPG
Yeast extract (Difco)	1%
Bacto tryptone (Difco)	4%
Galactose	2%
Ethanol	0.5%
$CuSO_4$	0.1 mM

Yeast transformation. Cells were picked up from a solid culture and grown in 50 ml of YPPG medium at 28° on a rotary shaker (150 rpm) up to 1.3-1.5 OD_{600}. Cells were sedimented by centrifugation at 3000 x g at 4°C for 10 min and washed twice with cold H_2O, and once with cold 1 M sorbitol. Cell pellet was then resuspendend in 0.3 ml of 1 M sorbitol, and an aliquot of this suspension (50 µl) was mixed with transforming DNA plasmid (up to 1 µg). K. lactis transformation was performed by electroporation with a Bio-Rad Micro-Pulser apparatus, as specified by the manufacturer. The cells were then spread on YPPG medium containing 100 µg ml^{-1} of geneticin G418, after an over night incubation at 28°C in YPPG. Agar plate assays on YPPG supplemented with 100 µg ml^{-1} of geneticin G418 and0.2 mM ABTS (2,2'–azinobis (3ethylbenzothiazoline-6-sulfonate)) were used for transformant selection. Plates were incubated inverted for 4 days at 28°C and checked for the development of green colour. 100 µl of ethanol was added each day to the lid of the plate to compensate its evaporation.

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)	0.67%
Casaminoacids (Difco)	0.5%
Glucose	2%
Adenine	30 mg l^{-1}
Tryptophane	40 mg l^{-1}
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 transformation. Yeast cells were scraped from an YPD plate and washed with 1 ml of H_2O. The cell pellet was then incubated in 1 ml of 0.1 M LiAc for 5 min at 30^o

C. Cells were sedimented by centrifuging at 12000 x g for 5 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^{-1})
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: plates were incubated inverted for 4 days at 28°C and checked for the development of green or orange colour.

Experiments of homologous recombination were performed following the previous protocol and transforming the yeast cells with different ratios (1: 1 and 1:3) of linearized plasmid (1.5 µg) and mutated cDNA.

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 OD_{600} 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 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 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 (Robzyk and Kassir, 1992). Yeast cells were grown in 3 ml of liquid SD medium at 28°C over night on a rotary shaker (150 r. p. m.). 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.

Nucleic acid manipulation

All DNA manipulations were performed according to Sambrook *et al.*, (1989).

The plasmids C-pSAL4, B-pSAL4 and pYA1bps (Piscitelli et al, 2005) were used to express library of mutated cDNA in *S. cerevisiae,* and , the last one, in *K. lactis.*

Libraries construction .

The GenBank accession numbers of the sequences of the P. ostreatus laccase cDNAs poxc (Giardina et al. 1996) and poxa1b (Giardina et al. 1999) used as templates are Z34848 and AJ005018, respectively.

DNA shuffling library construction. DNA shuffling was performed using the protocol described by van der Veen (Van der Veen at al, 2003) based on the shuffling method described by Zhao and Arnold (Zhao and Arnold 1997). This consists of fragmentation of the genes using restriction enzymes, a PCR1 without primers to reassemble the genes, and a PCR2 with primers for amplification of the full-length shuffled genes. *P. ostreatus* laccase cDNAs poxc and poxa1b were digested for production of fragments with sizes of 40÷800 bp, using NlaIV and TaqI to fragment poxc cDNA and HinfI and Taq I to fragment poxa1b cDNA. Fragmented genes were subjected to a PCR without primer (PCR1), according to the scheme below. In the second PCR, aliquots of the products of the above PCR were used as template with four different combination of primers: 1)POXA1bfw and POXa1brev; 2) POXA1bfw and POXCrev; 3) POXCfw and POXA1Brev; 4) POXCfw and POXCrev (Table 1).

Primer	**Nucleotide sequences**	**Ann. T**
POXA1b*fw*	ATAA*AAGCTTGAATTC***ATGGCGGTTGCATTCG**	90°C
POXA1b*rev*	TAA*GGATCCAAGCTT***TTATAATCATGCTTC**	82°C
POXC*fw*	TTT*AAGCTTGAATTC***ATGTTTCCAGGCGCACGG**	96°C
POXC*rev*	AAAA*GAATTCAAGCTT***TTAAGAAGTAGGAATAAGAAG**	94°C

Table 1 List of the primers used in amplification experiments. Nucleotides in bold are complementary to the laccase cDNA sequences. Nucleotides in italics are recognized by restriction enzymes

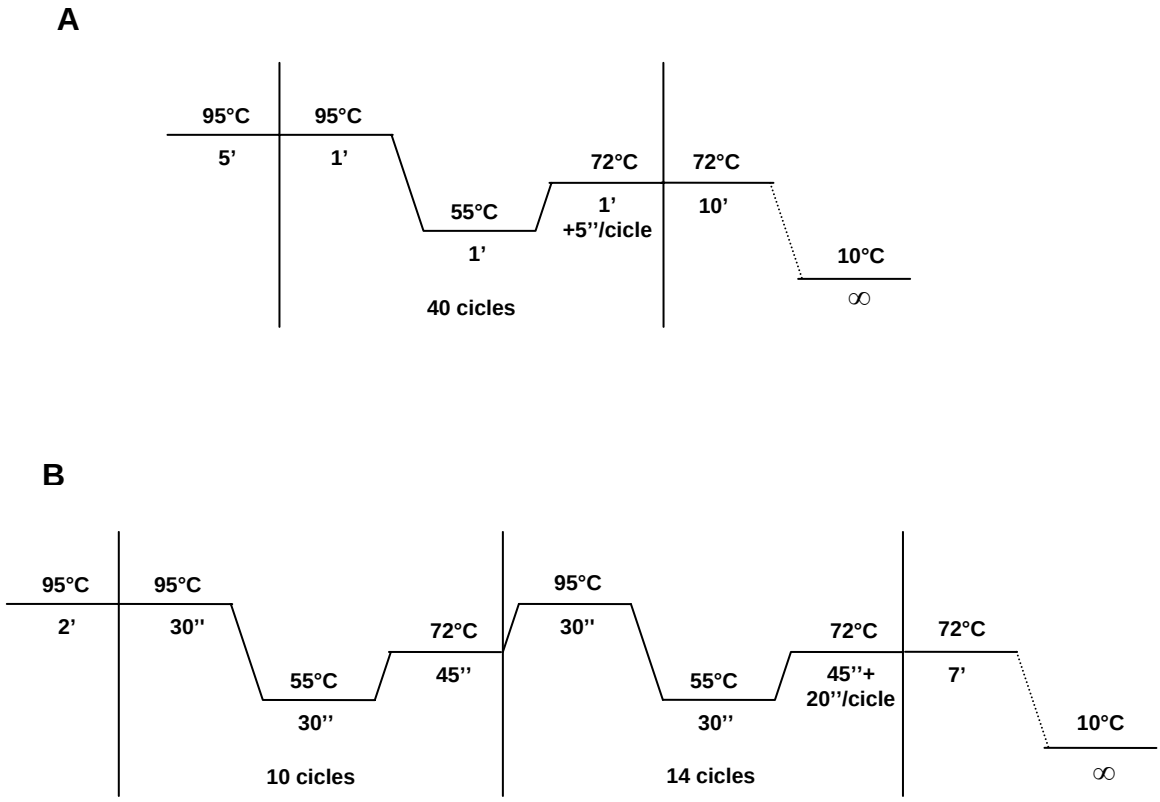


Figure 1 Scheme of PCR1 without primer (A) and PCR2 (B) programs.

Error-prone PCR library construction. Mutazyme® DNA polymerase (GeneMorph PCR mutagenesis kit, Stratagene) is the enzyme used for random prone PCR. The desired mutation frequency can be achieved selecting the amount of cDNA used as template, as higher mutation frequencies are reached simply by lowering input DNA template concentration and, consequently, increasing the number of replication cycles.

Table 2 Predicted mutation frequencies vs. initial amount of target DNA required to produce low, medium, or high mutation frequencies.

Mutation frequency (mutations/kb)	Initial target amount
0-3 (low range)	10-100 ng
3-7 (medium range)	10 pg- 10 ng
7-16 (high range)	Double or triple PCR, 10-100 pg in each PCR

poxc cDNA was mutated with low mutation frequencies (0-3 mutations/kb) and *poxa1b* cDNA was mutated with low, medium (3-7 mut/kb) and high (7-16 mut/kb) mutation frequencies. The reaction mix (50 µl) contained cDNA template, primers (250 ng μl^{-1} each), Mutazyme buffer 1x, dNTP 200µM, 2.5 U of Mutazyme and milliQ water. POXA1b*fw* and POXA1b*rev* primers were used to amplify *poxa1b* cDNA and

POXC*fw* and POXA1b*rev* to amplify *poxc* cDNA (Table 1). Polymerase chain reactions were performed in according to the scheme above:

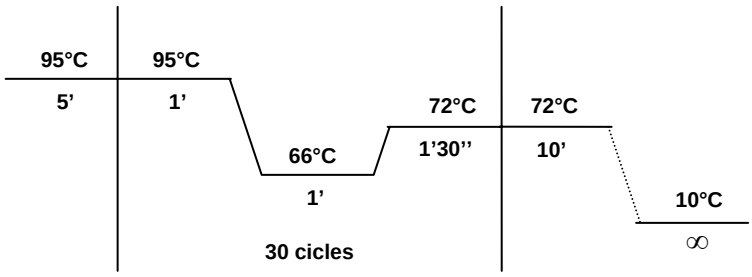


Figure 2 Scheme of PCR error prone program

All the polymerase chain reactions were performed in a thermal cycler PCR-sprint Hybaid (Hybaid Ltd., Ashford, UK).

Digestion with restriction enzymes. The PCR products, the cloning vectors and cDNAs were hydrolysed with opportune restriction enzymes (Promega). The reaction was performed using 5U of enzyme each µg of DNA incubating at restriction nuclease optimum temperature for 2 h and 30 min.

Ligation of foreign cDNA in a plasmid. The ligation was performed with ligase from T4 bacteriophage (Boehringer Mannheim) at 16°C over night. A maximum of 100 ng of plasmid was used for each reaction, and several molecular ratio plasmid: insert were tested (1:2, 1:3, 1:4), in a mix containing 1mM ATP, ligase buffer 1x and 1 U of enzyme.

Gel electrophoresis of DNA. The PCR products were separated and analysed on agarose gel 1% w/v. The electrophoresis was conducted in TAE (40 mM tris-acetate, 1mM EDTA pH 8) at 100 V. To visualize DNA 0.5 µg ml^{-1} ethidium bromide was added to the agarose gel. This substance contains a planar group that intercalates between the stacked bases of DNA. DNA fragments were visualized with ultraviolet light (365 nm).

PCR products and linear plasmid were extracted and purified from agarose gel with the kit Quiaex from Quiagen as specified by the manufacturer.

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}).

K_M values were estimated using the software GraphPad Prism on a wide range of substrate concentrations.

Effect of pH and temperature. The effect of pH on laccase activity towards 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-posphate buffer adjusted to pH 7.0

Stability at pH and temperature. Phenol oxidase stability at 60°C was measured in 50 mM 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.

Results

Selection of suitable host organism.

Both *K. lactis* and *S. cerevisiae* were used as expression systems for *P. ostreatus* laccases heterologous production, although *K. lactis* is much more effective than *S. cerevisiae* (Piscitelli et al., 2005). Unfortunately, in contrast to *S. cerevisiae*, the budding yeast *K. lactis* shows, as also described by Zeeman and Steensma, 2003, 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. On this basis *S. cerevisiae* was preferred to *K. lactis* as host for expressing laccase mutated cDNA libraries.

Development of a high-throughput screening procedure

Mutants able to produce laccase activity higher than that of wild type were looked for.

Qualitative screening

A suitable method of activity detection was required to identify clones able to produce more active enzymes. Preliminary experiments were performed on *S. cerevisiae* clones carrying plasmids containing wild-type or mutated cDNAs grown on polycarbonate filter laid on a PVDF filter (Fig.1).

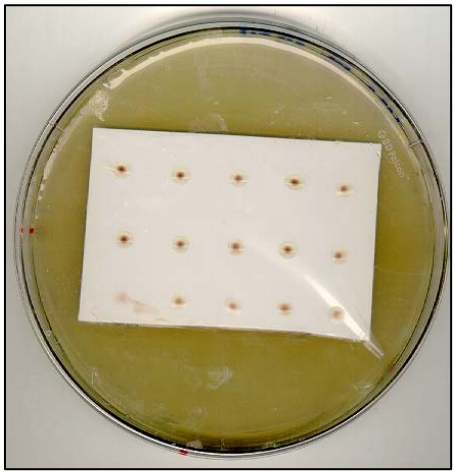


Figure 1 *S. cerevisiae* clones grown on filters laid on solid medium.

After four days, PVDF filters, with immobilized proteins, were used to detect laccase activity towards ABTS. PVDF filters were incubated on plate containing buffer and substrate gelled in agar, after 1÷2 hours halos produced by the oxidation of ABTS could be detected (Fig.2).

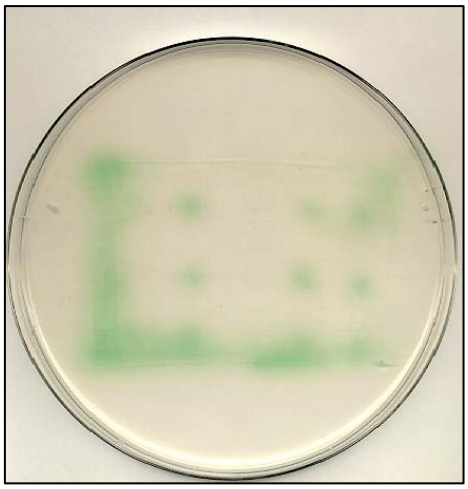


Figure 2 Halos produced by ABTS oxidation after incubation with PVDF filter.

This technique allows to separate micro organism growth phase and laccase activity revelation step and is quite repeatable. Nevertheless it is too laborious and above all it does not allow to perform any quantitative evaluation.

Quantitative screening

Microscale liquid cultures were performed in 96 well microtiter plates and conditions to follow laccase activity production were set up (see Materials and Methods). The riprodicibility of this procedure was verified. Cellular density and laccase activity production were followed for three days, showing only slight (20%) variations (Fig.3).

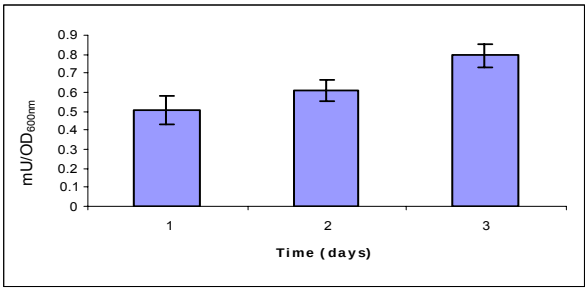


Figure 3 Variations associated with the screening procedure

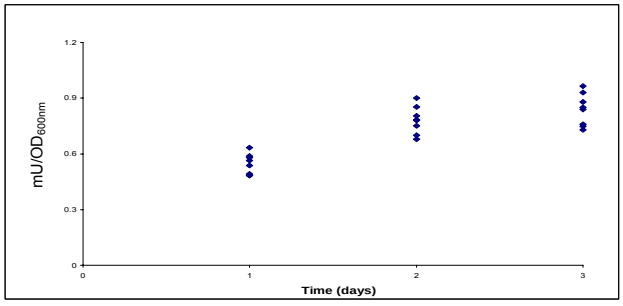


Figure 4 Specific activity production trend from different inocula.

Moreover we also verified that, using a clone expressing wild-type protein, different inocula, prepared starting from different values of cellular density, do not affect significantly laccase activity production trend (Fig.4).

Libraries construction and screening for higher laccase activity production

Genetic variation was introduced by error prone polymerase chain reactions on *poxc* and *poxa1b* cDNAs at different frequency of mutation. Libraries of "low range" (0÷3 mut/kbase), "medium range" (3÷7 mut/kbase) and "high range" mutants (over 7 mut/kbase) were generated by error-prone PCR using as template *poxa1b* cDNA; a library of "low range" mutants was generated by error-prone PCR using as template *poxc* cDNA. Genetic variants were cloned in expression vectors through homologous recombination and expressed in *S. cerevisiae*.

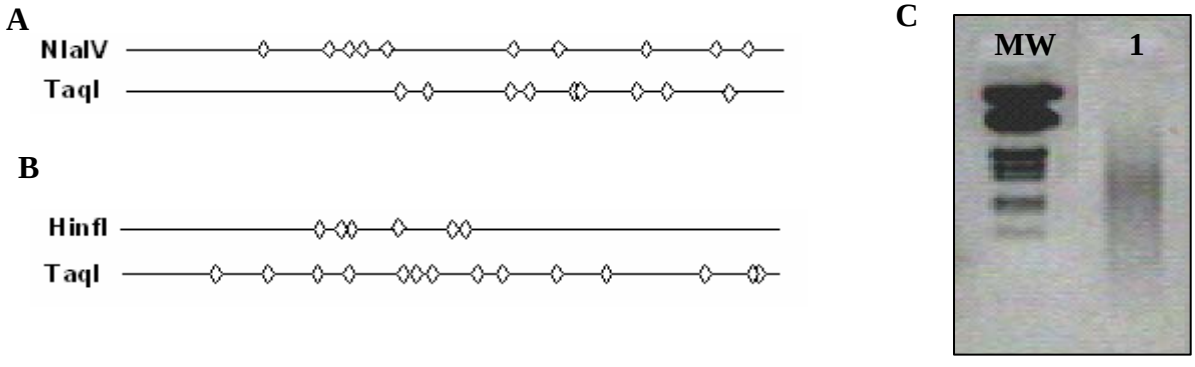
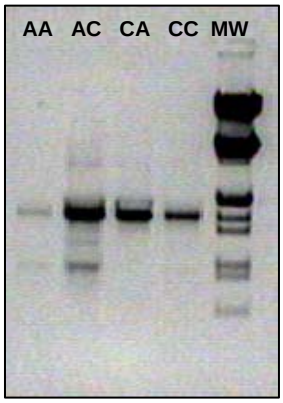


Figure 5 Restriction map of *poxc* (A) and *poxa1b* (B) for the enzymes used for fragmentation. C) Product of PCR self priming (lane 1) after cDNAs fragmentation.

Moreover a library from *poxc* and *poxa1b* cDNAs shuffling was prepared: cDNAs were digested for production of fragments with sizes of 40÷800 bp (Fig.5, A and B).



Fragmented genes were subjected to PCR without primer (PCR self priming, Fig.5, C). Aliquots of the products of the PCR self priming were used as template in a conventional PCR with four different combination of primers: AA) POXA1bfw and POXa1brev; AC) POXA1bfw and POXCrev; CA) POXCfw and POXA1Brev; CC) POXCfw and POXCrev (Fig. 6)

Figure 6 Product of the conventional PCR with different combination of primers.

Shuffled cDNAs were ligated in suitable expression vectors and expressed in *S. cerevisiae*.

All the mutants were screened in microtiter plate using ABTS as substrate. The obtained libraries and their curtailment are summarized in table 1.

S. cerevisiae clones carrying mutated or wild-type *poxc* plasmids, showed laccase activity production too low to compare productions by different clones with sufficient accuracy.

Analysis of mutants from DNA shuffling experiments (Fig.7) showed that only a low percentage (40%) of them expresses laccase activity, at low level.

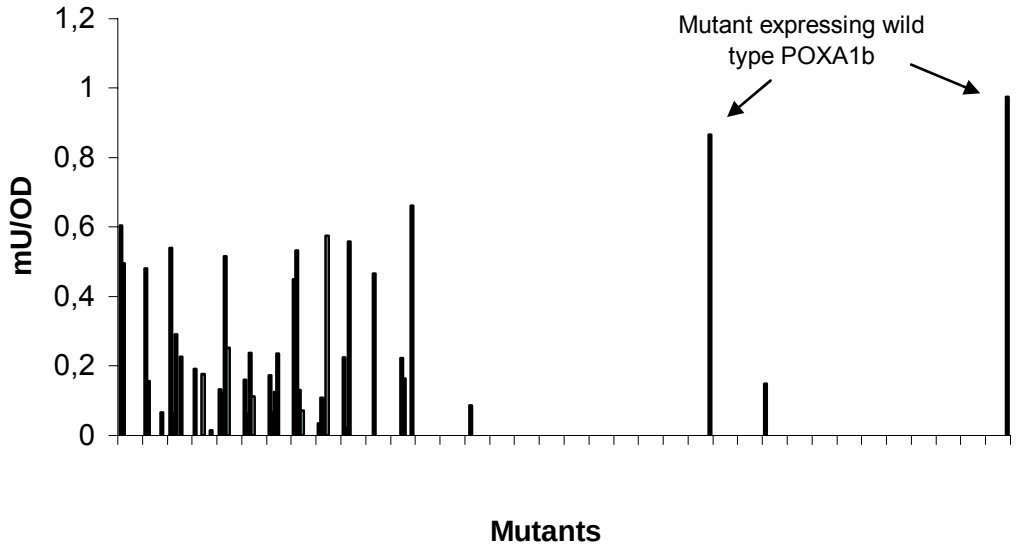


Figure 7 Analysis of the 300 mutants from DNA shuffling experiments

Figures 8 shows the results of the screening of mutants from *poxa1b.* Almost all the proteins obtained from Error Prone PCR, using *poxa1b* cDNA, with low frequency of mutation are active, some of them displaying also higher activity respect to the wild type protein.

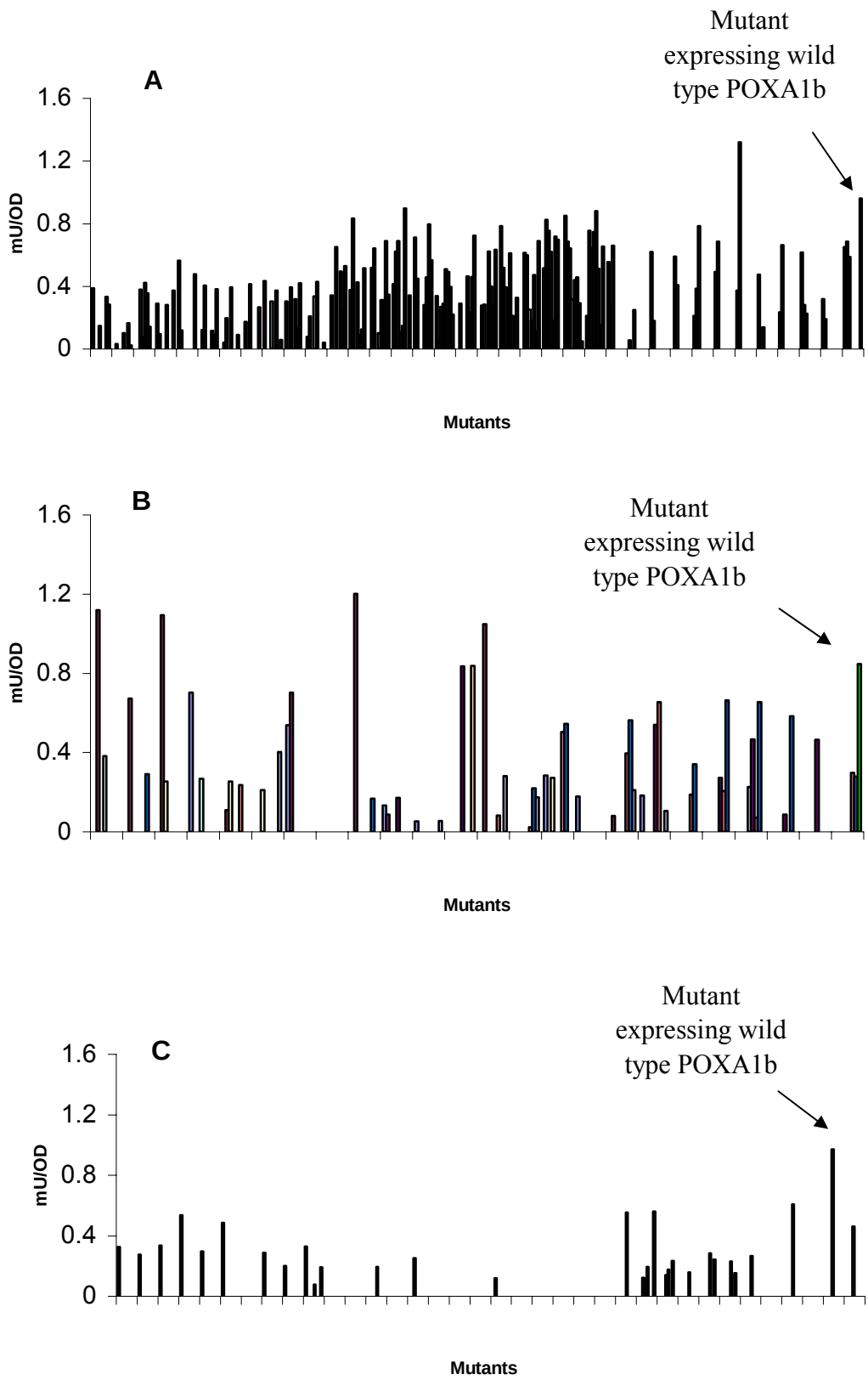


Figure 8 Analysis of the mutants obtained using *poxa1b* as template by error prone PCR at low (A), medium (B) and high (C) frequency of mutation.

It is worth to note that the number of mutants unable to produce laccase activity proportionally increases with the frequency of mutation (70% and 90% for medium and high frequency of mutation, respectively).

To further verify the results from the first screening, activity of the 38 positive clones were analysed for three days in multiwell plates. As a second screening the 38 clones exhibiting the highest laccase specific production were further analysed in microtiter plate (Fig. 9).

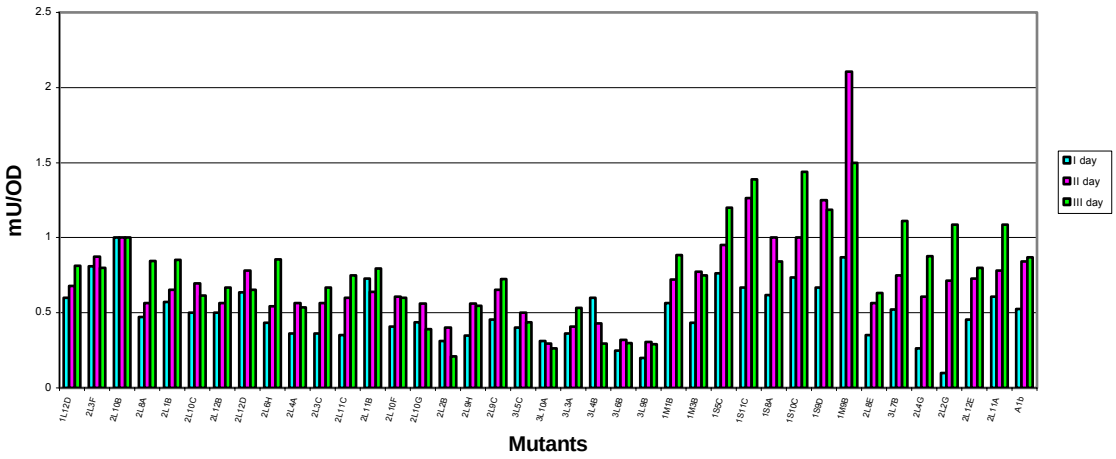


Figure 9 Analysis of the mutants selected during the first screening step

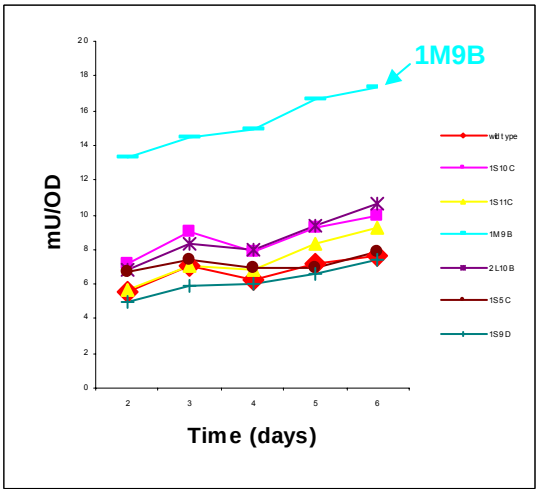


Figure 10 Specific activity production in shaken flasks of the selected mutants compared to that of the wild type protein expressing yeast.

Seven of the thirty-eight mutants from the first screening were further selected. Figure 10 reports the last step of the screening procedure, where the selected seven mutants were analysed in shaken flasks. One clone, 1M9B, was selected on the basis of its ability to over express laccase activity (1.6 fold respect to the yeast expressing wild type protein).

Table 1 Summary of obtained mutants and their curtailment

Libraries from first 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 *(poxa1b)*	200 mutants (frequency: 0÷3 mut/ kbase)	29 mutants	1 mutant	0
	200 mutants (frequency: 3÷7 mut/ kbase)	3 mutants	1 mutant	**1 mutant: 1M9B**
	300 mutants (frequency: more than 7 mut/ kbase)	0	0	0
EP-PCR *(poxc)*	100 mutants Frequency: (0÷3 mut/ kbase)	0	0	0
DNA shuffling (*poxa1b* plus *poxc*)	300 mutants	6 mutants	5 mutants	0

The cDNA from 1M9B mutant was used as template for producing a new collection of genetic variants: 500 “low range” and 700 “medium range” mutants were generated by error-prone PCR. The new libraries were screened with the same procedure (data summarized in table 2).

Table 2 Summary and curtailment of mutants obtained in the second generation

Libraries of second generation		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)	17 mutants	5 mutants	**1 mutant: 1L2B**
	700 mutants (frequency: 3÷7 mut/ kbase)	12 mutants	2 mutants	**2 mutants: 1M10B and 3M7C**

As a result of the screening of the second generation library of mutants, the clones, 1L2B, 1M10B and 3M7C, showing activity higher than 1M9B were selected (Fig 11).

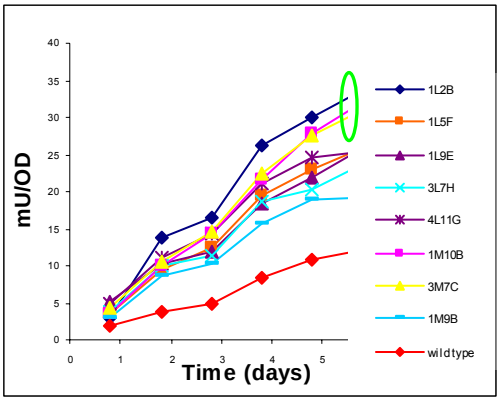


Figure 11 Specific activity production in shaken flasks of the mutants selected screening the second library, the clone isolated screening the first library (1M9B), and the clone expressing wild type protein (recombinant POXA1b).

Purification of wild-type and mutant laccases

A protocol for the enrichment of the recombinant protein was developed. Optical density and laccase activity measurements were performed during the time course of the preparative culture. Mutants and wild type expressing yeast growth curves do not differ significantly. Therefore the higher specific activity observed for the mutants (Fig 12) could be attributed to more effective enzymes production. Culture broth collected at the 6th growth day were used for laccase purification. The developed protocol provided for ultrafiltration, ammonium sulphate selective precipitation and size exclusion chromatography. Purification of the recombinant proteins are summarized in table 3.

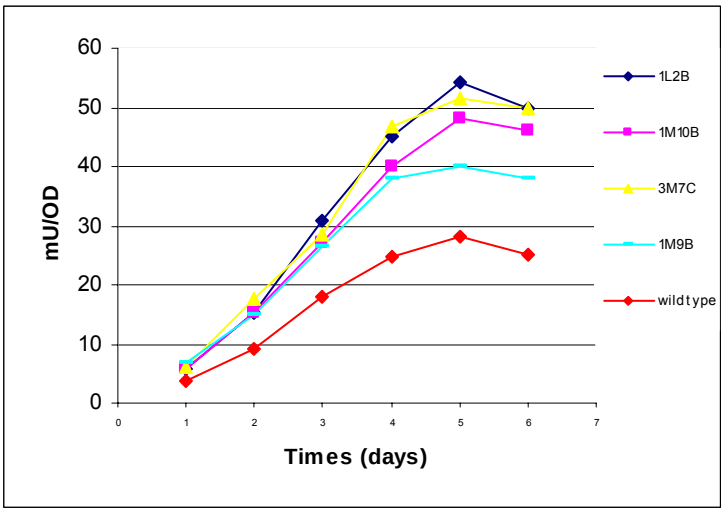


Figure 12 Laccase specific activity production in preparative cultures.

Table 3 Wild type and mutant proteins purification from *S. cerevisiae* cultures

	Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (*U*%)	*Recovery (mg%)*
Wild type	Broth	74.4	2.51	30	100	100
	$(NH4)_2SO_4$ * and SEC	52.2	0.29	183	70	12
1M9B	Broth	106.7	2.36	45	100	100
	$(NH4)_2SO_4$ * and SEC	67.7	0.22	325	63	9
1L2B	Broth	136.8	2,74	50	100	100
	$(NH4)_2SO_4$ * and SEC	96.5	0,23	420	70	8
1M10B	Broth	140	2.54	55	100	100
	$(NH4)_2SO_4$ * and SEC	88.4	0.21	421	63	8
3M7C	Broth	147.7	2.40	61	100	100
	$(NH4)_2SO_4$ * and SEC	79.5	0.18	454	54	7.5

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

Characterization of wild-type and mutant laccases

Kinetic and catalytic parameters determination

Some kinetic and catalytic parameters for three different substrates (ABTS at pH3, DMP at pH 5 and SGZ at pH 6) were determined and reported in Table 4.

Table 4 Kinetic and catalytic parameters of wild type and mutant proteins

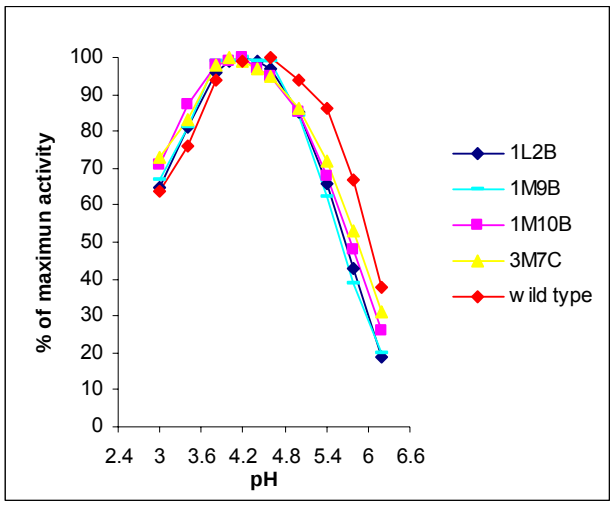
	SUBSTRATES								
Laccases	ABTS			DMP			SGZ		
	K_M (mM)	Specific activity (U/mg)	Catalytic efficiency (U/mg*mM)	K_M (mM)	Specific activity (U/mg)	Catalytic efficiency (U/mg*mM)	K_M (mM)	Specific activity (U/mg)	Catalytic efficiency (U/mg*mM)
wt	0,085±0.007	183±1	2153±15	0,54±0.02	187±1	346±7	0,048±0.006	23.2±0.8	483±2
1M9B	0,067±0.005	315±1	4701±23	0,36±0.02	168±1	467±9	0,038±0.007	9,3±0.5	245±1
1L2B	0,107±0.009	420±2	3925±71	0,31±0.01	295±2	952±19	0,025±0.004	24,3±0.5	972±2
1M10B	0,100±0.009	421±2	4210±76	0,35±0.01	281±1	803±8	0,040±0.006	24,3±0.6	607±2
3M7C	0,077±0.009	454±2	5896±106	0,24±0.01	337±2	1404±28	0,055±0.011	26,9±0.9	489±5

A common trend could be observed when ABTS or DMP were used as substrate. Proteins show increasing specific activities passing from wild type to the mutant selected screening the first library and a further increase was observed for the mutants selected screening the second library. The best identified mutant, 3M7C, show a specific activity 2.5 fold higher than wild type protein towards ABTS and 1.8 fold towards DMP.

None increase in the specific activity towards SGZ is observed.

Only slight differences were observed in Michaelis constant (K_M) values of mutants and wild type protein towards ABTS, DMP and SGZ.

Effect of pH on laccase activity



The effect of pH on the activity of laccases towards DMP was studied in the pH range 3,0÷6,2. A bell-shaped profile of the activity as a function of pH for all the laccases tested was observed. The wild type protein and its mutants show maximum activity at pH value of ~4.2. Slight differences among wt and mutants were observed only at pH above 4.4.

Figure 13 Effect of pH on the activity of wild type and mutated laccases.

Effect of Temperature on laccase activity

When laccase activity was studied as a function of the temperature all the proteins, including wild type, turned out to be almost fully active in the temperature range 40-70°C with maximum activity at temperatures from 50°C to 60°C.

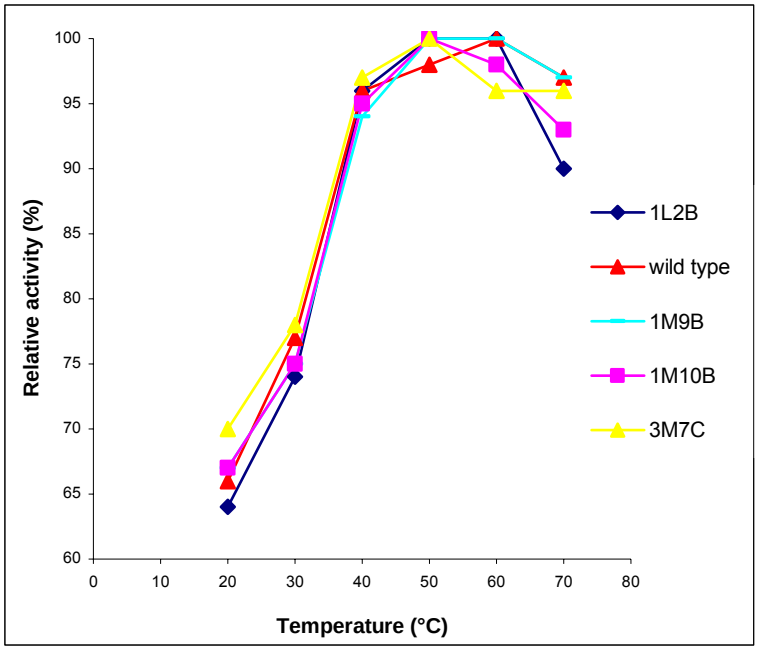


Figure 14 Effect of temperature on the activity of wild type and mutated laccases.

Stability at high temperature

The stability of the enzymes with respect to temperature at neutral pH was studied. The enzymes appeared to be reasonably stable with respect to temperature. All the mutants, except 1M9B, at 60°C showed similar or slightly higher half-life ($t_{1/2}$) respect to wild type recombinant protein (Table 5 and Fig 15).

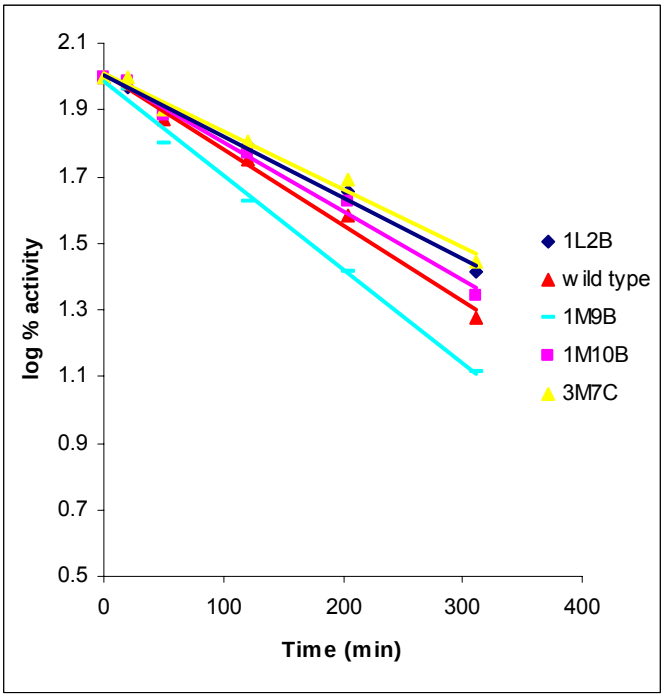


Figure 15 Stability of wild type and mutated laccases at 60°C

Table 5 Proteins half life values at 60°C

Laccases	$t_{1/2}$(hours)
wild type	2.2
1M9B	1.7
1L2B	2.8
1M10B	2.5
3M7C	3.1

Stability at pH

The stability of the laccases with respect to pH values and different buffers at room temperature was also studied. 1M9B is the less stable mutant, in all the examined conditions. The other mutants show $t_{1/2}$ values (Table 6 and Fig 16) similar or higher than that of wild type. At pH5 the mutant 3M7C is more stable (~2 fold) than the wt protein. At alkaline pH one mutant, 1M10B, displays higher stability (~1.5 fold) compared with that of wt protein.

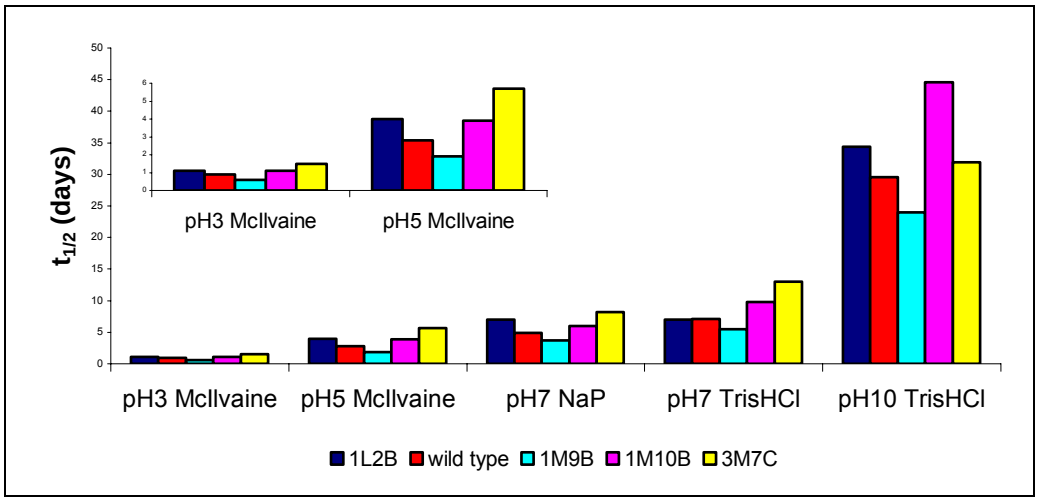


Figure 16 Stability of wild type and mutated laccases at different pH values

Table 6 Proteins half life values at different pH values

	$t_{1/2}$ (Days)					
Laccases	pH3, McIlvaine buffer	pH5, McIlvaine buffer	pH7, McIlvaine buffer	pH7, NaP buffer	pH7, Tris-HCl buffer	pH10, Tris-HCl
Wild type	0.9	2.8	7.6	4.9	7.1	29.6
1M9B	0.6	1.9	3.6	3.7	5.5	24.0
1L2B	1.1	4	6.3	7.0	7.0	34.4
1M10B	1.1	3.9	8.6	6.0	9.8	44.6
3M7C	1.5	5.7	n.d.	8.2	13	31.9

Structural characterization

Nucleotide sequences of the mutants 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

Table 7 Mutations found in mutants sequences

Mutants	Codon substitution*	Aminoacid substitution
1M9B	CTT (11%)→TTT (53%) ACA (26%)→ACG (12%)	L112F conservative
1L2B	CTT (11%)→TTT (53%) ACA (26%)→ACG (12%) AAC (46%)→TAC (50%) AAC (46%)→AAG (49%) GTT (44%)→ATT (50%)	L112F conservative N248Y N261K V350I
1M10B	CTT (11%)→TTT (53%) ACA (26%)→ACG (12%) AAG (49%)→CAG (26%) AAG (49%)→AAT (54%) GGA (15%)→GGT (61%)	L112F conservative K37Q K51N conservative
3M7C	CTT (11%)→TTT (53%) ACA (26%)→ACG (12%) ATC (30%)→ATT (50%) CCA (49%)→ACA (26%)	L112F conservative conservative P494T

*Percentage of codon usage is reported between brackets.

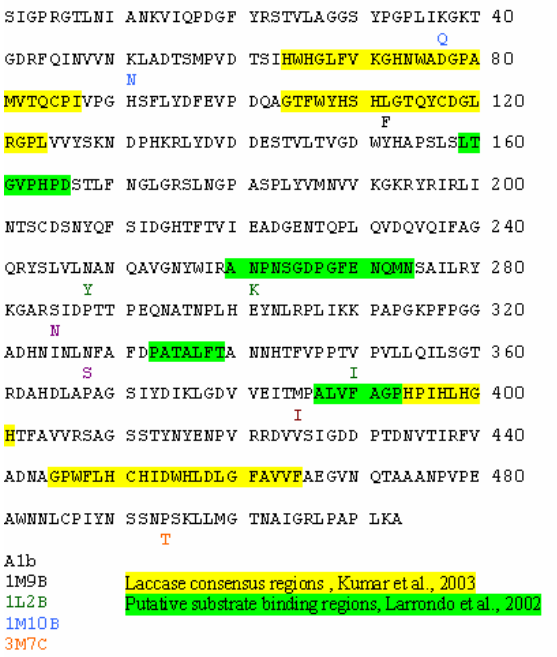


Figure 17 Primary structure of POXA1b wild type and mutated residues in isolated mutants.

Only the mutation L112F, common to all mutants, is located in a laccase consensus region, named L2 by Kumar et al, 2003 (Fig 17).

To locate the substitutions in protein tertiary structure, *in silico* mutants were constructed replacing the original amino acids with the new residues in POXA1b 3D-model.

Discussion

A library of new phenol-oxidases was constructed introducing mutations at different frequency in *poxa1b* and poxc cDNAs and shuffling *poxa1b* and *poxc* cDNAs. Mutated cDNAs were expressed in *S. cerevisiae*. 2300 clones were screened and four variants exhibiting activity higher than that of wild type were isolated and fully characterized.

The developed quantitative screening procedure to pick out more active mutants is endowed with three qualities: 1) it is rapid, to allow the analysis of a large number of mutants; 2) it is reliable, to avoid false data; 3) it is sensible when ABTS is used as substrate. The sensibility is of primary importance because of the chosen strategy of introducing a small number of mutations, bring about an evolution proceeding by short steps, so it is necessary to pick slight differences among clones. For this reason, in the first steps of screening, only ABTS was used as substrate.

A range of strategies for the introduction of diversity into the starting genes are generally available, and these can be broadly divided into two classes (i) non-recombinative and (ii) recombinative methods. We used Errore Prone PCR (EP-PCR), the most frequently used non recombinative method, and DNA shuffling, the most traditional among recombinative methods. It was worthy to note that, when EP-PCR was used, increasing the frequency of mutation the number of mutants unable to produce laccase activity drastically raises. This is consistent with the theory generally accepted that using a low mutation rate the probability of discovering beneficial mutations increases, since the majority of random mutations are either neutral or deleterious (Valetti and Gilardi, 2004). Therefore, after the first round of EP-PCR we made the choice of accumulating beneficial mutations using the best mutant from the first round (1M9B) as template for a second round of EP-PCR. As a fact three new positive clones were identified in the library obtained after the second round of EP-PCR.

As for DNA shuffling, only a low percentage (40%) of laccase expressing clones were obteined; this datum confirms the idea that, despite the most significant changes in enzyme function can be created using DNA shuffling (Williams et al., 2004), this methodology brings with it the frequent risk of damaging essential structural components during “gene reconstruction”, producing mainly cDNAs coding for inactive proteins. As a fact, no positive variant was obtained from DNA shuffling.

The four selected mutants, one (1M9B) from the screening of the library obtained after the first round of EP-PCR and three (1L2B, 1M10B and 3M7C) from the screening of the library obtained after the second round of EP-PCR, were partially purified, fully characterised and compared with the wild type protein (recombinant POXA1b).

Kinetic and catalytic parameters were determined using three substrates: 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS); 2,6-dimethoxy phenol (DMP); syringaldazine (SGZ). All the proteins show a specific activities towards ABTS higher then that of wt protein, thus confirming the reliability of the screening procedure. Three of the four enzymes show 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* (Giardina et al., 1999; Palmieri et al., 2003). The slightly lower K_M values observed for some of the proteins towards DMP

and SGZ suggests that some of mutations cause an alteration of the substrate-enzyme complex formation. As a fact, sequences alignments indicates thus only 1L2B mutant presents an aminoacidic substitution located in a potentially substrate binding loop. Thus, difficultly predictable structural arrangements probably occur near the substrate site as consequences of substitutions of residues distant from active site.

As for the effect of pH on laccase activity, all the proteins show a bell-shaped profile exhibiting maximum activity at pH value of 4.2, using DMP as substrate. All laccase isoenzymes work generally at acidic pH and the activity decreases when the pH increases. As a fact, the four proteins seem to feel more the effect of pH increase respect to wild type protein. This difference is imputable to L112F substitution, that is common to all the mutated proteins.

Stability of the new laccases with respect to pH and temperature has been studied and compared with that of wild type. As a fact, whereas 1L2B, 1M10B and 3M7C mutants display similar or better stability if compared with that of wild type, the 1M9B mutant is sensibly less stable in all conditions analyzed. Thus, the single substitution L112F can be considered responsible of improvement in specific activity and decrease in stability. The mutation in position 112 occurs in a consensus region where a Leucine residue is conserved. A Phe→Leu substitution is found only in the laccases codified by a gene from *Coriolopsis gallica* and by eight genes from *Coprinopsis cinerea.* On the other hand, the Phenilalanine in position 112 seems to be a conserved residue in all laccases from ascomycetes.

Fig 18 Sequence aligment of laccases from Ascomycetes *(Melanocarpus albomyces, Podospora anserina, Neurospora crassa and Colletotrichum lagenarium).*

Position 112 is located in the channel through which the solvent has access to the oxygen-reducing T3 site. Any substitution of the residue in this position could affect the stability of the coordination to copper ion and influence the stability of the enzyme.

Phenilalanine residue, characterised by a lateral chain larger than that of Leucine residue, could be able to interact with residues located in more distant positions. For example Phenilalanine 112 could interact with leucine residue in position 457, located in the α-helix carrying Histidine456 coordinating the type-1 copper. This interaction could cause a movement of this helical segment which could result in an elongation of the Cu1-N (His456) bond at the T1 site, rendering the copper more electron deficient. A similar movement was hypothesized in *Trametes versicolor* laccase, upon formation of a hydrogen bond between D460 and S113, to explain the high redox potential (Piontek et al., 2002).

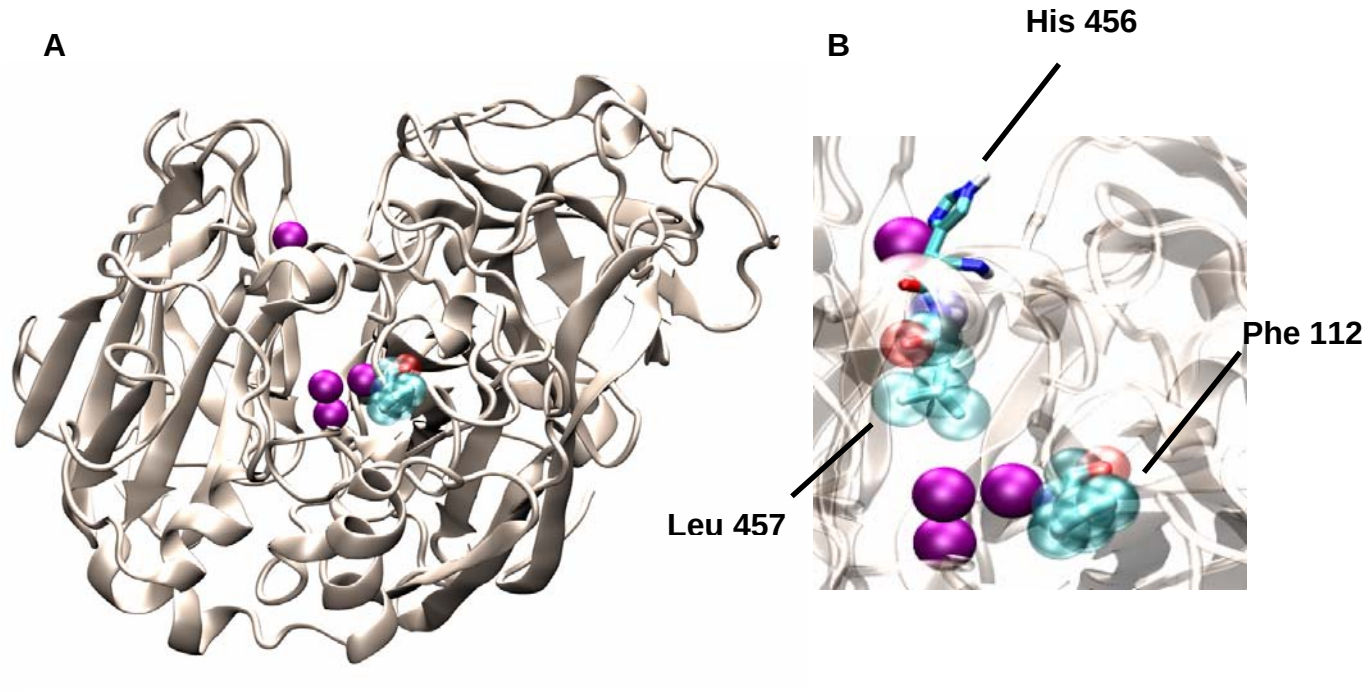


Figure 19 (A) Ribbon rappresentation of 1M9B model. Copper ions and mutated residue are dispayed in VDW rendering styles coloured in purple and cyano respectively. (B) Close up on T3 channel.

As for the three mutants generated from 1M9B (1L2B, 1M10B and 3M7C) besides the increased specific activity, an increase in stability was also observed.
1L2B mutant presents three substitutions (N248Y, N261K, V350I) besides L112F. Each of the three substitutions, independently or in a synergistic way, can be supposed to compensate for destabilizing effect of the L112F substitution.

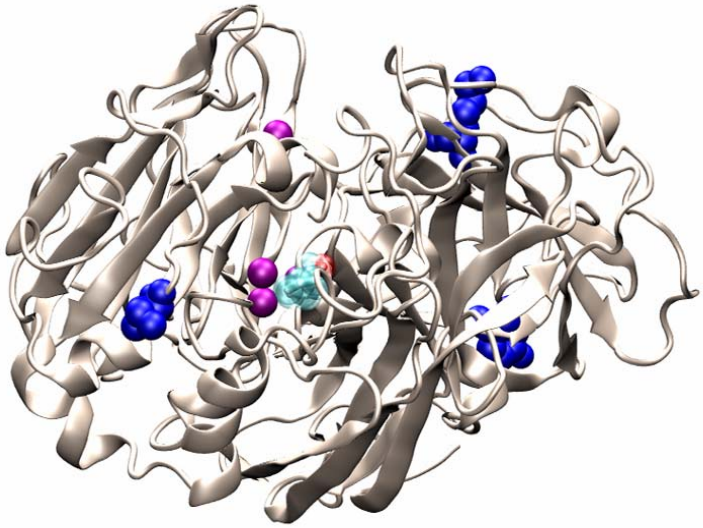


Figure 20 Ribbon rappresentation of 1L2B model. Copper ions and mutated residues are dispayed in VDW rendering styles. Copper ions are coluored in purple, Phe 112 is coloured in cyano and the other three mutated residues are coloured in blue.

The position 248 is quite variable: in the alignment of thirty laccases from basidiomyces different amino acids occur in this position instead of Asparagine (Glutamic acid, Threonine, Aspartic acid, Histidine and Lysine) but never Tyrosine, suggesting that this residue in this position is suppressed by natural evolution as deleterious. The other two mutated residues, Asparagine in position 261 and Valine in position 350, are very conserved: no laccase shows a lysine residue in 261 or a Isoleucine residue in 350. Moreover the position 261 is located in a loop potentially interacting with the substrate, according to Larrondo et al.,2003, that could explain the higher affinity of this mutant towards SGZ. However, no clear hypothesis can be foreseen on the role played by these mutations.

1M10B mutant presents two substitutions (K37Q, K51N) besides L112F. In laccase sequences, positions 37 and 51 are generally conserved and occupied by ammidic residues. The two found substitutions restore conserved residues in the protein sequence, suggesting that, during the process for producing better enzymes, the relatively fast directed evolution can get the same result of the slower natural evolution. Position 37 is located near the N-terminus loop carrying residues (Ser-Ile-Gly-Pro-Arg) with which a Glutamine residue can establish different interactions respect to Lysine residue, stabilizing the protein.

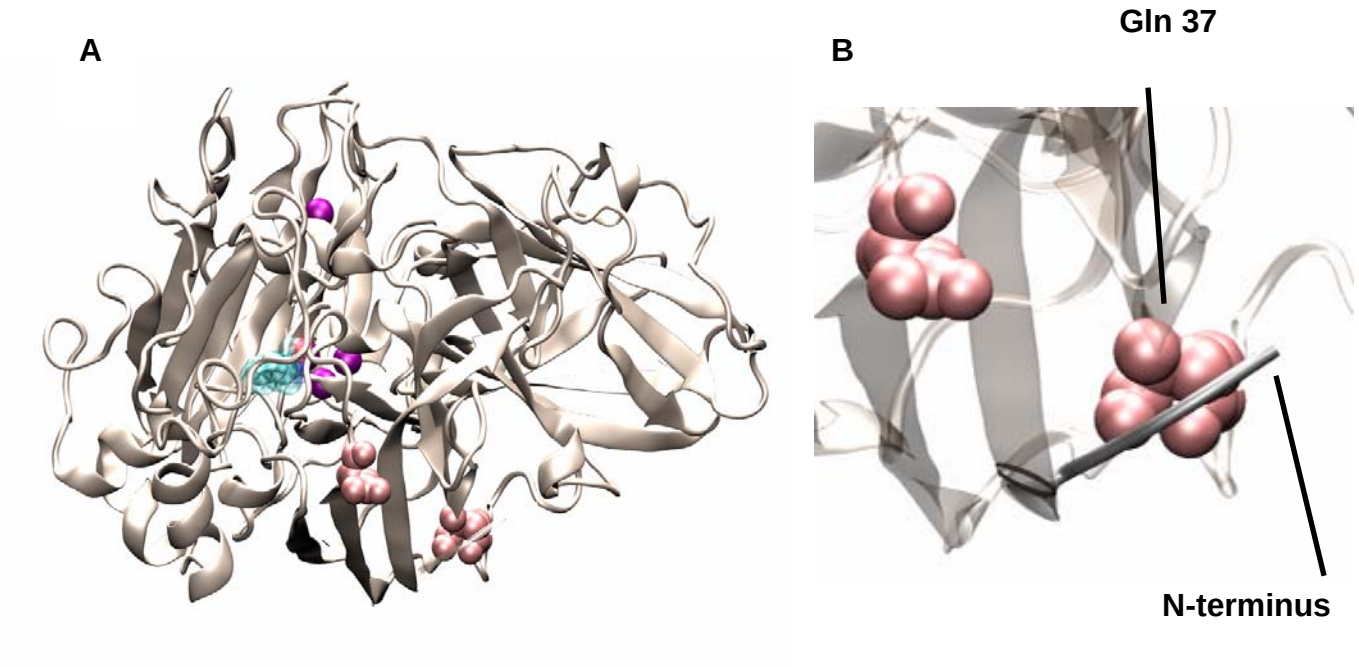


Figure 21 (A) Ribbon rappresentation of 1M10B model. Copper ions and mutated residues are dispayed in VDW rendering styles. Copper ions are coluored in purple, Phe 112 is coloured in cyano and the other two mutated residues are coloured in pink.

The last mutant analysed, 3M7C, presents only one substitution (P494T) besides L112F. The Proline494 is a quite conserved residue in a not very conserved region: the C-terminus. The mutation introduces, instead of Pro, a Threonine residue that is

a peculiarity among the aligned laccase sequences. The presence of Threonine instead of Proline residue in position 494 has to be responsible both of the increased stability and activity of this mutant. The position 494, is located in an variable and mobile loop, the C-terminus, that, in accordance with modelling data, forms a plug on the channel through which the solvent has access to type-3 copper sites, moreover the position 494 corresponds to the and of an α-helix, unable to grow up when the Proline residue is present. Hakulinen et al. (2002) proposed a probable conformational change in the C-terminus to open the tunnel for the entrance of an oxygen molecule and for the exit of a water molecule in laccase from the Ascomycete *Melanocarpus albomyces*; moreover Gelo-Pujic et al. (1999), have noticed that the redox potential of the type-1 Cu of a truncated laccase from *Trametes versicolor*, produced in *Pichia pastoris*, changed when 11 amino acids at the C-terminus were replaced with a single Cysteine residue. As a fact, in mutant 3M7C, specific activity and stability are improved changing a residue at the C-terminal sequence; this result is in line with that supposed by other authors about the capability of C-terminal amino acids to affect the function of all fungal laccases.

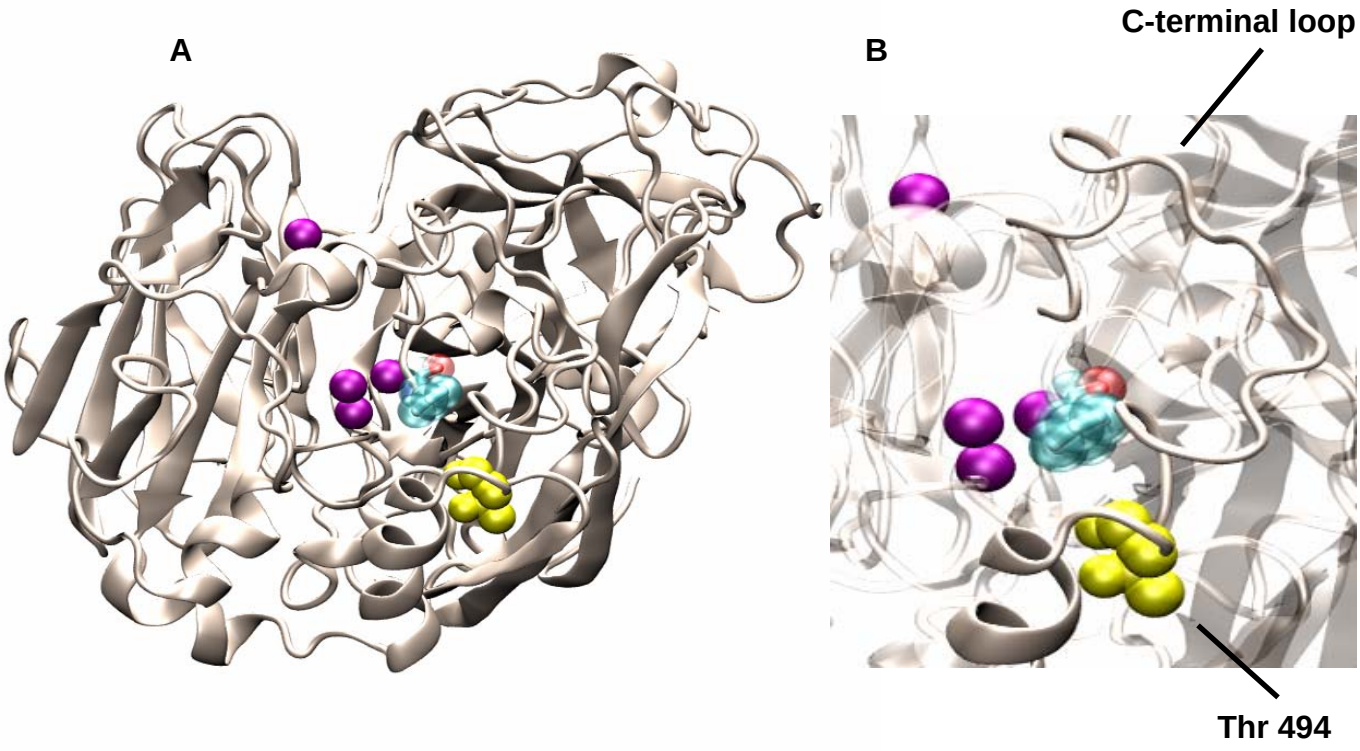


Figure 22 (A) Ribbon rappresentation of 3M7C model. Copper ions and mutated residues are dispayed in VDW rendering styles. Copper ions are coluored in purple, Phe 112 is coloured in cyano and the other mutated residue is coloured in yellow. (B) Close up on C-terminus.

In conclusion, adaptive molecular evolution is a fundamental biological process, yet it remains poorly understood, and directed evolution, based on the principles of natural evolution, is presently a lucky art rather than a science. Moreover laccases are proteins with very conserved, functionally essential, wide regions, thus, not many positions can be mutated without loosing activity. Present data report the construction of a library of 2300 mutated laccases and the identification and

characterization of four “new” laccases more active and more stable than the protein used as staring point. 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.

4. References

Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A & Gubitz GM. 2000. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. *Appl Environ Microbiol*. **66**:3357–3362.

Ahn M Y, Dec J, Kim JE & Bollag JM. 2002. Treatment of 2,4-dichlorophenol polluted soil with free and immobilized laccase. *J Environ Qual.* **31**:1509–1515.

Alcalde M, Bulter T, Zumarraga M, Garcia-Arellano H, Mencia M, Plou FJ, Ballesteros A. 2005 Screening mutant libraries of fungal laccases in the presence of organic solvents.*J Biomol Screen.* **10**(6):624-31.

Antorini M, Herpoel-Gimbert I, Choinowski T, Sigoillot JC, Asther M, Winterhalter K & Piontek K. 2002 Purification, crystallisation and X-ray diffraction study of fully functional laccases from two ligninolytic fungi. *Biochim Biophys Acta* **1594**: 109–114.

Argyropoulos DS. 2001. Oxidative Delignification Chemistry. ACS Symposium Series 785. American Chemical Society, Washington, DC.

Baiocco P, Barreca AM, Fabbrini M, Galli C & Gentili P. 2003. Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase-mediator systems. *Org Biomol Chem.* **1**:191–197.

Bajpai P. 1999. Application of enzymes in the pulp and paper industry. *Biotechnol Prog.* **15**(2):147-57.

Balakshin M, Capanema E, Chen CL, Gratzl J, Kirkman A & Gracz H. 2001. Biobleaching of pulp with dioxygen in the laccase-mediator system–reaction mechanisms for degradation of residual lignin. *J Mol Catal B-Enzym*. **13**:1–16.

Baldrian P. 2006. Fungal laccases - occurrence and properties. *FEMS Microbiol Rev.* **30**(2):215-42.

Baldrian P, Valaskova V, Merhautova V and Gabriel J. 2005. Degradation of lignocellulose by *Pleurotus ostreatus* in the presence of copper, manganese, lead and zinc. *Res Microbiol.* **156**, 670-6.

Bauer CG, Kuehn A, Gajovic N, Skorobogatko O, Holt PJ, Bruce NC, Makower A, Lowe CR, Scheller FW. 1999. New enzyme sensors for morphine and codeine based on morphine dehydrogenase and laccase. *Fresenius' J. Anal. Chem*. **364**:179–183.

Bertrand G. 1896. Sur la presence simultanee de la laccase et de la tyrosinase dans le suc de quelques champignons. C. R. Hebd. *Seances Acad. Sci.* **123**: 463–465.

Bollag JM, Chu HL, Rao MA & Gianfreda L. 2003. Enzymatic oxidative transformation of chlorophenol mixtures. *J Environ Qual.* **32**:63–69.

Bollag JM. 1991. Enzymatic binding of pesticide degradation products to soil organic-matter and their possible release. *ACS Symp Ser*. **459**:122–132.

Bollag JM & Myers C. 1992. Detoxification of aquatic and terrestrial sites through binding of pollutants to humic substances. *Sci Total Environ*. **118**:357–366.

Bourbonnais R, Leech D & Paice MG. 1998. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochim Biophys Acta*. **1379**:381–390.

Bourbonnais R & Paice MG. 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett.* **267**:99–102.

Bourbonnais R & Paice MG. 1996. Enzymatic delignification of kraft pulp using laccase and a mediator. *Tappi J.* **79**:199–204.

Bourbonnais R, Paice MG, Freiermuth B, Bodie E & Borneman S. 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl Environ Microbiol*. **63**:4627–4632.

Bourbonnais R, Paice MG, Reid ID, Lanthier P, Yaguchi, M. 1995. Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2-azinobis(3-ethylbenzthiazoline-6-sulphonate) in Kraft lignin depolymerisation. *Appl. Environ. Microbiol*. **61:**1876–1880.

Bulter T, Alcalde M, Sieber V, Meinhold P, Schlachtbauer C, Arnold FH. 2003. Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. *Appl Environ Microbiol.* **69**(2):987-95. Erratum in: *Appl Environ Microbiol.* 2003. **69**(8):5037.

Call HP & Mucke I. 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediatorsystems (Lignozyms-process). *J Biotechnol*. **53**:163–202.

Camarero S, Ibarra D, Mart´ınez MJ & Mart´ınez AT. 2005. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl Environ Microbiol*. **71**:1775–1784.

Campos R, Kandelbauer A, Robra KH, Cavaco-Paulo A & Gubitz GM. 2001. Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii*. *J Biotechnol*. **89**:131–139.

Carunchio F, Crescenzi C, Girelli AM, Messina A, Tarola AM. 2001. Oxidation of ferulic acid by laccase: Identification of the products and inhibitory effects of some dipeptides. *Talanta* **55**:189–200.

Carvalho MEA, Monteiro MC & Sant'Anna GL. 1999. Laccase from *Trametes versicolor* - stability at temperature and alkaline conditions and its effect on biobleaching of hardwood kraft pulp. *Appl Biochem Biotechnol.* **77**:723–733.

Chen DM, Bastias BA, Taylor AFS & Cairney JWG. 2003. Identification of laccase-like genes in ectomycorrhizal basidiomycetes and transcriptional regulation by nitrogen in *Piloderma byssinum*. *New Phytol* **157**: 547–554.

Chivukula M & Renganathan V. 1995 Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. *Appl Environ Microbiol*. **61**:4374–4377.

Cho SJ, Park SJ, Lim JS, Rhee YH & Shin KS. 2002. Oxidation of polycyclic aromatic hydrocarbons by laccase of *Coriolus hirsutus*. *Biotechnol Lett*. **24**:1337–1340.

Clarke AR, Atkinson T and Holbrook JJ. 1989. From analysis to synthesis – new ligand-binding sites on the lactate dehydrogenase framework. *Trends Biochem. Sci.* **14:** 145–148.

Claus H. 2003. Laccases and their occurrence in prokaryotes. *Arch Microbiol.* **179**(3):145-50.

Collar C, Martinez JC, Andreu P, Armero E. 2000. Effect of enzyme associations on bread dough performance. A response surface study**.** *Food Sci Technol Int*, **6**:217-226.

Claus H, Faber G & Konig H. 2002. Redox-mediated decolorization of synthetic dyes by fungal laccases. *Appl Microbiol Biotechnol*. **59**:672–678.

Coco WM, Levinson WE, Crist MJ, Hektor HJ, Darzins A, Pienkos PT, Squires CH, Monticello DJ. 2001. DNA shuffling method for generating highly recombined genes and evolved enzymes.*Nat Biotechnol*. **19**(4):354-9.

Collins PJ, Kotterman MJJ, Field JA & Dobson ADW. 1996. Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versiocolor*. *Appl Environ Microbiol*. **62**:4563–4567.

Curir P, Thurston CF, Daquila F, Pasini C & Marchesini A. 1997. Characterization of a laccase secreted by *Armillaria mellea* pathogenic for Genista. *Plant Physiol Biochem* **35**: 147–153.

D'Annibale A, Stazi SR, Vinciguerra V & Sermanni GG. 2000. Oxirane-immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater. *J Biotechnol.* **77**:265–273.

Dawel G, Kastner M, Michels J, PoppitzW, G¨untherW& Fritsche W. 1997. Structure of a laccase-mediated product of coupling of 2,4-diamino-6-nitrotoluene to guaiacol, a model for coupling of 2,4,6-trinitrotoluene metabolites to a humic organic soil matrix. *Appl Environ Microbiol*. **63**:2560–2565.

Dec J & Bollag JM. 2000. Phenoloxidase-mediated interactions of phenols and anilines with humic materials. *J Environ Qual.* **29**:665–676.

Derbyshire KM, Salvo JJ and Grindley NDF. 1986. A simple and efficient procedure for saturation mutagenesis using mixed oligodeoxynucleotides. *Gene.* **46**:145–152.

Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ostergaard P, Schneider P, Yaver DS, Pedersen AH & Davies GJ. 1998. Crystal structure of the type-2 Cu depleted laccase from Coprinus cinereus at 2.2 angstrom resolution. *Nat Struct Biol* **5**:310–316.

Durão P, Bento I, Fernandes AT, Melo EP, Lindley PF and Martins LO. 2006. Perturbations of the T1 copper site in the CotA-laccase from *Bacillus subtilis*: Structural, Biochemical, Enzymatic and Stability Studies. *J. Biol. Inorg. Chem.* **11**:514-526

Edens WA, Goins TQ, Dooley D & Henson JM. 1999 Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. tritici. *Appl Environ Microbiol* **65**: 3071–3074.

Eggen T. 1999. Application of fungal substrate from commercial mushroom production – *Pleuorotus ostreatus* – for bioremediation of creosote contaminated soil. *Int Biodeter Biodegrad*. **44**:117–126.

Eggert C, Temp U, Dean JFD, Eriksson KEL. 1996. A fungal metabolite mediates degradation of non-lignin structures and synthetic lignin. *FEBS Lett.* **391**:144–148.

Enguita FJ, Marcal D, Martins LO, Grenha R, Henriques AO, Lindley PF & Carrondo MA. 2004. Substrate and dioxygen binding to the endospore coat laccase from *Bacillus subtilis*. *J Biol Chem.* **279**:23472–23476.

Enguita FJ, Martins LO, Henriques AO & Carrondo MA. 2003. Crystal structure of a bacterial endospore coat component – a laccase with enhanced thermostability properties. *J Biol Chem*. **278**:19416–19425.

Fabbrini M, Galli C & Gentili P. 2002. Comparing the catalytic efficiency of some mediators of laccase. *J Mol Catal B*. **16**:231–240.

Falcone C, Saliola M, Chen XJ, Frontali L and Fukuhara H. 1986. Analysis of a 1.6-micron circular plasmid from the yeast *Kluyveromyces drosophilarum*: structure and molecular dimorphism. *Plasmid.* **15:**248-252.

Garavaglia S, Cambria MT, Miglio M, Ragusa S, Lacobazzi V, Palmieri F, D'Ambrosio C, Scaloni A & Rizzi M. 2004. The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair. *J Mol Biol* **342**:1519–1531.

Gelo-Pujic M, Kim H, Butlin NG, and Palmore GTR. 1999. Electrochemical Studies of a Truncated Laccase Produced in *Pichia pastoris. Appl Environ Microb*. **65**(12):5515-5521

Giardina P, Palmieri G, Scaloni A, Fontanella B, Faraco V, Cennamo G and Sannia G. 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. *Biochem. J.* **341**, 655-663.

Giardina P, Cannio R, Martirani L, Marzullo L, Palmieri G and Sannia G. 1995. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol*. **61**:2408-2413.

Gray H B & Malmstrom BG. 1983. On the relationship between protein-forced ligand fields and the properties of blue-copper centers, *Comments Inorg. Chem.* **2**:203-209.

Gupta R, Beg QK, Khan S, Chauhan B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl Microbiol Biotechnol*. **60**(4):381-95.

Hakulinen N, Kiiskinen LL, Kruus K, Saloheimo M, Paananen A, Koivula A & Rouvinen J 2002. Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. *Nature Struct Biol* **9**: 601–605.

Hiraga K and Arnold FH. 2003. General method for sequence-independent site-directed chimeragenesis. *J Mol Biol.* **330**(2):287-96.

Ito, S. 1997. Alkaline cellulases from alkaliphilic Bacillus: enzymatic properties, genetics, and application to detergents. *Extremophiles* **1**(2):61-6

Johannes C, Majcherczyk A & H¨uttermann A. 1996 Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. *Appl Microbiol Biotechnol*. **46**:313–317.

Johannes C, Majcherczyk A & H¨uttermann A. 1998. Oxidation of acenaphthene and acenaphthylene by laccase of *Trametes versicolor* in a laccase-mediator system. *J Biotechnol.* **61**:151–156.

Jolivalt C, Brenon S, Caminade E, Mougin C & Pontie M. 2000. Immobilization of laccase from *Trametes versicolor* on a modified PVDF microfiltration membrane: characterization of the grafted support and application in removing a phenylurea pesticide in wastewater. *J Membr Sci*. **180**:103–113.

Kang KH, Dec J, Park H & Bollag JM. 2002. Transformation of the fungicide cyprodinil by a laccase of *Trametes villosa* in the presence of phenolic mediators and humic acid. *Water Res*. **36**:4907-4915.

Kashyap DR, Vohra PK, Chopra S, Tewari R. 2001. Applications of pectinases in the commercial sector: a review. *Bioresour Technol.* **77**(3):215-27.

Keum YS & Li QX. 2004. Copper dissociation as a mechanism of fungal laccase denaturation by humic acid. *Appl Microbiol Biotechnol*. **64**:588–592.

Kiiskinen LL and Saloheimo M. 2004. Molecular Cloning and Expression in *Saccharomyces cerevisiae* of a Laccase Gene from the Ascomycete *Melanocarpus albomyces. Appl. Environ Microb.* **70**:137-144.

Kilaru S, Hoegger PJ and Kües U. 2006. The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide into two distinct subfamilies. *Curr. Genet.* **50,** 45-60.

Kissi M, Mountadar M, Assobhei O, Gargiulo E, Palmieri G, Giardina P, Sannia G. 2001. Roles of two white-rot basidiomycete fungi in decolorisation and detoxification of olive mill waste water. *Appl Microbiol Biotechnol.* **57**(1-2):221-6.

Kottermann MJJ, Rietberg HJ, Hage A & Field JA. 1998. Polycyclic aromatic hydrocarbon oxidation by the white rot fungus *Bjerkandera sp*. strain BOS55 in the presence of nonionic surfactants. *Biotechnol Bioeng*. **57**:220–227.

Kramer KJ, Kanost MR, Hopkins TL, Jiang H, Zhu YC, Xu R, Kerwin JL, and Turecek F. 2001. Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron* **57**: 385-392.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage *T4*. *Nature*. **227**:680–685.

Larrondo LF, González B, Cullen D and Vicuña R. 2004. Characterization of a multicopper oxidase gene cluster in *Phanerochaete chrysosporium* and evidence of altered splicing of the *mco* transcripts. *Microbiology*. **150:**2775-2783.

Larsson S, Cassland P, Jonsson LJ. 2001. Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. *Appl. Environ. Microbiol*. **67**:1163–1170.

Lau KL, Tsang YY & Chiu SW. 2003. Use of spent mushroom compost to bioremediate PAH-contaminated samples. *Chemosphere*. **52**:1539–1546.

Law WM, Lau WN, Lo KL, Wai LM & Chiu SW. 2003. Removal of biocide pentachlorophenol in water system by the spent mushroom compost of *Pleurotus pulmonarius*. *Chemosphere.* **52**:1531–1537.

Leemhuis H, Rozeboom HJ, Wilbrink M, Euverink GJW, Dijkstra BW and Dijkhuizen L. 2003. Conversion of cyclodextrin glycosyltransferase into a starch hydrolase by directed evolution: the role of alanine 230 in acceptor subsite+1. *Biochemistry*. **42:** 7518–7526.

Leung DW, Chen and Geddel DV. 1989.*Technique.* **1**:11–15.

Lodato P, Alcaino J, Barahona S, Retamales P and Cifuentes V. 2003. Alternative Splicing of Transcripts from *crtI* and *crtYB* genes of *Xanthophyllomyces dendrorhous*. *Appl. Environ. Microbiol*. **69**:4676-4682.

Lucas MC, Jacobson JW and Giles NH. 1977. Characterization and in vitro translation of polyadenylated messenger ribonucleic acid from *Neurospora crassa*. *J. Bacteriol*. **130**:1192-1198.

Lund M, Eriksson M & Felby C. 2003. Reactivity of a fungal laccase towards lignin in softwood kraft pulp. *Holzforschung* 57:21–26.

Lutz S, Ostermeier M, Moore GL, Maranas CD, Benkovic SJ. 2001. Creating multiple-crossover DNA libraries independent of sequence identity. *Proc Natl Acad Sci U S A.* 98(20):11248-53.

Madzak C, Mimmi MC, Caminade E, Brault A, Baumberger S, Briozzo P, Mougin C, Jolivalt C. 2006. Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. *Protein Eng Des Sel.* **19**(2):77-84.

Majcherczyk A & Johannes C. 2000. Radical mediated indirect oxidation of a PEG-coupled polycyclic aromatic hydrocarbon (PAH) model compound by fungal laccase. *Biochim Biophys Acta*. 1474:157–162.

Majcherczyk A, Johannes C & H¨uttermann A. 1998. Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microb Technol*. 22:335–341.

Malstrom BG. 1997. in Multi-Copper oxidases (Messerschmidt A ed), pp. 167-200, World Scientific, Singapore.

Martirani L, Giardina P, Marzullo L and Sannia G. 1996. Reduction of phenol content and toxicity in olive oil mill waste waters with the ligninolytic fungus Pleurotus ostreatus. *Water Res.* **30**:1914:1918.

Matsumura I. and Ellington AD. 2001. In vitro evolution of beta-glucuronidase into a beta-galactosidase proceeds through non-specific intermediates. *J. Mol. Biol.* **305:**331–339.

Mayer AM and Staples RC. 2002. Laccases: new functions for an old enzyme. *Phytochemistry* **60:**551–565.

Mazzoni C, Saliola M and Falcone C. 1992. Ethanol-induced and glucose-insensitive alcohol dehydrogenase activity in the yeast *Kluyveromyces lactis*. *Mol. Microbiol.* **6:**2279-2286.

Min KL, Kim YH, Kim YW, Jung HS & Hah YC. 2001. Characterization of a novel laccase produced by the woodrotting fungus *Phellinus ribis*. *Arch Biochem Biophys* **392**:279–286.

McCoy M. 2000. Novozymes emerges. *Chem Eng News.* **19**:23-25.

Messerschmidt A. 1997.Multi-Copper Oxidases. *World Scientific*, Singapore.

Messerschmidt A, Steigemann W, Huber R, Lang G and Kroneck PMH. 1992. X-Ray Crystallographic Characterisation of Type-2-depleted Ascorbate Oxidase from Zucchini, *Eur. J. Biochem.* **209**:597-602.

Min KL, Kim YH, Kim YW, Jung HS & Hah YC. 2001. Characterization of a novel laccase produced by the woodrotting fungus *Phellinus ribis*. *Arch Biochem Biophys* **392**:279–286.

Molitoris HP & Esser K. 1970 The phenoloxidases of the ascomycete *Podospora anserine*. V. Properties of laccase I after further purification. *Arch Mikrobiol* **72**: 267–296.

Monteiro MC & de Carvalho MEA. 1998. Pulp bleaching using laccase from *Trametes versicolor* under high temperature and alkaline conditions. *Appl Biochem Biotechnol*. **70**:983–993.

Moore JC, Jin HM, Kuchner O and Arnold F. 1997. Strategies for the in vitro evolution of protein function: enzyme evolution by random recombination of improved sequences. *J. Mol. Biol.* **272:**336–347

Moore JC and Arnold FH. 1998. Directed evolution of a para-nitrobenzyl esterase for aqueous-organic solvents. *Nat. Biotech.* **14:** 458–467.

Nagai M, Sato T, Watanabe H, Saito K, Kawata M & Enei H. 2002. Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolorization of chemically different dyes. *Appl Microbiol Biotechnol*. **60**:327–335.

Nannipieri P & Bollag JM. 1991. Use of enzymes to detoxify pesticide-contaminated soils and waters. *J Environ Qual.* **20**:510–517.

Ness JE, Kim S, Gottman A, Pak R, Krebber A, Borchert TV, Govindarajan S, Mundorff EC, Minshull J. 2002. Synthetic shuffling expands functional protein diversity by allowing amino acids to recombine independently. *Nat Biotechnol.* **20**(12):1251-5.

Ng TB &Wang HX. 2004. A homodimeric laccase with unique characteristics from the yellow mushroom *Cantharellus cibarius*. *Biochem Biophys Res Commun* **313**: 37–41.

Olivieri G, Marzocchella A, Salatino P, Giardina P, Cennamo G and Sannia G. 2006. Olive mill wastewater remedation by means of *Pleurotus ostreatus*: Kinetic characterization and Preliminary Tests in an Internal Loop Airlift Bioreactor. *Biochemical Engineering Journal*. In press.

Ostermeier M, Shim JH, Benkovic SJ. 1999. A combinatorial approach to hybrid enzymes independent of DNA homology. *Nat Biotechnol. Dec.* **17**(12):1205-9.

Paice MG, Bourbonnais R, Reid ID, Archibald FS & Jurasek L. 1995. Oxidative bleaching enzymes – a review. *J Pulp Paper Sci.* **21**:280–284.

Palmieri G, Cennamo G and Sannia G. 2005. Remazol Brilliant Blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. *Enz. Microb. Technol*. **36**, 17-24.

Palmieri G, Cennamo G, Faraco V, Amoresano A, Sannia G & Giardina P. 2003. Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. *Enzyme Microb Technol* 33: 220–230.

Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A & Sannia G. 1997 A novel white laccase from *Pleurotus ostreatus*. *J Biol Chem* **272**: 31301–31307.

Palmieri G, Giardina P, Marzullo L, Desiderio B, Nitti G, Cannio R and Sannia G. 1993. Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Microbiol. Biotechnol*. **39**, 632-636.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. 2000. Advances in microbial amylases. *Biotechnol Appl Biochem.* **31**:135-52.

Penning TM and Jez JM. 2001. Enzyme redesign. *Chemical Reviews* **101**:3027–3046.

Peralta-Zamora P, Pereira CM, Tiburtius ERL, Moraes SG, Rosa MA, Minussi RC & Dur´an N. 2003. Decolorization of reactive dyes by immobilized laccase. *Appl Catal B-Environ*. **42**:131–144.

Perry CR, Matcham SE, Wood DA &Thurston CF. 1993 The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*. *J Gen Microbiol* **139**: 171–178.

Piontek K, Antorini M & Choinowski T. 2002. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90- angstrom resolution containing a full complement of coppers. *J Biol Chem* **277**: 37663–37669.

Piscitelli A, Giardina P, Mazzoni C, Sannia G. 2005. Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol. **69**(4):428-39.

Pozdnyakova NN, Rodakiewicz-Nowak J & Turkovskaya OV. 2004. Catalytic properties of yellow laccase from *Pleurotus ostreatus* D1. *J Mol Catal B*. **30**:19–24.

Raillard S, Krebber A, Chen YC, Ness JE, Bermudez E, Trinidad R et al. 2001. Novel enzyme activities and functional plasticity revealed by recombining highly homologous enzymes. *Chem. Biol*. **8**: 891–898

Reid ID & Paice MG. 1994. Biological bleaching of kraft pulps by white-rot fungi and their enzymes. *FEMS Microbiol Rev*. **13**:369–376.

Rodriguez E, Pickard MA &Vazquez-Duhalt R. 1999. Industrial dye decolorization by laccases from ligninolytic fungi. *Curr Microbiol*. **38**:27–32.

Roper JC, Sarkar JM, Dec J & Bollag JM. 1995. Enhanced enzymatic removal of chlorophenols in the presence of cosubstrates. *Water Res*. **29**: 2720–2724.

Royarcand L & Archibald FS. 1991. Direct dechlorination of chlorophenolic compounds by laccases from *Trametes* (*Coriolus*) *versicolor*. *Enzyme Microb Technol*. **13**: 194–203.

Saliola M, Mazzoni C, Solimando N, Crisà A Falcone C Jung G and Fleer R. 1999. Use of the *KlADH4* promoter for ethanol dependent production of recombinant human serum albumin in *Kluyveromyces lactis*. *Appl. Environ. Microbiol*. **65**:53-60.

Schoemaker HE, Mink D, Wubbolts MG. 2003. Dispelling the myths--biocatalysis in industrial synthesis. *Science*. **299**(5613):1694-7.

Schultz A, Jonas U, Hammer E & Schauer F. 2001. Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. *Appl Environ Microbiol*. **67**:4377–4381.

Sealey J, Ragauskas AJ & Elder TJ. 1999. Investigations into laccase-mediator delignification of kraft pulps. *Holzforschung* **53**:498–502.

Seebeck FP and Hilvert D. 2003. Conversion of a PLP-dependent racemase into an aldolase by a single active site mutation. *J. Am. Chem. Soc.* **125:** 10158–10159.

Servili M, DeStefano G, Piacquadio P, Sciancalepore V. 2000. A novel method for removing phenols from grape must. *Am. J. Enol. Vitic.* **51**:357–361.

Shleev SV, Morozova O, Nikitina O, Gorshina ES, Rusinova T, Serezhenkov VA, Burbaev DS, Gazaryan IG & Yaropolov AL. 2004. Comparison of physico-chemical characteristics of four laccases from different basidiomycetes. *Biochimie* **86**:693–703.

Sieber V, Martinez CA, Arnold FH. 2001. Libraries of hybrid proteins from distantly related sequences. *Nat Biotechnol.* **19**(5):456-60.

Sigoillot C, Record E, Belle V, Robert JL, Levasseur A, Punt PJ, van den Hondel CAMJ, Fournel A, Sigoillot JC & Asther M. 2004. Natural and recombinant fungal laccases for paper pulp bleaching. *Appl Microbiol Biotechnol.* **64**:346–352.

Soares GMB, Amorim MTP, Hrdina R & Costa-Ferreira M. 2002 Studies on the biotransformation of novel disazo dyes by laccase. *Process Biochem.* **37**:581–587.

Solomon EI, Sundaram UM & Machonkin TE. 1996. Multicopper oxidases and oxygenases. *Chem Rev* **96**:2563–2605.

Souza CGM & Peralta RM. 2003. Purification and characterization of the main laccase produced by the white-rot fungus *Pleurotus pulmonarius* on wheat bran solid state medium. *J Basic Microbiol* **43**: 278–286.

Stemmer WPC. 1994. Rapid evolution of a protein in vitro by DNA shuffling. *Nature.* **370**:389–391.

Stemmer W. 1994. DNA shuffling by random fragment and reassembly: in vitro recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA*. **91**:10747–10751.

Stuardo M, Larrondo LF, Vásquez M, Vicuña R and González B. 2005. Incomplete processing of peroxidase transcripts in the lignin degrading fungus *Phanerochaete chrysosporium*. *FEMS Microbiol. Lett*. **242:**37-44.

Tatsumi K, Freyer A, Minard RD & Bollag JM. 1994. Enzymemediated coupling of 3,4-dichloroaniline and ferulic acid – a model for pollutant binding to humic materials. *Environ Sci Technol*. **28**:210–215.

Thakker GD, Evans CS & Rao KK. 1992. Purification and characterization of laccase from *Monocillium indicum Saxena*. *Appl Microbiol Biotechnol* **37**: 321–323.

Torres E, Bustos-Jaimes I & Le Borgne S. 2003. Potential use of oxidative enzymes for the detoxification of organic pollutants. *Applied Catal B-Environ*. **46**:1–15.

Tsioulpas A, Dimou D, Iconomou D & Aggelis G. 2002. Phenolic removal in olive oil mill wastewater by strains of *Pleurotus spp.* in respect to their phenol oxidase (laccase) activity. *Biores Technol*. **84**:251–257.

Ullah MA, Bedford CT & Evans CS. 2000. Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. *Appl Microbiol Biotechnol*. **53**:230–234.

Valetti F and Gilardi G. 2004. Directed evolution of enzymes for product chemistry. *Nat Prod Rep.* **21**:490 – 511.

van den Heuvel RHH, Fraaije MW, Ferrer M, Mattevi A and van Berkel WJH. 2000. Inversion of stereospecificity of vanillyl-alcohol oxidase. *Proc. Natl. Acad. Sci. USA* **97**:9455–9460.

Velazquez-Cedeno M A, Mata G & Savoie JM. 2002. Wastereducing cultivation of *Pleurotus ostreatus* and *Pleurotus pulmonarius* on coffee pulp: changes in the production of some lignocellulolytic enzymes. *World J Microbiol Biotechnol.* **18**:201–207.

Wesenberg D, Kyriakides I, Agathos SN. 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol Adv.* **22**(1-2):161-87.

Wahleithner JA, Xu F, Brown KM, Brown SH, Golightly EJ, Halkier T, Kauppinen S, Pederson A & Schneider P. 1996. The identification and characterization of four laccases from the plant pathogenic fungus *Rhizoctonia solani*. *Curr Genet* **29**:395–403.

Wang L, Brock A, Herberich B and Schultz PG. 2001. ,lExpanding the genetic code of *Escherichia coli*. Science **292:**498–500

Wong YX & Yu J. 1999. Laccase-catalyzed decolorization of synthetic dyes. *Water Res*. **33**:3512–3520.

Wood DA. 1980. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J Gen Microbio*l **117**:327–338.

Xu F, Kulys JJ, Duke K, Li KC, Krikstopaitis K, Deussen HJW, Abbate E, Galinyte V & Schneider P. 2000. Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compounds. *Appl Environ Microbiol.* **66**:2052–2056.

Xu F, Palmer AE, Yaver DS, Berka RM, Gambetta GA, Brown SH, Solomon EI. 1999 Targeted mutations in a *Trametes villosa* laccase: Axial perturbations of the T1 copper*. J. Biol. Chem.* **274:**12372-12375

Xu F, Berka RM, Wahleithner JA, Nelson BA, Shuster JR, Brown SH, Palmer AE, Solomon EI. 1998. Site-directed mutations in fungal laccase: Effect on redox potential, activity and pH profile*. Biochem. J.* **334**:63-70.

Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K & Dalboge H. 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. *Appl Environ Microbiol* **62**:834–841.

Yoshida H. 1883. Chemistry of lacquer (Urushi). Part 1. *J. Chem. Soc.* **43**: 472–486.

Yoshitake A, Katayama Y, Nakamura M, Iimura Y, Kawai S & Morohoshi N. 1993. N-Linked carbohydrate chains protect laccase-III from proteolysis in *Coriolus versicolor*. *J Gen Microbiol* **139**:179–185.

Zaitseva I, Zaitsev V, Card G, Moshkov K, Bax B, Ralph A, Lindley P. 1996.The X-ray structure of human serum ceruloplasmin at 3.1 Å: nature of the copper centres. *J. Bioinorg. Chem.* **1**:15-23

Zaks, A. 2001. Industrial biocatalysis. *Curr Opin Chem Biol*. **5**:130-136.

Zhao H, Giver L, Shao Z, Affholter JA, Arnold FH. 1998. Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat Biotechnol.* **16**(3):258-61.

Zeeman AM, Steensma HY. 2003. The acetyl co-enzyme A synthetase genes of *Kluyveromyces lactis*. Yeast. **20**(1):13-23.

Zille A, Tzanov T, Gubitz GM & Cavaco-Paulo A. 2003 Immobilized laccase for decolorization of Reactive Black 5 dyeing effluent. *Biotechnol Lett.* **25**:1473–1477.

Publications-Comunications

Publications

- **Faraco V, Palmieri G, Festa G, Monti M, Sannia G and Giardina P**. 2005. A new subfamily of fungal subtilases: structural and functional analysis of a *Pleurotus ostreatus* member. *Microbiology*. **151**:457-66.
- **Festa G, Giardina P, Faraco V, Piscitelli A and Sannia G**. 2006. Atypical laccases from the white-rot fungus *Pleurotus ostreatus* and their application for the treatment of industrial coloured effluents. *Genetics and Cellular Biology of Basidiomycetes VI*, ISBN 84-9769-107-5.
- **Giardina P, Faraco V, Festa G, Palmieri G, Piscitelli A and Sannia G.** 2006. Subunit role and interaction in heterodimeric laccases from *Pleurotus ostreatus. Biochemical Journal.* Submitted.
- **Festa G, Giardina P and Sannia G**. Directed evolution of *Pleurotus ostreatus* laccases. Manuscript in preparation.

Comunications

- **Sannia G, Palmieri G, Cennamo G, Faraco V, Festa G and Giardina P.**
  Pleurotus ostreatus heterodimeric laccase isoenzymes.
  2nd European Laccase Meeting, Oxizymes 2004, Naples, Italy.
- **Sannia G, Giardina P, Cennamo G, Festa G and Palmieri G.**
  Pleurotus ostreatus heterodimeric laccase isoenzymes: structure and application to degradation of recalcitrant molecules.
  International Conference on Biotechnology in the Pulp and Paper Industry 2004, Durban, South Africa.
- **Festa G, Giardina P, Cennamo G, Palmieri G and Sannia G.**
  Atypical laccase from the white-rot fungus *Pleurotus ostreatus* and its application for the treatment of industrial coloured effluents.
  Oral presentation at 6th Congress on Genetics and Cellular Biology of Basidiomycetes, 2005, Pamplona, Spain.
- **Sannia G, Giardina P, Cennamo G, Festa G and Palmieri G.**
  Oxidative biosystems: structure, function and application to bioremedation of industrial wastewater.
  COST E23 Conference, 2005, Baiona.
- **Piscitelli A, Giardina P, Festa G and Sannia G**
  Development of new oxidases.
  Royal Society of Chemistry Conference, 2005, Exeter.
- **Festa G, Giardina P, Piscitelli A, Autore F, Cestone R and Sannia G.**
  Directed evolution of *Pleurotus ostreatus* laccases.
  Oxizymes in Oeiras, 2006, Portugal.
- **Festa G, Piscitelli A, Faraco V, Giardina P, Autore F, Fraternali F and Sannia G.**
  Laccase Engineering by Rational and Random Mutagenesis.
  Oxizymes in Oeiras, 2006, Portugal.

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A new subfamily of fungal subtilases: structural and functional analysis of a *Pleurotus ostreatus* member

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Pleurotus ostreatus produces several extracellular proteases which are believed to be involved in the regulation of the ligninolytic activities of this fungus. Recently, purification and characterization of the most abundant *P. ostreatus* extracellular protease (*Po*SI) has been reported. The sequence of the *posI* gene and of the corresponding cDNA has been determined, allowing the identification of its pre- and pro-sequences. A mature protein sequence has been verified by mass spectrometry mapping, the *N*-glycosylation sites have been identified and the glycosidic moieties characterized. Mature *Po*SI shows a cleaved peptide bond in the C-terminal region, which remains associated with the catalytic domain in a non-covalent complex. Reported results indicate that this enzyme is involved in the activation of other *P. ostreatus* secreted proteases, thus suggesting its leading role in cascade activation mechanisms. Analyses of the *Po*SI sequence by homology search resulted in the identification of a DNA sequence encoding a new protease, homologous to *Po*SI, in the *Phanerochaete chrysosporium* genome. A new subgroup of subtilisin-like proteases, belonging to the pyrolysin family, has been defined, which includes proteases from ascomycete and basidiomycete fungi.

INTRODUCTION

Subtilisin-like serine proteases, subtilases, have been classified into six families on the basis of their amino acid sequences (Siezen & Leunissen, 1997). Members of these families have been extensively studied and the crystallographic structures of some of them have been determined (Bode *et al.*, 1987; Jain *et al.*, 1998; Wright *et al.*, 1969). Site-directed mutagenesis and protein engineering have provided intimate knowledge of structure–function relationships in this class of enzymes (Sroga & Dordick, 2001; Ness *et al.*, 2002). The majority of the subtilases are synthesized as a precursor with a pre- and pro-sequence extension of the N-terminus of the mature protein (Bryan *et al.*, 1995). The pre-sequence acts as a signal peptide, driving translocation through the cell membrane, whilst the pro-sequence acts both as an intramolecular chaperone that guides the correct folding of the mature protein and as a protease self-inhibitor (Yabuta *et al.*, 2001, 2003). The pro-sequence is usually cleaved from the mature protein by autoproteolysis to produce active mature protease. In addition to a myriad of prokaryotic subtilases, many members of this superfamily have been identified in eukaryotes such as fungi, plants, insects and mammals.

Pleurotus ostreatus and *Phanerochaete chrysosporium* are white-rot basidiomycetes, which belong to different subclasses of ligninolytic micro-organisms, producing distinct patterns of ligninolytic enzymes. They produce extracellular proteases, which are believed to be involved in the regulation of the ligninolytic activities of these fungi (Palmieri *et al.*, 2000, Dosoretz *et al.*, 1990). In particular, it has been demonstrated that different laccase isoenzymes from *Pl. ostreatus* can be specifically degraded or activated during fungal growth by proteases present in the culture broth (Palmieri *et al.*, 2000, 2003). In a recent study, we reported the purification and characterization of the main *Pl. ostreatus* extracellular protease *Po*SI (Palmieri *et al.*, 2001).

On the basis of structural and kinetic properties, *Po*SI appears to be a serine protease belonging to the subtilase family. This enzyme seems to play a key role in the regulation process of *Pl. ostreatus* laccase activities. A similar relationship was observed for lignin peroxidases (LiPs) in *Ph. chrysosporium*: in this case, the extracellular proteases caused an almost complete disappearance of LiP activity due to degradation of all LiP isoenzymes (Dosoretz *et al.*, 1990).

Abbreviations: MALDIMS, Matrix-assisted laser desorption ionization–mass spectrometry; PTH, phenylthiohydantoin.

The EMBL accession numbers for the nucleotide sequences reported in this paper are AJ634913 (*posI*) and AJ748587 (*pcsI*).

This paper reports evidence concerning the role played by *Po*Sl in the activation of *Pl. ostreatus* extracellular proteases. The *posl* gene and cDNA were cloned and sequenced, and mass spectrometric analysis was employed to validate the deduced amino acid sequence and to identify post-translational modifications. Furthermore, analyses by homology search allowed us to define a new subtilase subfamily which includes proteases from ascomycete and basidiomycete fungi.

METHODS

Organisms and culture conditions. White-rot fungi, *Pleurotus ostreatus* (Jacq.Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) and *Phanerochaete chrysosporium* Burdsall M1 (DSM 13583) were maintained through periodic transfer at 4 °C on potato glucose (2·4 %) agar plates (Difco) in the presence of 0·5 % yeast extract (Difco).

Pl. ostreatus cultures were carried out in the basal medium as previously described (Palmieri *et al.*, 1997), or with the addition of 150 μM copper sulphate or 1 mM vanillic acid (4-hydroxy-3-methoxybenzoic acid). *Ph. chrysosporium* cultures were carried out in 0·24 % potato glucose broth in the presence of 0·05 % yeast extract.

Protease assay. Protease activity was assayed using SucAAPFpNA (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; Sigma) or azoalbumin as substrates as follows:

a) The assay mixture contained 5 mM SucAAPFpNA, 10 mM $CaCl_2$ and 50 mM Tris/HCl buffer, pH 8·0, in a final volume of 1 ml. Hydrolysis of the substrate was followed by absorbance increase at 405 nm ($\varepsilon_{405}=8800\ M^{-1}\ cm^{-1}$).

b) A 400 μl volume of 15 mg ml^{-1} azoalbumin (Sigma) in 50 mM MOPS buffer, pH 7·0, was incubated with 250 μl of the enzyme sample at 37 °C for 30 min. To stop the reaction, 650 μl 20 % TCA was added and the undigested precipitated substrate was removed by centrifugation at 10 000 r.p.m. for 15 min. Six hundred and fifty microlitres of supernatant was added to 350 μl 10 M NaOH and the absorbance at 440 nm was measured. The control assay was performed without any enzyme in the reaction mixture and used as reference. One unit of enzyme activity was defined as the amount of enzyme needed to increase the A_{440} by 0·01.

Zymographic analysis. Samples underwent electrophoresis in 10 % gelatin-containing polyacrylamide gel at alkaline pH under non-denaturing conditions. The separating and stacking gels contained 12·5 % acrylamide solution in 50 mM Tris/HCl, pH 9·5, and 9 % acrylamide solution in 18 mM Tris/HCl, pH 7·5, respectively. The electrode reservoir solution was 25 mM Tris/190 mM glycine, pH 8·4. After electrophoresis, gels were incubated for 16 h at 37 °C in 50 mM Tris/HCl, pH 7·6, buffer containing 200 mM NaCl and 5 mM $CaCl_2$. Gels were then stained for 30 min with 30 % methanol/10 % acetic acid containing 0·5 % Coomassie Brilliant Blue R-250 and destained in the same solution without dye. Clear bands on the blue background represent areas of gelatinolysis.

Cloning of the *posI* gene. Amplification experiments of *Pl. ostreatus* genomic DNA were performed using the oligonucleotide pair 1, as primers (Table 1). The 800 bp fragment obtained was cloned into the pGEM-T Easy Vector (Promega) and sequenced. This fragment, labelled by the random priming method, was used as probe to screen a *Pl. ostreatus* genomic library (Giardina *et al.*, 1995). A further screening of the genomic library was performed using, as probe, a 700 bp fragment obtained by *Kpn*I digestion of the amplified cDNA. Colony hybridization experiments were carried out in 5× SSC at 65 °C (where 1× SSC is 0·15 M NaCl, 0·015 M sodium citrate).

Cloning of *posI* and *pcsI* cDNAs. Mycelia from *Ph. chrysosporium* and copper-supplemented *Pl. ostreatus* cultures were collected after 2 days of growth. Total RNA was extracted from lyophilized mycelia, as described by Lucas *et al.* (1977). Reverse transcription

Table 1. Oligonucleotide pairs used in amplification experiments

Y = T/C, R = G/A, V = G/C/A, B = G/T/C, D = G/A/T, E = T/C/A, P = A/G/C/T.

Oligonucleotide pair		Sequence	Annealing temperature
1	*posl* nterm	5′-GGECCPGAYGAYCCPGC-3′	56 °C
	posl 1 down	5′-TCVGTCCADCCRTCPGC-3′	
2	pep segn *posl*	5′-ATGAAGGGCGTACTCGTGTGG-3′	64 °C
	nter rev *posl*	5′-GAGCCGGATCGTCAGGGCC-3′	
3	*posl* spec nterm	5′-CCACCCGACTCAGAGTCC-3′	58 °C
	posl 1D down	5′-GCCTCCCAAAGAAAGGGTG-3′	
4	*posl* 1D up	5′-CACCCTTTCTTTGGGAGGC-3′	60 °C
	dT-*Not*I	5′-AATTCGCGGCCGCTTTTTTTTTTTTTTT-3′	
5	5′ *pcsl* phan	5′-CGGTCAGACCCATGAGGC-3′	58 °C
	pcsl phan rev	5′-GGACGAGGAATTCTCCGTCG-3′	
6	*pcsl* phan fw	5′-CGACGGAGAATTCCTCGTCC-3′	58 °C
	3′ *pcsl* phan	5′-TCAGGAGCTCGGGGCATTG-3′	
7	nter rev *posl*	5′-GAGCCGGATCGTCAGGGCC-3′	65 °C
	d(T)RACE	5′-GACCACGCGTATCGATGTCGACTTTTTT-TTTTTTTTTTV-3′	
8	nter rev *posl*	5′-GAGCCGGATCGTCAGGGCC-3′	65 °C
	Anchor-RACE	5′-GACCACGCGTATCGATGTCGAC-3′	

reactions were performed using Super Script II Rnase H$^-$ Reverse transcriptase (Invitrogen) and oligonucleotide dT-*Not*I as primer, following the manufacturer's instructions. Amplification experiments for each *posl* cDNA fragment were performed using oligonucleotide pairs 2, 3 and 4 at the corresponding annealing temperatures (Table 1). Amplification experiments for each *pcsl* cDNA fragment were performed using oligonucleotide pairs 5 and 6 at the corresponding annealing temperatures (Table 1). The amplified fragments were cloned in the pGEM-T Easy Vector and sequenced.

First-strand cDNA synthesized from *Pl. ostreatus* total RNA using the gene-specific primer *posl* 1D down (Table 1) was used to perform rapid amplification of 5′ cDNA end (5′-RACE). Terminal transferase (Roche) was used to add a homopolymeric A-tail to the 3′ end of the cDNA. Tailed cDNA was then amplified by PCR, using oligonucleotide pair 7 (Table 1), and the resulting product was reamplified using oligonucleotide pair 8 (Table 1), cloned in pGEM-T Easy Vector and sequenced.

DNA preparation, subcloning and restriction analyses were performed by standard methods according to Sambrook *et al.* (1989). Sequencing by the dideoxy chain-termination method was performed by the CEINGE Sequencing Service (Naples, Italy) using universal and specific oligonucleotide primers.

Amino acid sequence analysis. Automated N-terminal degradation of the protein was performed using a Perkin-Elmer Applied Biosystem 477A pulsed liquid protein sequencer equipped with a model 120A phenylthiohydantoin analyser for the on-line identification and quantification of phenylthiohydantoin (PTH) amino acids.

Matrix-assisted laser desorption ionization–mass spectrometry (MALDIMS) analysis. MALDIMS analyses were carried out with a Voyager DE MALDI Time of Flight mass spectrometer (PerSeptive Biosystems) on both the protein and on the peptide mixtures obtained from proteolytic hydrolyses.

Molecular mass determination of whole protease was performed by loading a mixture of sample solution and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) on a sample slide and drying the slide *in vacuo*. Mass range was calibrated using apomyoglobin from horse heart (mean molecular mass, 16 952·5 Da) and human serum albumin (mean molecular mass, 66 431·0 Da).

Peptide mixtures derived from each hydrolysis were lyophylized and successively dissolved in 0·2 % trifluoroacetic acid, and 1 µl sample solution was mixed with 1 µl 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (CHCA) solution on a sample slide and left to dry under vacuum. The CHCA solution was prepared in acetonitrile : 0·1 % TFA, 70 : 30 (v/v). Mass spectra were acquired in a linear mode, and calibrated from 379·35 and 5734·59, the *m*/*z* values of the CHCA dimer and bovine insulin, respectively.

Enzymic hydrolysis. A *Po*Sl sample (1 nmol) was reduced with 100 mM DTT at 37 °C for 2 h under a nitrogen atmosphere in 0·25 M Tris/HCl (pH 8·5), 1·25 mM EDTA, containing 6 M guanidinium chloride, and then alkylated for 30 min by using an excess of iodoacetamide at room temperature in the dark. The protein sample was desalted by loading the reaction mixture onto a PD-10 prepacked column (Pharmacia), equilibrated and eluted in 0·4 % ammonium bicarbonate, pH 8·5.

Enzymic digestions with trypsin and endoproteinase Glu-C were carried out in 0·4 % ammonium bicarbonate, pH 8·5, at 37 °C overnight using an enzyme/substrate molar ratio of 1 : 50.

The tryptic mixture was deglycosylated by incubation with 0·15 U of peptide *N*-glycosidase F (Boehringer Mannheim) in 0·4 % ammonium bicarbonate, pH 8·5, at 37 °C for 16 h. Samples were fractionated on a prepacked cartridge Sep-Pak C18 (Waters); peptide fractions were collected manually.

RESULTS

Protease induction and activity regulation

Different substances, already tested as inducers of *Pl. ostreatus* laccase production, were analysed with respect to their ability to induce protease production; among them, vanillic acid was shown to be the best protease inducer. Fig. 1 shows the time-course of extracellular protease activity from *Pl. ostreatus* culture broth supplemented with 1 mM vanillic acid compared to that of basal or copper-supplemented culture media. SucAAPFpNa, a subtilisin-like protease substrate, or azoalbumin, a non-specific

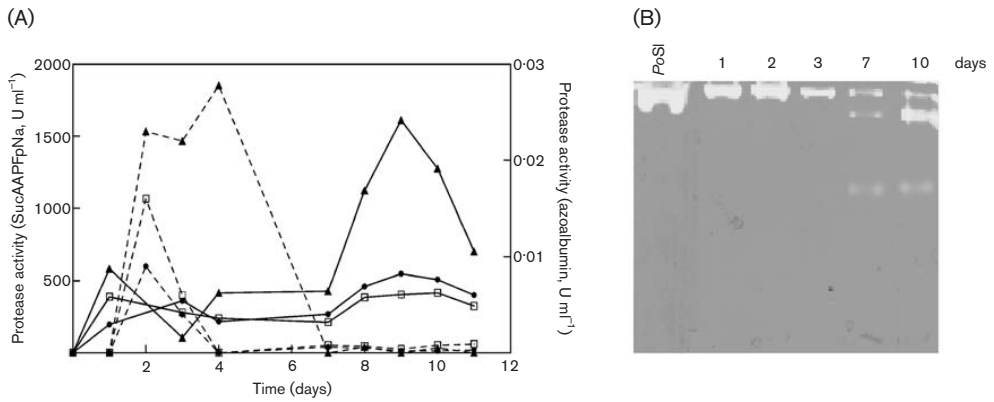

Fig. 1. (A) Time-course of *Pl. ostreatus* extracellular protease activity from cultures supplemented with copper sulphate (□) or vanillic acid (▲), in comparison to basal medium culture (●). Protease activity was determined using SucAAPFpNa as substrate (dashed line) or azoalbumin (solid line). (B) Gelatin zymography of samples collected at different growth times from vanillic acid-supplemented culture.

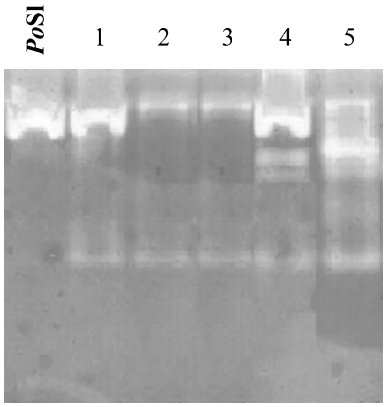

Fig. 2. Zymogram of filtered culture broth (supplemented with vanillic acid) incubated with 1 mM PMSF or 10 mM $CaCl_2$. Lanes: 1, 3-day-old filtered culture broth (sample A); 2, 3, sample A after PMSF addition (lane 2) and 24 h incubation (lane 3); 4, sample A after $CaCl_2$ addition and 24 h incubation; 5, 9-day-old filtered culture broth.

protease substrate, wwere used as substrates. The gelatin zymography of samples from vanillic acid culture is shown in the same figure. The protease activity determined using SucAAPFpNa as substrate, as well as the zymogram analysis, indicated that the maximum activity of *Po*Sl was reached in the first days of growth. New active protease bands appeared at longer growth times, and a remarkable increase of total protease activity (assayed with azoalbumin) was observed. The occurrence of these new protease activities at prolonged growth times could arise from their late production or from maturation of inactive precursors. In order to distinguish between these two hypotheses, samples of culture broth at the third day of fungal growth were filtered through a 0·2 μm pore-size membrane and incubated for 24 h at 28 °C in the presence of 1 mM PMSF (an irreversible inhibitor of serine proteases) or 10 mM $CaCl_2$, which strongly increases *Po*Sl activity (Palmieri *et al.*, 2001). Fig. 2 shows the zymogram analysis of these samples together with culture broth samples collected after 3 or 9 days of growth. No *Po*Sl activity signal was detected in the PMSF-treated sample, and the protease activity bands of this sample were unaltered after 24 h incubation. On the other hand, when the incubation was performed in the presence of $CaCl_2$, new protease activity bands were switched on, displaying a pattern similar to that observed at prolonged fungal growth time (i.e. the ninth day).

Cloning and sequencing of the *posl* gene and cDNA

Sequences of *Po*Sl N-terminus and of three tryptic peptides have previously been reported (Palmieri *et al.*, 2001). On the basis of the sequences of the N-terminus and that of a tryptic peptide (boxed peptides in Fig. 3), oligonucleotide/primer mixtures were designed (*posl* nterm and *posl* 1 down, Table 1) and used in amplification experiments performed using *Pl. ostreatus* genomic DNA as template. The amino acid sequence encoded by the 800 bp amplified fragment contained the sequence of another tryptic peptide, homologous to the conserved sequence around the Asp residue of the catalytic triad in the subtilase family. To amplify the *Po*Sl-encoding cDNA, two amplification experiments were performed using oligonucleotide pairs 3 and 4 (Table 1), designed on the basis of the nucleotide sequence of the amplified gene fragment. Two amplified fragments of 500 bp and 2000 bp, corresponding to 5′ and 3′ regions of *posl* cDNA, respectively, were cloned and sequenced.

A *Pl. ostreatus* genomic library was screened using the gene-amplified fragment. One of the positive clones analysed (named 6C4, 2500 bp) encompassed the 5′ coding sequence and extended 750 bp upstream from the codon corresponding to the N-terminal of the mature protein. To complete the 3′ coding region of the *posl* gene, a 700 bp fragment of the amplified 2000 bp cDNA was used as probe for further screening of the genomic library. One of the positive clones analysed (5–13) overlapped clone 6C4 for 1600 bp and extended 60 bp at the 3′ non-coding region. Thus, the whole *posl* gene sequence was determined. A putative start codon (ATG 166–168) was identified in the 5′ gene region, 600 bp upstream from the codon corresponding to the N-terminal of the mature protein. The corresponding cDNA fragment was amplified (oligonucleotide pair 2, Table 1), cloned and sequenced. The strategy used to clone the *posl* gene and cDNA is shown in Fig. 4. All known *Po*Sl peptide sequences were identified in the encoded sequence. Comparison of cDNA and gene sequences allowed determination of the gene structure, with the coding sequence interrupted by 20 introns (Fig. 4).

A 5′-RACE experiment was performed allowing the identification of the transcription initiation site at nucleotide 145, 21 bp upstream from the ATG 166–168, thus confirming it as the translation start codon.

Validation of the *Po*Sl primary structure and characterization of its glycoside moiety

Comparison of the *Po*Sl amino acid sequence with that of the mature protein N-terminus allowed us to identify the putative *Po*Sl pre-propeptide. The putative pre-peptide (−124 −108) was identified as a secretion signal by the program SignalP; consequently the propeptide was established as peptide (−107 −1).

The mature protein N-terminal sequence had previously been determined from the electroblotted purified protein (GPDDPALPPD...) (Palmieri *et al.*, 2001). Edman degradation of the purified native protein actually gave rise to two equimolar PTH amino acids for each step. Other than the above N-terminal sequence, a second one (AQVPTLGTVF-EL), corresponding to a polypeptide chain starting from A690 of *Po*Sl, was detectable. Hence, it can be inferred that the F689-A690 peptide bond is hydrolysed in the mature protein and the cleaved peptide remains associated with the

-124 MKGVLVWSIL SAGCALAVTP LSSVKRVTNL PIVANKFIVE VDSTSDIPTK

-74 RSNPHDALYE SLRKRAIGFK VDREFNTQGL FVGAALTLSD AQDVGQIQST

-24 TGVKSIRPVR AFDRPKPVKV QVVK**GPDDPA** **LPPDSESTHV ITGVDKLHAQ**

27 **GITGAGIKIG ILDTGIDYTH PTLGGAFGPG NKVIGGFDLV GDDYDGTNTP**

77 **VPDPDPLDQC AGHGTHVAGI IGANPDNAFN ISGVAFQASL SAYRVFGCVG**

127 **FVTDDVLVDA LLLGFNEGQD ILTLSLGGAD GWTESVSAVV ASRIAATGKV**

177 **VTIAAGNDGA SGAWYTSSPG NGIDVISVAS LDNTVVPLQT AQVHGVTHDP**

227 **IIYQDTFPLP INDTRPIFAT STDVTVVDDA CNPLPDSTPD LSGFVVIVRR**

277 **GTCTFVQKLG NVAAKGAKVT LIYDNGSGFG AIDTGAFVAS FIQAADGEFL**

327 **VQQFASGQNV SLSFPQSGGL IQFPDPAGGL ISSFTSYGPS NDFFFKPAVA**

377 **APGGNILSTL PVNLGLFGIE SGTSMATPFV AGSAALLFQA KGTSAAVGRS**

427 **ARTLFETTAQ RVPSTHTDGD PLQTLTQQGA GLINVFNAIH TTTIVSPGEL**

477 **ILNDTAHFKG TQQFTVRNAG SSAKTYTLRH IPAGTAVTVT PGTIFPADGP**

527 **VPLSTDFASV SLSTSKFTLA PGKTQTVTAR FTPPAAADPS TFPVFSGFIQ**

577 **IESGTEQVQV SYLGLKASLK DKQVIDNTDF FFGVPTPVLT DPNGEVQTSP**

627 **RNYSFLASDF PTLIFRLAFG SPKVVVDLVS PTINFKPTLN TRALGDTHGS**

677 **FFSFPHRVKT GSFAQVPTLG TVFELDFTSR NNDDPTDNGF NTVAIDPPTF**

727 **ANGTTIPNGQ YRLLLRALRV TGDPTNEADF ESFLSPIIGV NAP**

Fig. 3. *Po*SI sequence. The numbers represent the positions of the amino acid residues starting from the N-terminus of the mature *Po*SI protein. Regions verified by peptide mass mapping are underlined. *N*-Glycosylated asparagine residues are marked with one star, asparagine residues found both glycosylated and non-glycosylated are marked with two stars. Boxed peptide sequences were used to design oligonucleotide-primer mixtures for the amplification experiments. The arrow indicates the peptide bond cleaved in the mature protein.

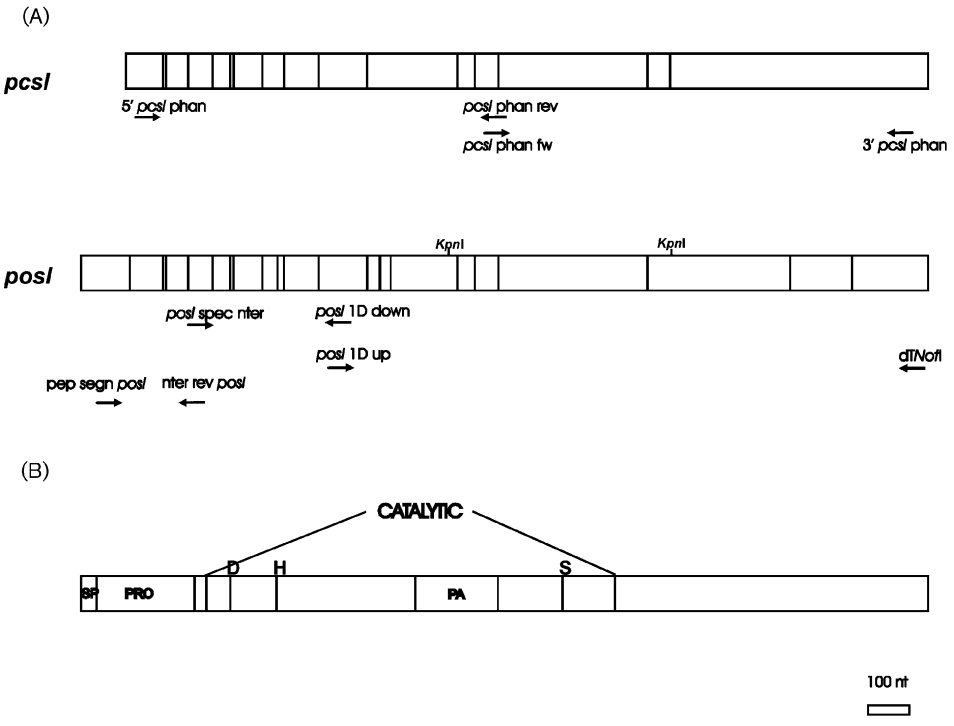

Fig. 4. (A) Schematic representation of *posI* and *pcsI* cDNAs. Arrows show the positions of oligonucleotide primers used for cDNA amplifications. *Kpn*I restriction sites at the ends of the probe used for genomic library screening are also shown. The positions of introns are shown by vertical bars. (B) *Po*SI domains. SP, signal peptide; PRO, propeptide; CATALYTIC, catalytic domain; PA, PA domain; D, H, S, catalytic active site residues.

catalytic domain in a non-covalent complex, since no Cys residues in the cleaved peptide are present.

The *Po*Sl amino acid sequence is characterized by seven putative glycosylation sites. As previously reported (Palmieri *et al.*, 2001), MALDIMS analysis of permethylated *N*-linked sugar released by hydrolysis with *N*-glycosidase F showed the occurrence of several high mannose moieties with molecular mass ranging from 1580·0 and 2603·6 *m*/*z* and identified as $Hex_5HexNAc_2$ and $Hex_{10}HexNAc_2$. The other molecular ions at *m*/*z* 1784·0, 1987·5, 2191·5 and 2394·6 were identified as homologous structures having between three and six mannose residues linked to the pentasaccharide core.

To verify the deduced *Po*Sl amino acid sequence, the *Po*Sl protein was reduced, alkylated and then digested with trypsin, and the peptide mixture was directly analysed by MALDIMS, allowing the identification of several peptides (Fig. 3). This analysis also showed three different clusters of signals each characterized by a pattern of *m*/*z* values differing in 162 Da, in agreement with the presence of the high mannose glycosidic moiety, as described above. The assignments of the different molecular masses to the corresponding peptides (Table 2) led to the identification of Asn106, Asn479 and Asn728 as *N*-glycosylation sites. As shown in Fig. 3, the two residues Asn479 and Asn728 were present in both glycosylated and unglycosylated state. A further picture of *Po*Sl *N*-glycosylation sites was obtained after deglycosylation of peptide mixture by *N*-glycosidase F. Indeed, new signals were detected by MALDIMS analysis at 3440·1, 5087·1, 5484·1 and 6361·2 *m*/*z*, corresponding to the expected molecular mass of peptides 707–738, 438–485, 59–112 and 59–120, respectively, each increased by 1 Da, since the *N*-glycosylated Asn residues are converted to Asp following *N*-glycosidase F treatment. Moreover, an additional peak at *m*/*z* 1793·0 was present after deglycosylation. This mass value is in agreement with the expected molecular mass of the peptide 628–642 (containing the potential *N*-glycosylation site Asn628) increased by 1 Da. This result led to the assignment of Asn628 as an *N*-glycosylation site, even though the MALDIMS spectrum of the tryptic peptide mixture did not allow us to detect the cluster of *m*/*z* peaks related to the Asn628 glycoforms. Hence, two out of seven putative *Po*Sl *N*-glycosylation sites were found to be glycosylated, Asn479 and Asn728 were found to be both glycosylated and unglycosylated, whilst Asn238 and Asn301 were only found unmodified. MALDIMS analyses did not allow mapping of the last putative *N*-glycosylation site, Asn335. This could be due to an incomplete extraction of the peptides from the gel or to the well-known MALDI suppression phenomena.

To validate further regions of the protein sequence, the tryptic peptide mixture was submitted to enzymic hydrolysis with the endoproteinase Glu-C. The data obtained allowed us subsequently to verify 84 % the *Po*Sl primary structure, as shown in Fig. 3.

Homology search analysis and *pcsl* cDNA cloning and sequencing

*Po*Sl shows a high level of identity with a hypothetical protein from *Neurospora crassa* (38 %, BLAST E score = $2{\cdot}1 \times 10^{-121}$), a subtilisin-like serine protease from the fungus *Metarhizium anisopliae*, Pr1C (39 %, E score = $2{\cdot}0 \times 10^{-114}$), and a minor extracellular serine protease from *Bacillus subtilis*, Vpr (31 %, E score = $7{\cdot}1 \times 10^{-41}$).

Ph. chrysosporium is the only basidiomycete whose genome has been sequenced to date. BLAST analysis of this genome

Table 2. Theoretical and measured molecular mass values for PoSl glycopeptides

MH^+, singly protonated ion. Values in the theoretical and measured MH^+ columns are in Da.

Peptide	Glycosylation site	Theoretical MH^+	Measured MH^+	Glycoforms
628–642	Asn628	1792·0	–	–
707–738	Asn728	4655·5	4657·7	$Hex_5HexNAc_2$
		4817·6	4820·4	$Hex_6HexNAc_2$
		4979·7	4982·7	$Hex_7HexNAc_2$
		5141·8	5143·6	$Hex_8HexNAc_2$
59–112	Asn106	6699·9	6702·0	$Hex_5HexNAc_2$
		6862·0	6864·1	$Hex_6HexNAc_2$
		7024·1	7025·6	$Hex_7HexNAc_2$
438–485	Asn479	6788·9	6790·4	$Hex_8HexNAc_2$
		6951·0	6952·8	$Hex_9HexNAc_2$
		7113·1	7114·6	$Hex_{10}HexNAc_2$
59–120	Asn106	7576·8	7579·1	$Hex_5HexNAc_2$
		7738·9	7741·1	$Hex_6HexNAc_2$
		7901·0	7902·9	$Hex_7HexNAc_2$
		8063·1	8064·1	$Hex_8HexNAc_2$

(http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) resulted in the identification of two *Po*Sl homologous sequences, about 112 kb from each other. One of these, designated pc.18.58.1, appeared to encode a protein which was very similar to *Po*Sl. RNA was extracted from *Ph. chrysosporium* and specific cDNA was amplified using oligonucleotide pairs designed on the basis of the genomic sequence and protein homology. The amplified cDNA was longer than pc.18.58.1 at its 5′ terminus, even if it did not contain the start translational codon. Moreover, the alignment between pc.18.58.1 and AJ748587 showed a 6 aa insertion in the former, due to an incorrect exon end. The deduced amino acid sequence corresponding to the amplified cDNA showed an identity of 65 % with *Po*Sl. Fourteen out of 15 introns in the gene sequence were in the same positions as those of the *posl* gene (Fig. 4).

DISCUSSION

The extracellular subtilisin-like protease *Po*Sl from *Pl. ostreatus* was believed to be involved in the activation/degradation mechanism of laccase isoenzymes (Palmieri *et al.*, 2000, 2001). This mechanism seemed to be indirectly controlled by *Po*Sl, whose activity appeared to be necessary but not sufficient for laccase post-translational regulation. In this paper we investigated the effect of *Po*Sl on other *Pl. ostreatus* extracellular proteases. The addition of vanillic acid to the fungal culture led to an increase both of total protease activity and of *Po*Sl activity, with respect to any of the other conditions examined, such as copper-supplemented culture (Fig. 1). In vanillic acid-amended cultures, some protease activities were only detectable at long fungal growth times (Fig. 1). These proteases were also found to be activated after incubation of the early growth time culture broth in the absence of mycelia, but in the presence of active *Po*Sl. Therefore these proteases were secreted as inactive forms and subsequently activated in a process governed by *Po*Sl.

The *Po*Sl coding sequence was determined and the deduced protein primary structure confirmed by MALDIMS mapping. This approach has proven useful for the identification of the *N*-glycosylation sites and characterization of the glycoside moiety of the protein. *Po*Sl is homologous to many other serine proteases, especially in the areas surrounding the amino acids that are known to be involved in the active site of subtilisin (D, H and S).

The first amino acid (G1) of the mature protein was identified, allowing determination of the *Po*Sl pro-sequence. Pro-sequences are usually removed from the catalytic domain by self-digestion or by another protease upon completion of protein folding. The peptide bond K^{-1}–G^{1}, which connects the *Po*Sl pro-sequence to its catalytic domain, should be cleaved by a protease with trypsin-like specificity different from the known *Po*Sl substrate preference (Palmieri *et al.*, 2001). Moreover, no significant activity of *Po*Sl towards the chromogenic peptide D-Val-Leu-Lys-*p*-nitroanilide was observed (data not shown). On the other hand, *Po*Sl has a D residue in the position corresponding to E156 in subtilisin BPN′. Subtilases with a negative charge on this residue are reported to have an additional ability to cleave after K at the P1 position (Gron *et al.*, 1992; Voorhorst *et al.*, 1997). Furthermore, all the interactions of the substrate P4–P4′ residue side chains with the S1 pocket can contribute to binding; thus it cannot be excluded that *Po*Sl zymogen can undergo autolysis to self-remove the pro-peptide from unprocessed protein during the maturation process.

*Po*Sl shows a high identity with hypothetical proteins from *N. crassa* and *Trichoderma reesei*, and Pr1C, a 'bacterial-type' subtilisin-like serine protease from the fungus *M. anisopliae*. Eleven subtilisin-like proteins were identified in the latter fungus (Bagga *et al.*, 2004), ten of which displayed identities ranging from 93 to 98 %, being classified as proteinase K-like subtilisins. The unique exception was the 'bacterial-type' subtilisin Pr1C, which is largely divergent from the others. Among the bacterial subtilases, Vpr, a minor extracellular serine protease from *B. subtilis* (Sloma *et al.*, 1991), shows the highest identity with *Po*Sl. This bacterial subtilase belongs to the pyrolysin family, according to the classification of Siezen & Leunissen (1997).

Interestingly, a *Ph. chrysosporium* genomic sequence encoding a hypothetical protein, *Pc*Sl, showing the highest identity with *Po*Sl (65 %), has been identified and the corresponding cDNA was amplified and sequenced, thus demonstrating the expression of the *pcsl* gene in this fungus. The *posl* and *pcsl* genes shared 57 % of their sequences and their intron/exon structures were very similar (Fig. 4). Furthermore, all the glycosylated Asn identified in *Po*Sl was located in consensus sequences conserved in *Pc*Sl, suggesting common structural features between the two proteins. Indeed, a 76 kDa protease, inhibited by PMSF, has previously been reported in *Ph. chrysosporium* culture broth. This enzyme is the most abundant protease produced in the presence of both excess and limiting nitrogen source (Dass *et al.*, 1995). These data suggest that this protease is the one encoded by the *pcsl* gene. Hence, a new subgroup of subtilisin-like proteases from ascomycete and basidiomycete fungi, belonging to the pyrolysin family, can be defined, including the proteases *Po*Sl and *Pc*Sl from the basidiomycete fungi *Pl. ostreatus* and *Ph. chrysosporium*, the hypothetical protein from *N. crassa*, and Pr1C from *M. anisopliae*. In Fig. 5 the alignment of these protease sequences is shown.

The spacing between the catalytic H and S residues in *Po*Sl, as well as for several other members of the pyrolysin family and all those of the new fungal pyrolysin subfamily, is approximately 150 aa longer than that of subtilisins (Voorhorst *et al.*, 1996). This insert region has significant similarity to sequences of other protein classes (M8/M33 zinc peptidases, Ring-type Zinc Finger proteins, vacuolar sorting receptor, transferrin receptor), and has been named as a protease-associated (PA) domain (Mahon & Bateman, 2000). The role of the PA domain remains unclear; however, it has been supposed that the position and the size

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PoSl            MKGVLVWSILSAGCALAVTPLSSVKRVTNLPIVANKFIVEVDSTSDIPTKRSNPHDALYE -65
PcSl            ---------------------------------------------------KIGQTHEALYS
N. crassa       ----MVRLGLATTLLAAASFAQAAHQKAPAVVPGAYIVEYEDSHDPTSILASIKGDATIR
Pr1C            ------------------------------------------------------------

PoSl            SLRKRAIGFKVDREFNTQGLFVGAALTLSDAQDVGQIQSTTGVKSIRPVRAFDRPKPVKV -5
PcSl            HMRKRNVGFQVDKEFNSPGLFVGAAVTLQNADDKNQVASMPGVQAIRPVTLIPPPKPVFS
N. crassa       KDIRHELFKGASFQFKDLNKADDLASKVAAMSGVKALYPVRRYSIPEHTVH-----STGS
Pr1C            MKLDYELFKGVSVQLHDVDKAHEKAAKLADTPAVKVVYPVQLFNMPKPKVEWIAKDGTKA
                   1                                        *
PoSl            QVVKGPDDPALPPDSESTHVITGVDKLHAQGITGAGIKIGILDTGIDYTHPTLGGAFGPG 56
PcSl            HVVTGKSDPAVPADTESTHVITGVDKLHAQGITGKGIKIGIIDTGIDFTHPDLGGGIGPG
N. crassa       AVQEVVAKRDTGNDTFSPHLMTQVNKFRDSGITGKGIKIAVIDTGVDYLHPALGGCFGPG
Pr1C            PGGLLSSRADDGADIFSPHVMTQVDKLRAKGVTGKGVKIAVVDTGIDYKHPALGGCFGHG
                                              *
PoSl            NKVIGGFDLVGDDYDGTNTPVPDPDPLDQCAGHGTHVAGIIGANPDNAFNISGVAFQASL 116
PcSl            FKIIGGFDFVGDAFNGSNTPVPDPSPLDQCNGHGTHVAGIIGANPGNAFNISGVAFDASI
N. crassa       CLVSYGTDLVGDDFNGSNTPVPDSDPMDTCNGHGSHVLGLLSANTNNPYGIIGAAPDVTL
Pr1C            CLVAFGTDLVGDAYDGSNTPHPDPDPMD-CGGHGSHVAGIVAAQPN-TFGFTGAAPGATL

PoSl            SAYRVFGCVGFVTDDVLVDALLLGFNEGQDILTLSLGGADGWTESVSAVVASRIAATGKV 176
PcSl            TSYRIFGCSGSTTDDVIVDALLRGVSEGQDILTMSLGGPDGWTENTASVVSSRISDMGKI
N. crassa       GAYRVFGCSGDVGNDILIEAYLKAYDDGSDIITASIGGASGWPEDSWAAVVSRIVEKGVP
Pr1C            GAYRVFGCKGQAGNDVLIAAFNQAYQDGANIITASIGGPSGWSEDPWAEAVSRIVDKGVP

PoSl            VTIAAGNDGASGAWYTSSPGNGIDVISVASLDNTVVP-LQTAQVHGVTHDPIIYQDTFPL 235
PcSl            VTIAAGNDGADGMFFTSGPGNAIDAISVASLDNTVIP-LQNATVHGVQHDPITYFDALPL
N. crassa       CLVSAGNDGATGIFYASTAANGKRVTAVASVDNILAPALLSEASYSVANGSLSTFGFTAG
Pr1C            CTVSAGNEGAEGIFYASTAANGRRVSAIASYDNVQTPSLIYNAKYQIDNGADTKFGYVPS

PoSl            P----INDTRPIFATSTDVTVVDDACNPLPDSTPDLSGFVVIVRRGTCTFVQKLGNVAAK 291
PcSl            P----ITDTLPIFATSTNTTIDDDACDPLPDSTPDLSKFIVIVRRGTCTFVQKLTNIAAK
N. crassa       SPSAWANVSLPVWSVNFNTADAANGCEAFPDDTPDLSKYIVLIRRGTCTFVQKAQNAAAK
Pr1C            EPASWDGVTLPAWSNSLDPTIPDDGCDFFPANTPDLSKYIVLIRRGSCSFAQKVNNAVAK

PoSl            GAKVTLIYDN-GSGFGAIDTGAFVA------SFIQAADGEFLVQQFASGQNVSLSFPQ-- 342
PcSl            GGNVSLIYDN-GNGFADIDVGNFTS------ALIQAADGEFLVQQFASGANVSLSFPQ--
N. crassa       GAKYIIYYNN-ASGSTKVDVSAVAD--VKAAAMVTSETGAAWIKALQAGTQVTVNMADPE
Pr1C            GAKYVIVYNNNAVGAIPMDLTGVPAGSIKAASMIDGTTGATFINALKDGKKLTLKMVSPQ

PoSl            ---SGGLIQFPDPAGGLISSFTSYGPSNDFFFKPAVAAPGGNILSTLPVNLGLFGIESGT 399
PcSl            ---TGASTQFPDPTGGLISSFTSYGPTNDMFFKPAVSAPGGNILSTFPVPLGSFAVLSGT
N. crassa       TAPKNLNNFPNTATPGFLSTYTSWGPTYEVDVKPQISSPGGMILSTYPRALGSYAVLSGT
Pr1C            KTDSEVSTSNNTITGGALSTFTSWGPTWEMDTKPQFGAVGGNVLSTYPRALGSYAVLSGT
                *
PoSl            SMATPFVAGSAALLFQAKGTSAAVGRSARTLFETTAQRVPSTHTDGDPLQTLTQQGAGLI 459
PcSl            SMATPFMAGVSALLFGLKGNTPEVGRSARDLFETTAQLVPSSHTDGDPLQTVAQQGAGLV
N. crassa       SMACPLAAATWALVMQKRGTKDPKVLENLFSATAHPNLFNDGTKTYPMLAPVAQQGAGLI
Pr1C            SMSCPQTAGIIALIHQVRGTYDPELIQNLLSANANPQLFNDGTKFYDFLAPVPQQGGGLV

PoSl            NVFNAIHTTTIVSPGELILNDTAHFKGTQQFTVRNAGSSAKTYTLRHIPAGTAVTVT-PG 518
PcSl            NAFQALTTDIIITPGELLTNDTANFKSLHTFKIKNTGKTAESFKISHVPAGTAISLQ-NG
N. crassa       QAWDAANANALLSVSSISFNDTEHFKPLQSFEVTNTGKKAVTYQLGHTSAATAYTFANDT
Pr1C            QAYDAAYATTLLSPSSLSFNDTDHFVETLSFKLQNTDSKGITYKITHTPAMTMYALG-DG

PoSl            TIFPADGPVPLSTDFASVSLSTSKFTLAPGKTQTVTARFTPPAAADPSTFPVFSGFIQIE 578
PcSl            TAFFNDGPVPLTSQFATVKFSETSVTVHPGQTQEITAHITPPSGVDPSVLPVFSGFIQVE
N. crassa       SIGPAAFPNELVDAKATLVLTPAKLTLNPGQKKTVTVLAIPPLGLDAKRLPVYSGYITLN
Pr1C            SPSVQQFPNDAVQAAATIKLSETSVTLNGGQSKSISVSPTPPQGLDAKRLALWSGYIVIN

PoSl            SGTEQ-VQVSYLGLKASLKDKQVIDNTDFFFGVPTPVLTDPNGEVQTSPRNYS------- 630
PcSl            SATET-LHVTYLGVAAALKDARVVDDTDVFFGVDLPVLTDPAGDFLTNATNFT-------
N. crassa       GTDSTGYSLPYQGVVGSMRSVTVLDKQNSYLSQSSDATYAPVAAGTTFTLPPAGKANDTL
Pr1C            GTDGTSLSLPYQGLTGSLHNSAVLGANNTWISKSTDKKSNPVPPNSTFVIPAPGNAG---

PoSl            FLASDFPTLIFRLAFGSPKVVVDLVSPTINFKPTLNTRALGDTHGSFFSFPHRVKTGSFA 690
PcSl            FVGTDVPQLLMRLAFGTPLLRADLVDPAEKIATTLNKRAD------------AAKGGSAA
N. crassa       YATTVYPTIVLTLSMGSAEVHADVVNSKG--------------------------------
Pr1C            -SNDTLPQLTVSLYLGSRKIRADIVPLTTCPSKNL--------------------TTEFQ

PoSl            QVPTLGTVFELDFTSRNNDDPTDNGFNTVAIDPPTFANGTTIPNGQYRLLLRALRVTGDP 750
PcSl            AIKTLGSLFEFDFQPRNTDEDDGTDFNVFELDTPVFANGTTIPNGLYKILLRVLKVTGDP
N. crassa       --KTIGQVLTFPARWNPR------GTFEWNWDG-ALSDGTYAPADTYKITLKALKIYGNS
Pr1C            GIKTIGQPYNFPALWGTR------GLNTFPWDG-RLDSGNYAPPGKYKFVVRALRIFGDE

PoSl            TNEADFESFLSPIIGVNAP--------- 769
PcSl            NNEADFESWLSPIIGVNAPSS-------
N. crassa       KWPLDWETQTTEPFTIKYAAKSKRAFTA
Pr1C            KKKDDWDVSTSPALHIKYQ---------
```

Fig. 5. Sequence alignment. The amino acid sequence of *Po*Sl is compared with those of *Pc*Sl from *Ph. chrysosporium*, a putative protein from *N. crassa* (accession no. Q7RZV0) and PR1C from *M. anisopliae* (accession no. Q8X1Y7). Gaps are denoted by dashes. The regions highlighted in grey are conserved among *Po*Sl and the analysed proteins. The amino acid residues that form the catalytic triad are denoted by an asterisk. The numbers represent the positions of the amino acid residues starting from the N-terminus of the mature *Po*Sl protein.

of the inserted domain might allow interference with substrate access and thus it could be involved in substrate specificity (Luo & Hofmann, 2001).

All the members of the new fungal pyrolysin subfamily have a long C-terminal extension, which is less conserved than the catalytic region (Siezen & Leunissen, 1997; Voorhorst *et al.*, 1996). Mature *Po*Sl shows a cleaved peptide bond in this region, probably caused by an autocatalytic event, on the basis of its specificity. The cleaved peptide remains associated with the catalytic domain in a non-covalent complex, as demonstrated by the two N-terminal sequences obtained from the native protein and confirmed by the MALDIMS characterization of the protein. This finding suggests that the cleaved bond belongs to an exposed loop of the protein and that, after the proteolytic event, the newly generated peptide remains tightly bound to the core of the protein.

The occurrence of this class of proteases in a wide variety of fungal genera could be suggestive of the crucial physiological role played by these proteins in the micro-organisms which produce them. With regard to *Pl. ostreatus*, the role proposed for *Po*Sl, a member of this subfamily, would be to start a cascade of proteolytic reactions, leading to the activation of other extracellular proteases.

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REFERENCES

Bagga, S., Hu, G., Screen, S. E. & St Leger, R. J. (2004). Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. *Gene* **324**, 159–169.

Bode, W., Papamokos, E. & Musil, D. (1987). The high-resolution X-ray crystal structure of the complex formed between subtilisin Carlsberg and eglin c, an elastase inhibitor from the leech *Hirudo medicinalis*. Structural analysis, subtilisin structure and interface geometry. *Eur J Biochem* **166**, 673–692.

Bryan, P., Wang, L., Hoskins, J., Ruvinov, S., Strausberg, S., Alexander, P., Almog, O., Gilliland, G. & Gallagher, T. (1995). Catalysis of a protein folding reaction: mechanistic implications of the 2·0 A structure of the subtilisin-prodomain complex. *Biochemistry* **34**, 10310–10318.

Dass, S. B., Dosoretz, C. G., Reddy, C. A. & Grethlein, H. E. (1995). Extracellular proteases produced by the wood-degrading fungus *Phanerochaete chrysosporium* under ligninolytic and non-ligninolytic conditions. *Arch Microbiol* **163**, 254–258.

Dosoretz, C. G., Dass, B., Reddy, A. & Grethlein, H. E. (1990). Protease-mediated degradation of lignin peroxidase in liquid cultures of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **56**, 395–400.

Giardina, P., Cannio, R., Martirani, L., Marzullo, L., Palmieri, G. & Sannia, G. (1995). Cloning and sequencing of a laccase from the lignin degrading basidiomycete *Pleurotus ostreatus*. *Appl Environ Microbiol* **61**, 2408–2413.

Gron, H., Meldal, M. & Breddam, K. (1992). Extensive comparison of the substrate preferences of two subtilisins as determined with peptide substrates which are based on the principle of intramolecular quenching. *Biochemistry* **26**, 6011–6018.

Jain, S. C., Shinde, U., Li, Y., Inouye, M. & Barman, H. M. (1998). The crystal structure of an autoprocessed Ser221Cys-subtilisin E-propeptide complex at 2·0 A resolution. *J Mol Biol* **284**, 137–144.

Lucas, M. C., Jacobson, J. W. & Giles, N. H. (1977). Characterization and in vitro translation of polyadenylated messanger ribonucleic acid from *Neurospora crassa*. *J Bacteriol* **130**, 1192–1198.

Luo, X. & Hofmann, K. (2001). The protease-associated domain: a homology domain associated with multiple classes of proteases. *Trends Biochem Sci* **26**, 147–148.

Mahon, P. & Bateman, A. (2000). The PA domain: a protease-associated domain. *Protein Sci* **9**, 1930–1934.

Ness, J. E., Kim, S., Gottman, A., Pak, R., Krebber, A., Borchert, T. V., Govindarajan, S., Mundorff, E. C. & Minshull, J. (2002). Synthetic shuffling expands functional protein diversity by allowing amino acids to recombine independently. *J Nat Biotechnol* **20**, 1251–1255.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A. & Sannia, G. (1997). A novel white laccase from *Pleurotus ostreatus*. *J Biol Chem* **272**, 31301–31307.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B. & Sannia, G. (2000). Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. *Appl Environ Microbiol* **66**, 920–924.

Palmieri, G., Bianco, C., Cennamo, G., Giardina, P., Marino, G., Monti, M. & Sannia, G. (2001). Purification, characterization, and functional role of a novel extracellular protease from *Pleurotus ostreatus*. *Appl Environ Microbiol* **67**, 2754–2759.

Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G. & Giardina, P. (2003). Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. *Enzyme Microb Technol* **33**, 220–230.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Siezen, R. J. & Leunissen, J. A. (1997). Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci* **6**, 501–523.

Sloma, A., Rufo, G. A., Jr, Theriault, K. A., Dwyer, M., Wilson, S. W. & Pero, J. (1991). Cloning and characterization of the gene for an additional extracellular serine protease of *Bacillus subtilis*. *J Bacteriol* **173**, 6889–6895.

Sroga, G. E. & Dordick, J. S. (2001). Generation of a broad esterolytic subtilisin using combined molecular evolution and periplasmic expression. *Protein Eng* **14**, 929–937.

Voorhorst, W. G., Eggen, R. I., Geerling, A. C., Platteeuw, C., Siezen, R. J. & Vos, W. M. (1996). Isolation and characterization of the hyperthermostable serine protease, pyrolysin, and its gene from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **271**, 20426–20431.

Voorhorst, W. G., Warner, A., de Vos, W. M. & Siezen, R. J. (1997). Homology modelling of two subtilisin-like proteases from the hyperthermophilic archaea *Pyrococcus furiosus* and *Thermococcus stetteri*. *Protein Eng* **10**, 905–914.

Wright, C. S., Alden, R. A. & Kraut, J. (1969). Structure of subtilisin BPN′ at 2·5 angstrom resolution. *Nature* **221**, 235–242.

Yabuta, Y., Takagi, H., Inouye, M. & Shinde, U. (2001). Folding pathway mediated by an intramolecular chaperone: propeptide release modulates activation precision of pro-subtilisin. *J Biol Chem* **276**, 44427–44434.

Yabuta, Y., Subbian, E., Oiry, C. & Shinde, U. (2003). Folding pathway mediated by an intramolecular chaperone. A functional peptide chaperone designed using sequence databases. *J Biol Chem* **278**, 15246–15251.

Atypical laccases from the white-rot fungus *Pleurotus ostreatus* and their application for the treatment of industrial coloured effluents

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1. Introduction

1.1 Laccases

White-rot fungi are the most efficient decomposers of lignocellulose because of their capability to synthesize the relevant hydrolytic (cellulases and hemicellulases) and oxidative (laccases, lignin-peroxidases and Mn-peroxidases) extracellular enzymes required to degrade the major components of substrates (cellulose, hemicellulose, and lignin) into low-molecular-weight compounds that can be assimilated in fungal nutrition [1]. Recently, extensive research on these fungi has been conducted with the aim of isolating new organisms able to secrete new enzymes with capability to be used in industrial applications, such as bioremediation of polluted soils and industrial waste-waters, biobleaching and biopulping in pulp and paper industries, textile and food industries, *etc*.

Fungal laccases (benzenediol: oxygen oxidoreductases; EC1.10.3.2) are ligninolytic enzymes that have been isolated from various fungi [2]. They belong to the class of the blue oxidases containing 4 copper atoms/molecule distributed in three different copper binding sites [3, 4]. The type-1 site is responsible for the intense blue colour of the enzyme due to a maximum absorbance at 605 nm; the type-2 site does not exhibit signals in the visible absorbance spectrum; and the type-3 site incorporates two copper centres and is responsible for a band near 330 nm. All these copper ions are involved in the catalytic mechanism. Laccases reduce oxygen to water and simultaneously perform a one electron oxidation of aromatic substrates (polyphenols, methoxysubstituted monophenols, aromatic amines, etc.). These enzymes are present in multiple isoforms, depending on the fungal species and environmental growth conditions [5, 6] .

1.2 *Pleurotus ostreatus* laccases

Laccase isoenzymes produced by *Pleurotus ostreatus*, a white rot basidiomycete fungus, have been extensively studied. Five different isoenzymes have been purified and characterized: POXC, POXA1b, POXA1w, POXA3a and POXA3b; seven different genes and the corresponding cDNAs have been cloned and sequenced [7, 8, 9].

POXC is the most abundantly isoenzyme produced under all the growth conditions examined [7] (maximum production level 25 mg/l); it shows all typical laccase characteristics: four copper ions/molecule, acidic pI, and stability to proteolytic degradation. All the others *P. ostreatus* laccases (POXA1w, POXA1b, POXA3a and POXA3b) are atypical phenol-oxidases [10, 8, 11].

POXA1w shows a remarkable high stability with respect to both pH and temperature if compared with that of POXC and of other known laccases, and it exhibits a neutral pI (6.7). The most striking characteristic of this protein is the lacking of the typical blue colour and its unusual metal content. UV/visible spectrum, atomic absorption and polarography proved that this enzyme contains only 1 copper atom/molecule- instead of the usual 4 atoms- and furthermore, 2 zinc atoms and 1 iron atom. Nevertheless, the classification of this enzyme as laccase was based on: i) the high degree of identity of the determined stretches of primary structure with the corresponding sequences of known laccases; ii) the use of O_2 as oxidative substrate and the lack of formation of H_2O_2 as a product in the catalysed reaction; iii) the almost standard pattern of substrate specificity.

POXC production is strongly increased by the presence of copper ions, whilst POXA1w production is substantially unaffected under this condition; furthermore a new neutral laccase isoenzyme (POXA1b) is produced in copper supplemented cultures. POXA1b shows the same characteristics of POXA1w concerning the stability with respect to pH and temperature. Furthermore POXA1b is only partly secreted [12].

In this study we review purification and characterisation of two closely related isoenzymes, POXA3a and POXa3b, and their role in decolourisation of Remazol Brilliant Blue R (RBBR), a

molecule frequently used as starting material in the production of many polymeric commercial dyes [13]. We also describe optimal conditions for using selected purified laccases or immobilized crude laccase mixtures in the treatment of this model dye [14].

2. Materials and Methods

2.1. Organism and culture conditions

White-rot fungus, *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) was maintained through periodic transfer at 4 ◦C on PDY agar plates (2.4 % potato dextrose and 0.5 yeast extract, Difco Laboratories, Detroit, MI). Incubations were carried out as previously described [10] and laccase production was induced by addition of 150μM copper sulphate.

2.2. Enzyme purification

Secreted proteins were precipitated from the filtered medium by addition of $(NH_4)_2SO_4$ up to 80% saturation and, after extensive dialysis, loaded onto a DEAE Sepharose Fast Flow (Pharmacia Biotech Inc.) column as previously described [10]. Two fractions containing laccase activity, recovered with the equilibrating buffer, were separately pooled, concentrated on an Amicon PM-10 membrane and equilibrated in Tris–HCl 50mM pH 8.0. Each pool was loaded onto an anion exchange Mono Q HR5/5 (Pharmacia) column in a fast protein liquid chromatography system (FPLC, Pharmacia) equilibrated with the same buffer. The active fractions were pooled, concentrated and loaded onto a gel filtration Superdex 75 PC 3.2/30 column in a SMART System (Pharmacia); the active fractions were pooled and desalted.

2.3. Enzyme assays

Laccase activity was assayed at 25 ◦C, using 2,2'-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), DMP and syringaldazine as substrates as previously described [10].

2.4. Remazol Brilliant Blue R transformation by laccase

The decolourising experiments were performed using the dye RBBR 50 μM, and the purified laccase POXC or POXA3 (1 U/ml) in sodium acetate 20 mM, pH 4.5 buffer. The same incubation was performed using a laccase mixture of POXC and POXA3 (1:1, U:U ratio) from 1 to 10 U/ml final activity. Control samples without enzyme, were run in parallel under identical conditions. All reactions were incubated at 20 ◦C for 100 min.

2.5. Effect of temperature and pH on dye decolourisation

The effect of temperature on dye decolourisation was studied incubating the reaction mixture, prepared as above described, at 20, 30 or 40 ◦C. The effect of pH on dye decolourisation was studied performing the experiments in the pH range 4–7 using the Mc Ilvaine buffer. In all cases, dye decolourisation was monitored as above described.

2.6. Immobilization method

The immobilization procedure was carried out at 4 °C. Different amounts of crude laccase preparation (from 0.2 to 660 U) were mixed with 5 ml of 3% sodium alginate solution (low viscosity, Sigma), 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 agitation. 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 5 ml of sodium alginate solution was about 3.5 g. The beads were stored wet at 4 °C. The immobilization yield was calculated as the fraction of laccase activity found after dissolution of alginate beads (by incubation for 3 h at 4 °C in 50 mM sodium phosphate buffer pH 7.0, 50 mM EDTA) with respect to the activity of the enzyme added to the soluble alginate preparation.

2.7. Continuous packed-bed reactor

A glass column (130 mm x 17 mm, working volume 25 ml) was filled with 24 g of copper alginate beads. The flow rate was 0.2 ml/min, and the feed solution was 50μM RBBR in 20 mM sodium acetate buffer, pH 4.5. Samples were collected after the passage of at least 25 ml of feed solution to

reach the concentration equilibration (time 0). All reactor systems were operated at room temperature (approximately 20 °C).

2.8. Chitosan treatment of alginate beads

A 24 g portion of copper alginate beads was transferred into a solution of 0.15% (w/v) chitosan, in 20mM sodium acetate buffer pH 4.5 containing 0.1 M CuSO4, and incubated for 20 h at 4 °C on a rotary shaker. After that, the alginate chitosan beads were extensively washed with 20 mM sodium acetate buffer pH 4.5 and used to fill the glass vessel reactor as described above.

3. Results

3.1. Purification of laccase isoenzymes POXA3a and POXA3b

Two active different protein fractions (POXA3a and POXA3b) were recovered after chromatography (fig 1) of the ammonium sulphate precipitate of culture broth collected after 10 days. Both isoenzymes were purified to homogeneity (gel filtration chromatography, fig 2, native PAGE and isoelectrofocusing). Isoelectric points of POXA3a and POXA3b are 4.3 and 4.1, respectively, and the molecular mass, determined by gel filtration chromatography, is 56 kDa for both proteins. Specific activities towards ABTS of POXA3a and POXA3b are 2000 and 1050 U/mg, respectively. However purified isoenzymes displayed a more complex pattern when analysed by SDS–PAGE, in fact three bands (MW 67, 18 and 16 kDa, respectively) were observed for both proteins (fig 3).

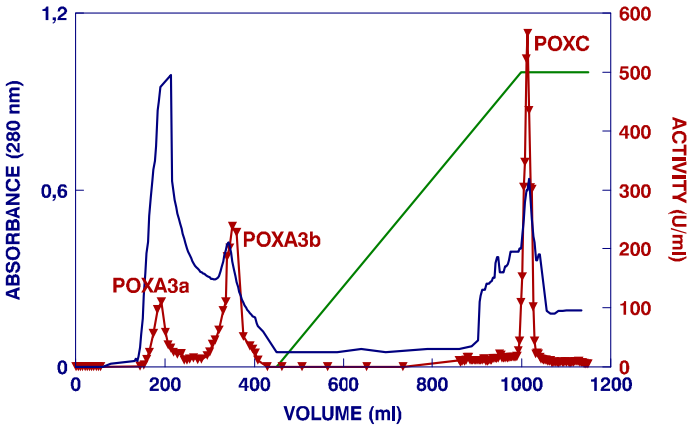

Figure 1: DEAE-Sepharose chromatography of *P. ostreatus*-secreted proteins. DEAE-Sepharose fast flow elution profile of proteins and laccase isoenzymes secreted by *P. ostreatus* is shown with three different active protein fractions (POXA3a, POXA3b, and POXC).

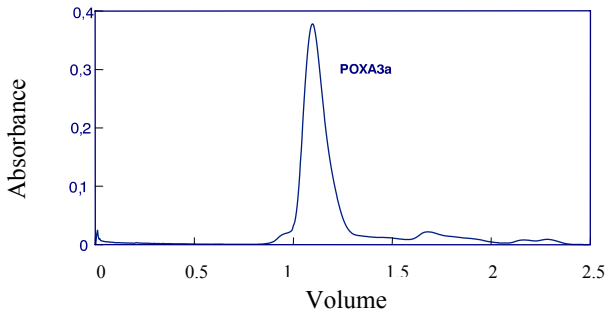

Figure 2: Gel filtration chromatography of POXA3a fraction from MONOQ: a single peak is detectable

3.2. Metal content analyses

UV-Vis absorption spectra of POXA3a and POXA3b display the characteristic absorbance peak at 600 nm, due to the presence of type I copper; copper content, determined by atomic absorption, results to be 3.3 copper/protein (mol/mol) for both proteins.

3.3. Structural analyses

The two isoenzymes were analysed by MALDI-MS: the mass spectra showed a peak exhibiting a molecular mass centred at about 60,860.4 Da for POXA3a and 60,700.6 for POXA3b; moreover the spectra showed the occurrence of two other components whose molecular masses were measured as 16,870.5 and 18,103.8 Da for POXA3a and 16,838.1 and 18,090.1 Da for POXA3b. The N-terminal sequences of the 60 kDa subunits are identical for both POXA3a and POXA3b, while the same analyses, carried out on the four 16 and 18 kDa subunits, revealed that all subunits have a blocked N-terminus.

Mass spectrometric analyses (peptide fingerprints) and N-terminus sequencing did not reveal any significant differences between POXA3a and POXA3b large subunits. Furthermore these analyses show that differences between the 18 and 16 kDa subunits of both isoenzymes are only due to the

presence of a glycosidic moiety on the 18 kDa subunits. None of the sequenced peptides from small subunits shows significant homology with proteins in data banks.

3.4. Cloning and sequencing of poxa3 gene and cDNA

Oligonucleotide-primer mixtures were designed on the basis of POXA3a tryptic peptide sequences. The 500 bp amplified fragment, homologous to known laccase genes, was used to screen a *P. ostreatus* genomic library. Two oligonucleotides were designed using the predicted N and C termini of the protein and were used to amplify POXA3 encoding cDNA. The 1500 bp amplified fragment was cloned and sequenced allowing the determination of *poxa3* gene whole structure. The coding sequence is interrupted by 21 introns.

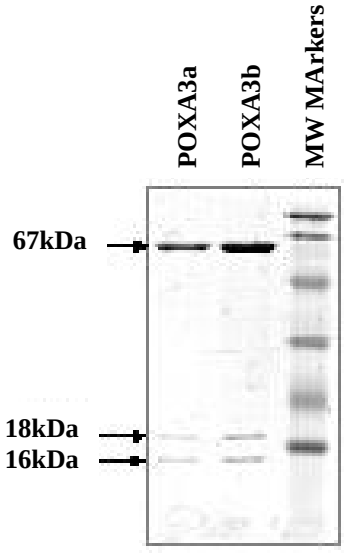

Figure 3: Coomassie stained SDS–PAGE of purified POXA3a and POXA3b laccase isoenzymes.

3.5. POXA3a and POXA3b activation

POXA3 specific activity increases during fungal growth, probably due to proteolytic activation. POXA3 isoenzymes, purified from broth supplemented with Phenylmethanesulfonyl fluoride (PMSF), were incubated with the serine protease from *P. ostreatus* (*Po*Sl) (fig 4). Under this condition, an increase of POXA3 activity was obtained (about 30%), confirming the generation of more active POXA3 isoform(s) due to *Po*Sl-induced proteolysis.

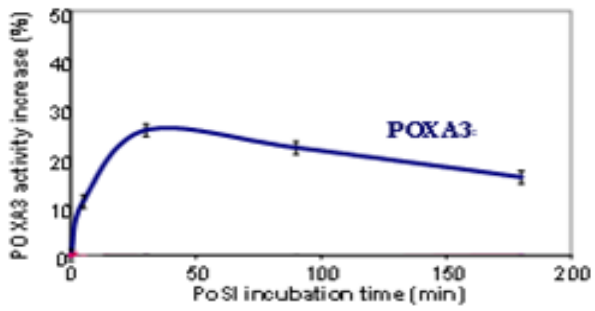

Figure 4: *In vitro* incubation of POXA3 with an extracellular subtilisin-like *Pleurotus ostreatus* protease (*Po*sl)

3.6. Biotransformation of RBBR by *P. ostreatus* laccases

Decolourisation experiments by *P. ostreatus* in solid and liquid media were performed using PDY broth in the presence of veratryl alcohol, added with RBBR at two different concentrations (5 and 50µM). *P. ostreatus* is able to decolourise the dye in all cultural conditions examined, with a temporal relationship between laccase production and dye biotranformation (fig. 5).

In order to elucidate the role of the oxidative activities produced during biodegradation of RBBR by *P. ostreatus*, purified POXA3 (1 U/ml) or POXC (1 U/ml) isoenzymes were incubated at 20°C with RBBR: after 100 min of incubation, 30 and 50% of decolourisation was obtained by POXC and POXA3, respectively. No more than 60% of decolourisation was obtained even when the enzyme concentration or the incubation temperature was increased. Kinetic parameters of the two laccases were also determined using RBBR as substrate: the *K*m values (0.054 and 0.051 mM for POXA3 and POXC, respectively) are very similar whilst the catalytic efficiency (*k*cat/*K*m) of POXA3 (9.0×10^6 mM^{-1} min^{-1}) is six-fold higher than that of POXC (1.5×10^6 mM^{-1} min^{-1}). Therefore, the difference in decolourisation efficiency of the two laccases could be due to a variation in enzymatic turnover rather than in substrate affinity.

The effect of a 1 U/ml (final laccase activity) POXC/POXA3 mixture (1:1; U:U) on RBBR was tested. As shown in figure 6, the enzymatic mixture determines an increase both in the rate and in the final level of dye decolourisation (74%) with respect to each isoenzyme working separately. Also enzyme concentration and incubation temperature affected dye decolourisation.

3.7. RBBR decolourisation by crude laccase complex mixture.

Crude enzymatic mixture obtained after ammonium sulphate precipitation of culture broth supplemented with $CuSO_4$ and ferulic acid was found to be more efficient in RBBR decolourisation than any single component.

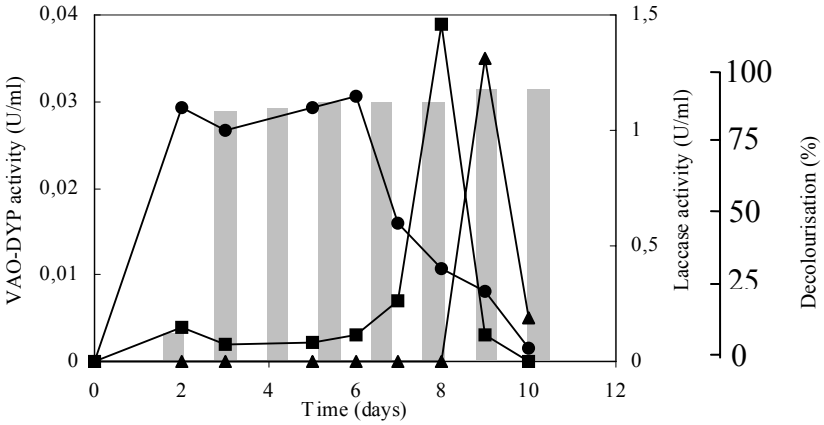

Figure 5: Decolourisation of RBBR in *P. ostreatus* culture supplemented by 50 μM of Remazol Brilliant Blue R. Symbols: laccase activity (●); DYP activity (▲); AAO activity (■); percentage of decolourisation ( ).

The effect of enzyme concentration on dye degradation was studied: increasing the amount of the crude preparation used, from 2 to 100 U/ml of laccase activity a more efficient dye decolourisation (up to 70%) was observed (fig 7).

3.8. Immobilization of crude laccase mixture in copper alginate beads and its performance in dye decolourisation.

Optimal conditions for crude laccase mixture immobilization by entrapment in copper alginate beads were set up. Immobilization yield was 65% when laccase concentrations ranging from 15 to 120 U/ml of sodium alginate solution were used, obtaining beads whose laccase activity ranged from 20 to 100 units per gram of beads (U/g). Immobilized protein mixture in copper alginate beads is more stable if compared with free enzyme mixtures. Optimal conditions for batch decolourisation process were determined (pH 4.5, temperature 20°C, 100 Units per gram of beads).

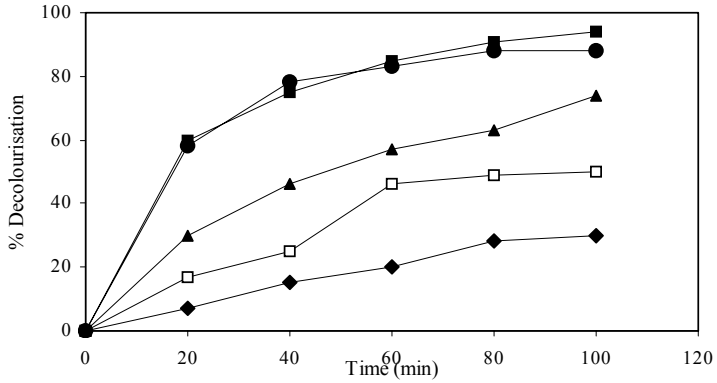

Figure 6: Remazol Brilliant Blue R decolourisation after treatment with laccase isoenzymes in different reaction conditions; POXC (1 U/ml; 20 °C) (□); POXA3 (1 U/ml; 20 °C) (□); POXC/POXA3 mixture (1:1, U:U; 1 U/ml; 20 °C) (▲); POXC/POXA3 mixture (1:1; U:U; 10 U/ml; 20 °C) (■); POXC/POXA3 mixture (1:1, U:U; 1 U/ml; 30 °C) (●).

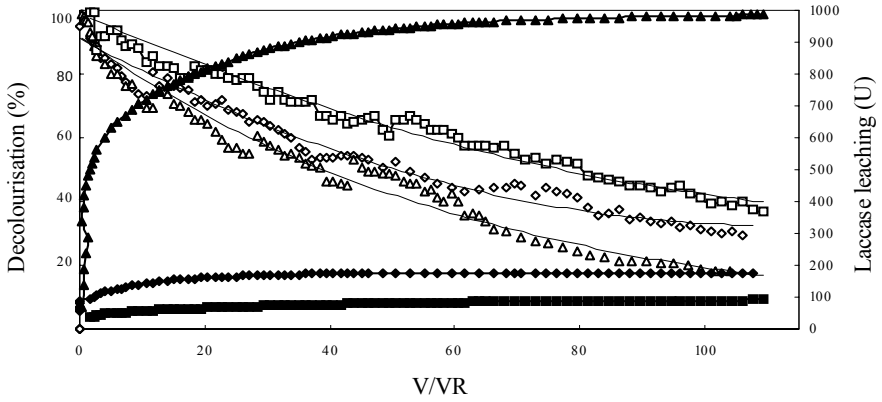

Figure 8: RBBR decolorization in continuous experiments by packed bed bioreactors. Bioreactor 1 (Δ): 100 U laccase activity/g copper alginate beads. Bioreactor 2 (◊): 50 U/g. Bioreactor 3 (□): 50 U laccase activity/g copper alginate beads treated with chitosan. Laccase leaching was measured (▲, bioreactor 1; ♦ bioreactor 2; ■ bioreactor 3).

3.9. Performance of immobilized laccase mixture in a fixed-bed reactor.

The immobilized enzyme mixture was used over 7 days in a fixed-bed reactor, under the optimal conditions set up in batch experiments, operating at a RBBR loading rate of 20.9 mg L^{-1} h^{-1} (fig 8). In these conditions (bioreactor 1) the final decolourisation percentage was 20% after elution of 100 V/V_R (volumes of dye solution respect to the reactor retention volume, V_R=18 ml). The leached laccase activity, determined during this continuous operation, quickly increased at the beginning of the decolourisation process, and the total activity washed out amounted to 1000 U. Attempts were performed to decrease enzyme release.

An experiment (bioreactor 2) was performed using a lower amount of immobilized enzyme mixture (50 U/g). As shown in figure 8, the final decolourisation percentage obtained by this bioreactor was enhanced (about 30%) and laccase leaching was 5-fold decreased with respect to the previously mentioned continuous experiment (bioreactor 1). Further improvements in the final decolourisation level (40%) and in laccase retention were obtained using chitosan treated beads (bioreactor 3). As a fact, chitosan is known to improve beads mechanical resistance and significantly to reduce leaching [15, 16].

4. Discussion

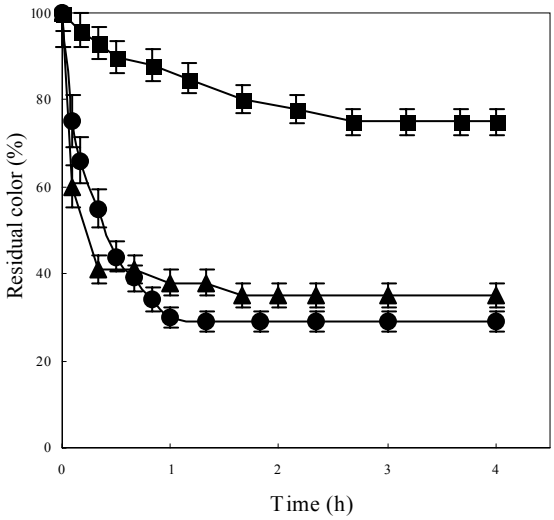

Figure 7: RBBR decolourisation obtained by incubation with different amounts of crude laccase mixture. ■, 2 U/ml laccase activity, 20 °C; ● 20 U/ml laccase activity, 20 °C; ▲100 U/ml laccase activity , 20 °C.

POXA3a and POXA3b, two closely related laccase isoenzymes, are produced in *P. ostreatus* copper supplemented cultures. SDS–PAGE and MALDI-MS analyses of purified POXA3a and POXA3b reveal the presence of three different polypeptides of 67, 18 and 16 kDa, whereas the native proteins behave homogeneously (as demonstrated by gel filtration chromatography, isoelectrofocusing and native-PAGE analysis). None of the previously characterised *P. ostreatus* laccase isoenzymes shows similar behaviour, and all of them are monomeric proteins. On the other hand, it has been reported that some laccase enzymes from *Phellinus ribis* [17], *Trametes villosa* [18] and *Rhizoctonia solani* [19] show homodimeric structure.

An unique *poxa3* gene has been identified and protein sequence deduced by cDNA has been verified by means of MALDI-MS mapping against the POXA3a and POXA3b large subunits. It is not possible to univocally associate this gene to either POXA3a and/or POXA3b, or to exclude the existence of another *poxa3* gene. Deduced amino acidic sequence contains all putative copper-binding residues, as well as the five Cys residues found in all the known laccase sequences.
Sequence data from the 18 kDa POXA3a subunit, accounting for about 50% of entire sequence, did not give information on the nature of this subunit because of the absence of significant homology with other known proteins. Furthermore, no sequence encoding these peptides have been recognised in the 3' and 5' flanking region of the *poxa3* gene, thus excluding that small subunit could be originated from maturation of a single polypeptide chain containing the largest one.
Due to their enzymatic properties and relatively low production cost, laccases represent a promising tool for applications in the textile industry effluent bioremediation.
POXC and POXA3 laccases are able to perform RBBR transformation *in vitro*. In particular, POXA3 shows higher decolourisation efficiency with respect to POXC, as also confirmed by kinetics constants determined using RBBR as substrate. It has been also verified that a more efficient process take place in the presence of a mixture of POXC and POXA3 suggesting that RBBR degradation *in vivo* could be due to a concerted action of the two isoenzymes. Hence, we demonstrated that a complete RBBR transformation can be obtained using a simple mixture of two laccase isoenzymes in the absence of any redox mediators and selecting optimal enzyme concentration, temperature and pH values. These findings differ from those reported for other laccases, which also transform RBBR in the absence of redox mediators, but in this case only an incomplete decolourisation is obtained [20, 21, 22]. On the other hand, other authors [23, 24] reported that RBBR is decolourised only when small molecular weight redox mediators are added to the laccase enzymes. These results suggest a strict correlation between decolourisation and detoxification. Reasonable basis for development of a cheaper biotechnological colour reduction process have been provided: a crude laccase mixture preparation was used to decolourise RBBR. Once the potentiality of the enzymatic system had been assessed, the mixture was immobilized by entrapment in copper alginate beads. RBBR decolourisation efficiency was about 70% even after 20 cycles of stepwise dye additions in batch operations. Different strategies for continuous decolourisation in a fixed-bed bioreactor were analysed. The best performances were obtained by decreasing enzyme loading and improving laccase retention by coating the alginate beads with chitosan.

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5. References

[1] Kirk TK, Farrell RL. Enzymatic "combustion", the microbial degradation of lignin. *Ann Rev Microbiol* 1987; 41:465–505.
[2] Yaropolov AI, Skorobogatko,OV, Vartanov SS, and Varfolomeyev SD. *Appl. Biochem. Biotechnol* 1994; 49: 257–280.
[3] Reinhammar B, and Malstrom BG Copper Proteins (Metal Ions in Biology) (Spiro, T. G., ed) 1981 pp. 109–149, John Wiley & Sons, Inc., New York
[4] Solomon E.I, Sundaram UM, and Machonkin TE *Chem. Rev.* 1997; 96: 2563–2605.
[5] Collins PJ, and A. D. W. Dobson.. Regulation of laccase gene transcription in *Trametes versicolor*. *Appl. Environ. Microbiol*. 1997; 63:3444–3450.
[6] Mansur M, Suarez T, Fernandez-Larrea J, Brizuela MA, and Gonzalez AE.. Identification of a laccase gene family in the new lignindegrading basidiomycete CECT 20197. *Appl. Environ. Microbiol*. 1997; 63:2637–2646.
[7] Giardina P, Aurilia V, Cannio R, Marzullo L, Amoresano A, Siciliano R, et al. The gene, protein and glycan structures of laccase from *Pleurotus ostreatus*. *Eur J Biochem* 1996; 235:508–15.z
[8] Giardina P, Palmieri G, Scaloni A, Fontanella B, Faraco V, Cennamo G, et al. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. *Biochem J* 1999; 34: 655–63.

[9] Giardina P, Cannio R, Martirani L, Marzullo L, Palmieri G, Sannia G. Cloning and sequencing of a laccase gene from the lignin degrading basidiomycete *Pleurotus ostreatus*. *Appl Environ Microbiol* 1995; 61:2408–13.
[10] Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G. A novel white laccase from *Pleurotus ostreatus*. *J Biol Chem* 1997; 272:31301–7.
[11] Palmieri G, Cennamo G, Faraco V, Amoresano A, Sannia G, Giardina P. Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. *Enzyme Microb Technol* 2003; 33:220–30.
[12] Palmieri, G, Giardina P, Bianco C, Fontanella B, Sannia G. Copper induction of laccase isoenzymes in the ligninolytic fungus Pleurotus ostreatus. *Appl. Environ. Microbiol.* 2000; 66:920-924.
[13] Palmieri G, Cennamo G, Sannia G. Remazol Brilliant Blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. *Enzyme and Microbial Technology* 2005;36:17–24.
[14] Palmieri G, Giardina P, Sannia G. Laccase-Mediated Remazol Brilliant Blue R Decolorization in a Fixed-Bed Bioreactor. *Biotechnol. Progress* 2005; 21:1436-1441.
[15]Li, X. The use of chitosan to increase the stability of calcium alginate beads with entrapped yeast cells. *Biotechnol Appl Biochem.* 1996; 23, 269-72.
[16]Gaserod, O, Sannes, A, Skjak-Braek, G. Microcapsules of alginate-chitosan. II. A study of capsule stability and permeability. *Biomaterials*. 1999; 20, 773-83.
[17] Min KL, Kim YH, Kim YW, Jung HS, Hah YC. Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. *Arch Biochem Biophys* 2001; 392:279–86.
[18] Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, et al. Purification characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. *Appl Environ Microbiol* 1996; 62:834–41.
[19] Munoz C, Guillen F, Martinez AT, Martinez MJ. Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. *Appl Environ Microbiol* 1997; 63:2166–74.
[20] Lonergan GT, Panow A, Jones CL, Schliephake K, Ni CJ, Mainwaring DE. Physiological and biodegradative behaviour of the white rot fungus *Pycnoporus cinnabarinus* in a 200 litre packed bedbioreactor. *Ferment Technol* 1995; 5:107–11.
[21] Claus H, Faber G, Konig H. Redox-mediated decolourisation of synthetic dyes by fungal laccases. *Appl Microbiol Biotechnol* 2002;59:672–8.
[22] Soares GMB, Costa-Ferreira M, Pessoa de Amorim MT. Decolourisation of an anthraquinone- type dye using a laccase formulation. *Bioresour Technol* 2001;79:171–7.
[23] Nagai M, Sato T, Watanabe H, Saito K, Kawata M, Enei H. Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolourisation of chemically different dyes. *Appl Microbiol Biotechnol* 2002; 60:327–35.
[24] Soares GMB, Pessoa de Amorim MT, Costa-Ferreira M. Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. *J Biotechnol* 2001; 89:123–9.