DEVELOPMENT OF MICROBIAL AND ENZYMATIC BIOSYSTEMS FOR THE TREATMENT OF COLOURED WASTEWATERS

Claudia Del Vecchio

Dottorato in Scienze Biotecnologiche – XXIII ciclo
Indirizzo Biotecnologie Industriale e Molecolare
Università di Napoli Federico II

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Dottoranda:	Claudia Del Vecchio
Relatore:	Prof. Giovanni Sannia
Coordinatore:	Prof. Giovanni Sannia

In ogni cosa ho voglia di arrivare
sino alla sostanza.
Nel lavoro, cercando la mia strada,
nel tumulto del cuore.

Sino all'essenza dei giorni passati,
sino alla loro ragione,
sino ai motivi, sino alle radici,
sino al midollo.

Eternamente aggrappandomi al filo
dei destini, degli avvenimenti,
sentire, amare, vivere, pensare,
effettuare scoperte.
Boris Pasternak

A te papà

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RIASSUNTO

Negli ultimi anni le biotecnologie hanno avuto un formidabile impatto in campo industriale, soprattutto per quanto concerne la valutazione e la conservazione della qualità ambientale. L'inquinamento è un fenomeno antico, ma ha raggiunto aspetti drammatici in questi ultimi anni in seguito all'esplosione industriale, urbanistica e demografica. La situazione sta diventando sempre più grave, in quanto le naturali capacità autodepurative dell'ambiente sono insufficienti a 'sostenere' tutte le sostanze tossiche che quotidianamente sono immesse nell'ecosistema.

Una delle principali cause del crescente livello di inquinamento ambientale, in particolare di quello idrico, è da ricercarsi nell'uso intensivo, in diversi settori dell'industria, di coloranti sintetici. Infatti, benché classificati come rifiuti pericolosi (direttiva 91/689/CEE del Ministero dell'Ambiente e della Tutela del Territorio), i coloranti sono largamente impiegati in diverse attività industriali quali quella alimentare, farmaceutica, cosmetica, tessile, conciaria, della plastica, della gomma, del legno, ecc, e si stima che oggigiorno ve ne siano più di 100.000 tipi commercialmente disponibili [1]. L'industria tessile è tra i maggiori consumatori di acqua e produttori di reflui colorati; considerando che per ogni kg di colorante utilizzato nelle tradizionali tecniche di colorazione dei tessuti sono richiesti da 100 a 200 litri di acqua e stimando una produzione annua di 40 milioni di tonnellate di fibre tessili, il rilascio di acqua contaminata da coloranti risulta essere circa 7 milioni di metri cubi per anno [2-4].

Il rilascio di queste molecole tossiche e colorate comporta notevoli problemi ambientali: spesso le sostanze tossiche contenute in questi scarichi rinforzano reciprocamente i propri effetti dannosi e, quindi, il danno complessivo risulta maggiore della somma dei singoli effetti. Inoltre, molti dei convenzionali metodi di trattamento chimico-fisici si rivelano inadeguati o troppo costosi se rapportati agli ingenti volumi di scarichi da trattare nonché alla complessa struttura chimica delle molecole colorate, appositamente studiata per conferirne elevata stabilità alla luce, all'acqua e agli agenti ossidanti [5, 6]. La maggior parte dei trattamenti fisico-chimici permettono la rimozione del contaminante dai reflui, ma non la sua distruzione; perciò il colorante recuperato viene in seguito collocato altrove, oppure distrutto per incenerimento. Alcuni, inoltre, prevedono l'impiego di composti chimici a loro volta pericolosi per la salute umana e/o l'ambiente, quali agenti sbiancanti come composti clorurati, ozono o perossidi [7].

Per tali ragioni negli ultimi anni l'attenzione per le problematiche ambientali ha dato un notevole impulso allo sviluppo del biorisanamento. Il biorisanamento costituisce un insieme di nuove tecnologie, economicamente sostenibili e a ridotto impatto ambientale da applicare in alternativa ai tradizionali processi di smaltimento, per ottenere l'eliminazione di sostanze inquinanti attraverso sistemi di purificazione di tipo biologico. Inoltre, a differenza delle metodologie tradizionali, la soluzione biologica al problema dell'inquinamento ambientale presenta il vantaggio della grande 'versatilità': la manipolazione genetica degli organismi consente di ampliare ulteriormente le capacità degradative verso substrati specifici.

Due differenti strategie di biorisanamento possono essere applicate al trattamento dei reflui industriali: la biodegradazione e il bioassorbimento. Un numero sempre crescente di microrganismi viene caratterizzato in base alla capacità di decolorare, sequestrare (bioassorbimento) o degradare (biodegradazione) un'ampia varietà di composti organici naturali o sintetici e coloranti artificiali.

La biodegradazione si basa sul principio secondo il quale gli organismi sono capaci di assimilare sostanze tossiche dall'ambiente e utilizzarle per la crescita e lo

sviluppo. Sfruttando, quindi, il naturale potenziale degradativo degli organismi (batteri, lieviti, funghi), i contaminanti vengono convertiti in composti meno tossici o vengono demoliti completamente fino alla formazione di anidride carbonica, acqua e/o altri prodotti finali inorganici (mineralizzazione).
Diverse specie batteriche e fungine, in particolare i funghi *white-rot,* trovano numerose applicazioni nel biorisanamento in virtù della loro capacità di degradare un ampio *range* di composti xenobiotici. La loro potenzialità risiede nella capacità di secernere un complesso sistema di enzimi ossidativi, che per la bassa specificità di substrato che li caratterizza trovano numerose applicazioni sia in processi di degradazione, che di biosintesi [8, 9]. In particolare le laccasi, cuproproteine appartenenti alla classe delle fenolo-ossidasi, che catalizzano l'ossidazione di fenoli, polifenoli e ammine aromatiche variamente sostituite con relativa riduzione di ossigeno molecolare ad acqua, sono commercialmente utilizzate nella demolizione di materiali ligninolitici, nella produzione di etanolo e nei processi di *bioremediation*, essendo in grado di degradare diverse classi di inquinanti, inclusi molti coloranti sintetici.
Il bioassorbimento, invece, consiste nel 'sequestro' delle sostanze tossiche presenti nei reflui da parte delle biomasse microbiche, vive o inattivate. Alcuni funghi, ad esempio, grazie alla peculiare composizione chimica e strutturale della loro parete cellulare si sono rivelati particolarmente efficaci nell'assorbire sostanze inquinanti di varia natura, tra cui coloranti e metalli pesanti presenti nei reflui dell'industria tessile, con rese di rimozione in molti casi maggiori rispetto ai convenzionali materiali adsorbenti [9, 10].

In tale contesto si è inserito il seguente progetto di tesi, finanziato dalla Compagnia di San Paolo e svolto in collaborazione con l'Università degli Studi di Torino [Dipartimento di Biologia Vegetale, Dott.ssa Cristina Varese; progetto "Sviluppo di procedure di biorisanamento di reflui industriali (BIOFORM)], il cui obiettivo è stato quello di identificare nuovi biosistemi ossidativi –microbici ed enzimatici- per il risanamento dei reflui dell'industria del colore.
In particolare nella Sezione 1 del progetto, nell'ambito dello sviluppo di biosistemi microbici ed enzimatici, sono state analizzate, rispettivamente, le capacità decoloranti di ceppi batterici appartenenti al genere *Streptomyces spp* e di enzimi ad attività fenolo-ossidasica quali le laccasi dal fungo *Pleurotus ostreatus* espresse eterologamente nelle forme native e mutate.
Affinchè sia possibile applicare le laccasi fungine per il trattamento di reflui colorati su scala industriale, due obiettivi devono essere perseguiti: il miglioramento delle loro prestazioni e l'ottimizzazione della loro produzione. A tale scopo lo studio è stato esteso, nelle Sezioni 2 e 3, rispettivamente alla caratterizzazione dei determinanti molecolari responsabili delle peculiari proprietà della laccasi POXA1b di *P. ostreatus* e all'ottimizzazione della produzione delle laccasi prodotte dal fungo *P. ostreatus* mediante la tecnica dell'incrocio classico.

Sezione 1: Analisi di biosistemi per il trattamento dei reflui colorati
Questa sezione del progetto ha mirato allo sviluppo di biosistemi per il risanamento dei reflui dell'industria del colore, basati su catalizzatori microbici ed enzimatici. Al fine di selezionare biosistemi in grado di decolorare in modo efficiente le acque reflue industriali, si è reso necessario uniformare i metodi di analisi, in modo che i diversi sistemi venissero analizzati e confrontati nelle stesse condizioni.
In una fase preliminare del progetto, nell'ambito del progetto europeo SOPHIED (Novel Sustainable Bioprocesses for European Colour Industries" -FP6-NMP2-CT-

2004-505899) sono state, quindi, definite le composizioni di quattro reflui modello (*Acid Bath*, *Optional Acid*, *Direct* e *Reactive*) che riflettono le caratteristiche dei principali reflui dell'industria tessile: in relazione al tipo di lavorazione da cui essi derivano - lana, seta, cotone, pellame- essi differiscono per valori di pH, forza ionica, nonché per la struttura e la reattività dei coloranti contenuti.
Il lavoro è stato articolato così come descritto di seguito:

- Sistema 1: I batteri Streptomiceti

Nell'ultimo decennio la ricerca ha posto particolare attenzione sulle capacità di decolorazione dei batteri Streptomiceti. Gli Streptomiceti sono batteri Gram-positivi del sottosuolo a crescita filamentosa appartenenti all'ordine degli *Actinomycetales.* Questi batteri svolgono un ruolo primario nel processo di mineralizzazione della sostanza organica e sono in grado di degradare, in aerobiosi, sostanze resistenti come la pectina, la lignina, la chitina, la cheratina, il lattice e diversi composti aromatici, riservandosi pertanto interessanti settori applicativi tanto in campo agronomico quanto nel biorisanamento di suoli contaminati [11]. Dati recenti hanno dimostrato, ad esempio, la capacità di questi microrganismi di degradare differenti tipi di coloranti sintetici, sia in presenza che in assenza di mediatori redox, grazie alla produzione di enzimi *laccasi-like*, simili a quelli fungini [12, 13].
Gli Streptomiceti e gli enzimi ossidativi da essi prodotti rappresentano, quindi, dei sistemi promettenti per il trattamento dei reflui industriali.

In particolare in questa parte del lavoro di tesi sono state analizzate la capacità decoloranti di 11 ceppi Streptomiceti, isolati da terreno contaminato da gasolio e pertanto potenziali degradatori di molecole aromatiche come i coloranti sintetici.
Un primo *screening* delle capacità decoloranti dei ceppi analizzati è stato condotto su terreno solido, allestendo crescite in presenza dei tredici singoli coloranti componenti i quattro reflui modello, alla concentrazione di 500 ppm. Le piastre sono state incubate a 28°C, per 21 giorni e al procedere della crescita batterica, è stata monitorata la progressiva scomparsa (o cambiamento) di colore, misurando sia il diametro di crescita del batterio, che quello dell'alone di decolorazione, laddove possibile.
Sulla base dell'efficacia, della rapidità di decolorazione e della capacità del ceppo di decolorare il maggior numero di coloranti nelle stesse condizioni di crescita (versatilità degradativa) sono stati selezionati i ceppi Streptomiceti dalle migliori '*performances*' decoloranti. In particolare sono stati selezionati, da questo primo *screening* su mezzo solido, sei ceppi *Streptomyces*: ceppo 3, 5, 6, 7, 8, 9, che hanno mostrato massima decolorazione di almeno sei singoli coloranti (Fig. 1).

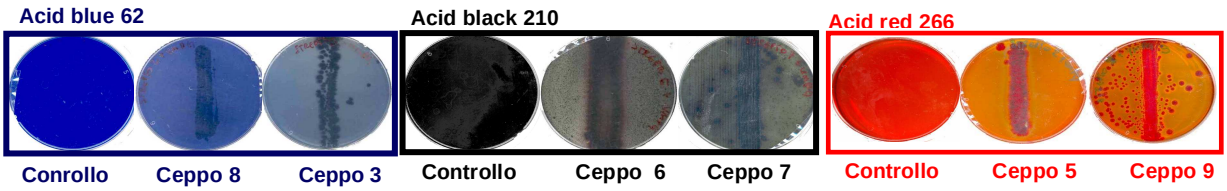

Figura 1: Esempi di aloni di decolorazione su mezzo solido ottenuti con alcuni ceppi in presenza di coloranti acidi.

Una volta verificata la capacità di decolorazione dei batteri in esame su terreno solido, sono state analizzate le capacità di decolorazione dei sei ceppi selezionati anche in terreno liquido. La decolorazione è stata seguita registrando ad intervalli prestabiliti (giorni 1, 7, 14, 21) lo spettro di assorbimento tra 280 e 800 nm dei diversi reflui trattati e valutata come la diminuzione dell'area sottesa dagli spettri dei campioni trattati rispetto al controllo (refluo non inoculato). I risultati ottenuti dagli

esperimenti in mezzo liquido hanno mostrato che i sei ceppi Streptomiceti selezionati dal primo *screening* riescono a decolorare, anche se con diversa specificità, i quattro reflui modello, mostrando una percentuale di decolorazione media del 50% già dal 7° giorno di crescita. Particolarmente interessante è il fatto che i sei ceppi selezionati hanno mostrato una alta percentuale di decolorazione (80%) anche del refluo REACTIVE: un refluo particolarmente recalcitrante ai trattamenti finora effettuati con altre classi di microrganismi [14].
L'osservazione delle biomasse al termine del trattamento (tutte le biomasse risultano colorate) e l'analisi degli spettri di assorbimento prodotti durante il processo (che evidenziavano in alcuni casi un abbattimento uniforme dello spettro di assorbimento e in pochi casi la diminuzione selettiva di specifici picchi di assorbimento) hanno indotto ad attribuire la decolorazione osservata all'azione di due processi: la biodegradazione ed il bioassorbimento. Pertanto al fine di valutare l'effetto del bioassorbimento sull'intero processo di decolorazione tali risultati sono stati completati con le prove di decolorazione condotte con biomasse inattivate.
I sei ceppi selezionati hanno mostrato risultati simili contro tutti i reflui modello analizzati: gli spettri di assorbimento dei campioni trattati con la biomassa attiva e inattivata mostravano profili paragonabili. Inoltre entrambi i trattamenti hanno prodotto le stesse percentuali di decolorazione su tutti i reflui analizzati e con tutti i ceppi selezionati. Questo risultato, insieme al fatto che non sono state rivelate attività ossidasiche nel mezzo extracellulare, ha avvalorato l'ipotesi che la decolorazione dei reflui modello trattati con i ceppi streptomiceti in esame sia attribuibile unicamente a un processo di adsorbimento da parte della biomassa batterica.

In conclusione, quindi, i ceppi *Streptomyces spp* selezionati hanno mostrato buone proprietà di adsorbimento, con percentuali decolorazione in media pari al 50%, anche utilizzando biomasse inattivate. Queste biomasse possono, quindi, essere considerate potenziali candidate per il bioassorbimento dei coloranti dalle acque di scarico industriali.
Inoltre, in accordo con Aksu [15] le biomasse inattivate sono preferibili in quanto non richiedono un contributo costante di nutrienti e non sono influenzate dalla tossicità dei coloranti e delle altre molecole 'tossiche' che caratterizzano i reflui. Infine, tali biomasse possono anche essere rigenerate e riutilizzate in molti cicli, così come già sviluppato per la biomasse fungine [16].

- Sistema 2: Laccasi da *Pleurotus ostreatus*

Al fine di arricchire la gamma di biosistemi ossidativi applicabili nel biorisanamento dei reflui colorati, l'attività di ricerca è stata incentrata sull'analisi delle capacità decoloranti delle laccasi prodotte dal fungo basidiomicete *Pleurotus ostreatus var. florida* ATCC- MYA 2306.
P. ostreatus desta particolare interesse per la vasta gamma di isoforme enzimatiche ad attività laccasica prodotte. Nel laboratorio in cui è svolta questa ricerca sono stati infatti isolati, negli ultimi anni, diversi isoenzimi laccasici [17] interessanti per l'eterogeneità delle loro proprietà (stabilità, pH ottimale, specificità di substrato). Questi isoenzimi risultano efficaci nella decolorazione di diverse classi di coloranti sintetici. In particolare, è stato dimostrato che le laccasi POXC e POXA3 sono capaci di degradare il colorante RBBR *in vitro*, anche in assenza di mediatori redox, con un'efficienza catalitica superiore per POXA3 ed un effetto sinergico osservato utilizzando una miscela dei due isoenzimi [18]. Più recentemente è stata inoltre verificata la capacità di una miscela enzimatica extracellulare prodotta dal fungo e caratterizzata da elevati livelli di attività laccasica di decolorare e detossificare efficacemente il refluo Acid [14].

Da qui l'interesse ad approfondire le capacità decoloranti delle singole isoforme enzimatiche nei confronti dei reflui modello, finalizzato all'identificazione di biosistemi più idonei (diversa stabilità al pH e alle concentrazioni saline) applicabili al trattamento di ciascun refluo. A tal fine le isoforme enzimatiche POXC, POXA1b e POXA3 sono state espresse in forma ricombinante nell'ospite *Kluyveromyces lactis* e le loro capacità decoloranti analizzate sui quattro reflui modello.
Le prove di decolorazione sono state condotte incubando diverse quantità di enzima ricombinante (0,1U, 1U e 3U) in ciascun refluo modello (volume finale 1mL di reazione). I risultati più significativi, ottenuti con i biosistemi analizzati su ciascun refluo modello, sono riassunti in termini di percentuale di decolorazione nella tabella seguente:

% decolorazione (ore)				
		POXC *K .lactis*	POXA3 *K. lactis*	POXA1b *K. lactis*
Acid bath	0,1U	13 (24h)	7 (24h)	5 (2h)
	1U	22 (24h)	10 (24h)	9 (2h)
	3U	22 (24h)	11 (24h)	11 (3h)
Optional Acid	0,1U	8 (24h)	0	0
	1U	7 (24h)	10 (24h)	5 (24h)
	3U	10 (24h)	13 (24h)	6 (2h)

Gli enzimi analizzati si sono rilevati attivi su i due reflui acidi (dati mostrati in tabella), mentre nessuna decolorazione significativa è stata ottenuta nei confronti degli altri due reflui analizzati (*Direct* e *Reactive*). Probabilmente la diversa struttura dei coloranti, le condizioni estreme di pH e la concentrazione salina caratteristiche di questi ultimi due reflui impediscono l'efficiente funzionamento delle laccasi nel processo di decolorazione. Dall'analisi dei risultati mostrati in tabella 1 si evidenzia che la laccasi POXC ricombinante mostra una maggiore % di decolorazione del refluo *Acid Bath*, circa il doppio, in confronto alle altre due laccasi analizzate.
Le differenze in termini di efficienza decolorazione possono essere attribuite alle differenze strutturali dei coloranti e alle diverse specificità per il substrato degli isoenzimi laccasici. D'altra parte, per quanto riguarda il trattamento del refluo *Optional Acid*, non si evidenziano differenze significative tra i tre isoenzimi, che raggiungono un percentuale di decolorazione del 10% circa.

In previsione di un utilizzo delle fenolo ossidasi per la decolorazione di acque reflue colorate, è di fondamentale importanza che esse siano attive su un più ampio *range* di substrati e che siano stabili, e soprattutto attive, in condizioni estreme di pH, normalmente presenti nei reflui industriali.
La disponibilità di una collezione di 3300 varianti enzimatiche ad attività laccasica, ottenute mediante mutagenesi *random* dell'isoenzima POXA1b ed espresse in forma ricombinante nel lievito *Saccharomyces cerevisiae* [19, 20], ha reso possibile la selezione di nuovi biosistemi enzimatici dalle migliori *performances* (migliorata stabilità alla temperatura e al pH, e aumentata affinità ai substrati rispetto all'enzima *wild type*) da applicare al trattamento dei reflui. In particolare sono stati selezionati 7 mutanti dalle migliori '*performances*' e sono state analizzate le loro capacità decoloranti sui quattro reflui modello. I risultati più significativi, ottenuti con i biosistemi analizzati su ciascun refluo modello, sono riassunti in termini di percentuale di decolorazione nella tabella seguente:

% decolorazione (ore)									
		1M9B	3M7C	R4	1H6C	3L7H	2L4A	4M10G	POXA1b
Acid bath	0,1U	3 (3h)	6 (3h)	6 (24h)	15(24h)	0	0	0	9 (3h)
	1U	18 (3h)	30 (2h)	31 (24h)	30(24h)	30(24h)	15 (3h)	30 (24h)	12 (2h)
	3U	31 (24h)	38 (3h)	24 (24h)	24(24h)	30(24h)	20 (3h)	28 (24h)	13 (3h)

I mutanti selezionati, anche se con specificità diverse, decolorano in modo significativo il refluo *Acid Bath*. Essi mostrano una capacità di decolorazione, in molti casi, doppia rispetto a quella dell'enzima POXA1b *wild-type*. In particolare, il mutante 3M7C mostra una percentuale di decolorazione del 38% dopo 3 ore di incubazione con 3U.
Nessun effetto significativo di decolorazione è stato invece ottenuto nei confronti degli altri modelli analizzati, quali *Optional Acid*, *Reactive* e *Direct.* In accordo con Faraco *et al*. [14], i dati riportati indicano la preferenza da parte delle laccasi di decolorare coloranti di tipo antrachinonici e azoici, componenti il refluo *Acid bath*. Inoltre, la percentuale di decolorazione ottenuta con i singoli enzimi risulta paragonabile a quella ottenuta con una miscela di isoenzimi laccasici da *P. ostreatus* [14], pari circa a 40%.
Concludendo, dato che gli isoenzimi analizzati mostrano diversa specificità, anche nei confronti dello stesso refluo, si potrebbero preparare miscele degli isoenzimi analizzati (sia *wilde-type* che mutati), '*ad hoc*' per il trattamento dei reflui colorati dell'industria tessile. Questo consentirebbe di sfruttare l'effetto sinergico tra i vari enzimi; già osservato nelle miscele di laccasi fungine [21].

Sezione 2: Caratterizzazione dei determinanti molecolari responsabili delle peculiari proprietà della laccasi POXA1b da *Pleurotus ostreatus*:
Le laccasi sono enzimi appartenenti alla classe delle fenolo-ossidasi che, data la bassa specificità di substrato che le caratterizza, sono in grado di degradare, oltre a polimeri naturali come la lignina, anche numerose molecole tossiche di natura xenobiotica, come composti fenolici e aromatici. Esse trovano applicazione in svariati settori industriali: 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. L'applicazione delle laccasi nei 'bio-processi' richiede una profonda conoscenza delle relazioni struttura/funzione degli enzimi nativi, fondamentale allo sviluppo di enzimi progettati "su misura" per le specifiche applicazioni industriali. Differenti strategie possono essere applicate per modificare le proprietà enzimatiche, tra cui l'evoluzione guidata e la mutagenesi razionale. La prima richiede la conoscenza della sequenza del gene ma non necessita della conoscenza della funzione e della struttura tridimensionale della proteina codificata; la seconda, invece, che prevede la pianificazione di esperimenti di mutazione sito-specifica, richiede la conoscenza della sequenza, della struttura tridimensionale, e ove possibile, anche del meccanismo d'azione enzimatica. Nel laboratorio in cui si è svolto questo progetto sono state caratterizzate da un punto di vista termodinamico e catalitico diverse laccasi isolate dal fungo basidiomicete *white-rot Pleurotus ostreatus* [17], le cui strutture tridimensionali sono però ancora sconosciute. Questa parte del mio lavoro di tesi ha previsto la predizione dei modelli tridimensionali delle laccasi da *P. ostreatus* e la successiva caratterizzazione dei determinanti molecolari dell'attività catalitica. L'analisi e la comparazione delle sequenze primarie delle laccasi da *P. ostreatus* con le sequenze delle laccasi da basiodiomiceti a struttura nota, accoppiata con lo studio dei modelli 3D ottenuti, ha permesso di individuare delle singolari caratteristiche delle

laccasi in esame. In particolare, dall'allineamento con le principali laccasi da basidiomiceti è emerso che la laccasi POXA1b presenta un prolungamento nella sequenza del C-terminale di sedici amminoacidi. E' stato dimostrato che la regione C-terminale influenza le proprietà delle laccasi, suggerendo un suo possibile ruolo funzionale nell'attività laccasica. I modelli tridimensionali ottenuti sono stati utilizzati per predire complessi enzima-substrato permettendo di individuare i residui in diretto contatto con il substrato (acido violurico), probabilmente responsabili dell'affinità dell'enzima per quest'ultimo. Da queste ulteriori analisi è stato identificato un residuo di arginina nel sito del legame al substrato nella laccasi POXA3 al posto di un residuo di acido aspartico altamente conservato nella maggior parte delle laccasi da basidiomiceti. Il residuo di acido aspartico nella zona del sito catalitico risulta probabilmente coinvolto nella stabilizzazione del catione ottenuto dall'ossidazione del substrato fenolico. La presenza di un'arginina, d'altra parte, porterebbe alla repulsione del radicale cationico e quindi ad una minore efficienza catalitica.
Al fine di investigare il ruolo della regione C-terminale delle laccasi da *P. ostreatus* sono stati progettati e realizzati i mutanti tronchi POXA1bΔ4 e POXA1bΔ16 deleti rispettivamente di 4 e 16 amminoacidi. Inoltre, allo scopo di caratterizzare i determinati molecolari coinvolti nell'interazione con il substrato, sono stati realizzati i mutanti POXA1b(D205R) e POXC(D210R). Successivamente tutti i mutanti sono stati espressi nel lievito *S. cerevisiae.* Per quanto riguarda POXA1b e i suoi tre mutanti (POXA1bΔ16, POXA1Δ4 and POXA1b(D205R)), è stato possibile purificare le tre proteine ed è stato possibile effettuare una caratterizzazione cinetica e catalitica dalla quale è emerso che: la costante di Michaelis-Menten (K_M) dei mutanti tronchi è paragonabile a quella dell'enzima *wild-type* rispetto a tutti i substrati saggiati, mentre il valore di K_M di POXA1b(D205R) è circa il doppio rispetto a quella della proteina ricombinante POXA1b. Questi dati evidenziano l'importanza del residuo Asp nell'interazione con il substrato, essendo questa l'unica differenza nelle relative sequenze. Inoltre, dall'analisi della stabilità a differenti pH è risultato che i mutanti tronchi sono più stabili del *wild-type* a pH acidi, mentre tutti i mutanti in esame perdono drasticamente la peculiare stabilità a pH alcalini di POXA1b. Inoltre, la termoresistenza effettuata a 60°C ha mostrato che POXA1bΔ16 e POXA1b(D205R) sono sensibilmente meno resistenti di POXA1b.
Dai dati ottenuti si può dedurre che il residuo di acido aspartico presente nel sito attivo è direttamente coinvolto nell'interazione con il substrato e che la sostituzione con un residuo di arginina potrebbe generare un riarrangiamento della struttura che avrebbe ripercussioni sulla stabilità della proteina. Pertanto, l'estremità C-terminale sembrerebbe essenziale nello stabilizzare la proteina in condizioni estreme di temperatura e di pH.

Sezione 3: Ottimizzazione delle rese di produzione delle laccasi di *P. ostreatus* mediante la tecnica del *classical breeding.*

Nonostante l'enorme attrattiva esercitata dalle potenzialità applicative degli enzimi laccasici, sia per gli studi fondamentali di caratterizzazione che per l'utilizzo pratico di questi sistemi ossidativi in un processo industriale, sono necessarie grandi quantità di enzima. Tuttavia, le quantità generalmente prodotte dagli organismi *wild-type* possono non essere adatte per tali scopi soprattutto a causa della scarsa resa o dei costi elevati delle procedure di preparazione.
Il fungo basidiomicete *white rot Pleurotus ostreatus*, come la maggior parte delle specie fungine, produce più di un enzima ad attività fenolo ossidasica. Diversi metodi

vengono generalmente utilizzati per incrementare i livelli di produzione di enzimi laccasici tra cui la selezione, la trasformazione genica e l'ibridazione.
Data l'assenza di un efficiente metodo di trasformazione genetica per i funghi *white-rot* e data l'impossibilità di utilizzare trattamenti mutageni, mediante i quali si producono ceppi non più GRAS, per ottenere una variabilità e un miglioramento delle specie "naturali" di *P. ostreatus* sono stati condotti esperimenti classici di cross-ibridazione.
Le basidiospore prodotte per meiosi possono essere isolate e indotte alla germinazione: i corrispondenti monocarionti, caratterizzati per le loro capacità produttive, vengono poi incrociati per determinare i gruppi di compatibilità. Le coppie di ceppi compatibili danno vita ad un nuovo dicarionte le cui proprietà derivano da una combinazione di caratteri multigenetici e/o multiallelici dei ceppi parentali.
In questa sezione della ricerca, al fine di selezionare nuovi ceppi maggiori produttori di laccasi è stata prodotta e caratterizzata una collezione di 28 funghi monocarionti derivanti da due ceppi parentali di *P. ostreatus* (ATCC 2306 e D1208). A partire da questa collezione sono stati isolati 6 ceppi monocarionti che esibivano un profilo di produzione laccasica maggiore di quello di rispettivi ceppi parentali. Tra i dicarionti ottenuti dagli incroci dei ceppi monocarionti selezionati sono stati identificati tre nuovi ceppi, i cui livelli di produzione, in coltura liquida in condizioni basali (PDY+$CuSO_4$ 150 µM), raggiungono livelli notevolmente maggiori dei ceppi parentali. La composizione isoenzimatica delle laccasi prodotte dai ceppi migliori produttori è stata esaminata mediante cromatografia a scambio anionico.
I risultati ottenuti nel giorno di massima produzione sono riassunti nella tabella seguente:

Ceppi dicarionti	**Attività laccasica (U/L)**	**Rapporto isoenzimatico**	
5AxD3	**47,000 ±18,000**	POXA3	<1%
		POXC	99%
6AxD11	**53,000 ± 13,000**	POXA1b	2%
		POXA3	15%
		POXC	48%
		POX1	35%
5AxD11	**45,000 ± 8,000**	POXA3	2,2%
		POXC	97.8%
Parentale	**17,000 ± 2,000**	POXA3	20%
		POXC	80%

Nelle condizioni di crescita basali, i tre ceppi selezionati raggiungono livelli di espressione che superano le 45,000 U/L, rispetto alle 17,000 U/L del ceppo parentale. Inoltre, al fine di valutare l'effetto dell'acido ferulico (2mM concentrazione finale), un noto induttore laccasico [21], sono state allestite per ciascun ceppo, crescite in coltura liquida in presenza dell'induttore. L'aggiunta dell'induttore al brodo di coltura incide positivamente sul profilo di produzione laccasica dei tre ceppi analizzati. In particolare il ceppo 5AxD3 in presenza dell'induttore raggiunge livelli di produzione doppi rispetto alla condizione basale (110,000 U/L) e circa 4 volte

superiori rispetto al ceppo parentale; mentre gli altri due ceppi (6AxD11 e 5AxD11) raggiungono livelli di produzione che sfiorano le 70,000 U/L.
L'analisi dei *pattern* isoenzimatici dei tre ceppi dicarionti, mediante elettroforesi in condizioni native, insieme ai dati di frazionamento ottenuti mediante cromatografia a scambio ionico, hanno permesso di identificare, sia in condizioni basali che di induzione, un'aumentata produzione delle isoforme presenti nei ceppi parentali da parte dei ceppi 5AxD3 e 5AxD11 e una nuova isoforma laccasica, secreta solo dal ceppo 6AxD11. La banda corrispondente alla nuova isoforma è stata analizzata mediante tecniche di spettrometria di massa *tandem* ed è stata identificata la laccasi POX1, di cui era stato precedentemente isolato il cDNA [22] ma non era stato ancora identificato il corrispondente prodotto proteico in forma attiva.
In conclusione, quindi, la cross-ibridazione ha portato a un miglioramento delle capacità produttive e a un aumento della variabilità del fungo *white-rot P.ostreatus* rivelandosi un sistema "naturale", economico ed ecocompatibile per il miglioramento di bioprocessi industriali.

Bibliografia:

[1] Holme I. Recent developments in colorants for textile applications. *Surface coating international part-B coatings transactions*. (2002); 85:243-264.
[2] Abadulla E., Robra K.H., Gubitz G.M., Silva L.M., Cavaco-Paulo A. Enzymatic decolorization of textile dyeing effluents. *Textile Research Journal.* (2000); 70:409-414.
[3] Dias A.A., Bezerra R.M., Lemos P.M., Pereira A.N. In vivo and laccase catalysed decolourization of xenobiotic azo dyes by a basidiomycetous fungus: characterization of its ligninolytic system. *World Journal of Microbiology and Biotechnology.* (2003); 19:969-975.
[4] Fu Y. and Viraraghavan T. Fungal decolorization of dye wastewaters: a review. Bioresource Technology. (2001); 79:251-262. **[5] Reddy C. A., Mathew Z.** Bioremediation potential of white-rot fungi. *In : G.M. Gadd. Fungi in bioremediation*. (2001); p. 52-71.
[6] Crini G. Non-conventional low-cost adsorbents for dye removal: A review. *Bioresource Technology* .(2006); 97:1061-1085.
[7] Knapp S.J., Vantoch-Wood E., Zhang F. Use of wood-rotting fungi for the decolorisation of dyes and industrial effluents. *In: G.M. Gadd. Fungi in bioremediation.* (2001); p. 27-51.
[8] Wesenberg D., Kyriakides I., Agathos S.N. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol Adv*. (2003); 22(1-2):161-87.
[9] Kaushik P., Malik A. Fungal dye decolourization: Recent advances and future potential. *Environ. Int.* (2009); 35(1):127-41.
[10] Prigione V., Varese G.C., Casieri L., Filipello Marchisio V. Biosorption of simulated dyed effluents by inactivated fungal biomasses. *Bioresource Technology.* (2008); 99(9):3559-3567.
[11] Miyadoh S. Research on antibiotic screening in Japan over the last decade: a producing microorganism approach. *Acinomyce-tologica* (1993); 9:100-106.
[12] Arias M.E., Arenas M., Rodríguez J., Soliveri J., Ball A.S. and Hernández M. Kraft Pulp Biobleaching and Mediated Oxidation of a Nonphenolic Substrate by Laccase from *Streptomyces cyaneus* CECT3335**.** *Applied and Environmental Microbiology*. (2003); 69:1953-1958.
[13] Dubé E., Shareck F., Hurtubise Y., Beauregard M. and Daneault C. Decolourization of recalcitrant dyes with a laccase from Streptomyces coelicolor

under alkaline conditions. *J of Industrial Microbiology & Biotechnol*. (2008); 35:1123-1129.
[14] Faraco V., Pezzella C., Miele A., Giardina P., Sannia G. Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochete chrysosporium* and *Pleurotus ostreatus* and their enzymes. *Biodegradation*. (2009); 20(2): 209-20.
[15] Aksu, Z. Application of biosorption for the removal of organic pollutants: a review. *Process Biochem*. (2005); 40:997–1026.
[16] Prigione V., Varese G.C., Casieri L., Marchisio V.F. Biosorption of simulated dyed effluents by inactivated fungal biomasses. *Bioresource Technology.* (2008); 99:3559–3567.
[17] Pezzella C., Autore F., Giardina P., Piscitelli A., Sannia G. and Faraco V. The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. *Curr. Genet.* (2009); 55: 45-57.
[18] Palmieri G., Cennamo G., Sannia G. Remazol Brilliant Blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. *Enzyme Microb Technol*. (2005); 36:17–24.
[19] Festa G., Autore F., Fraternali F., Giardina P., Sannia G. Development of new laccases by directed evolution: Functional and computational analyses*. Proteins.* (2008); 72 (1): 25–34.
[20] Miele A., Giardina P., Sannia G., Faraco V. Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation. *J Appl Microbiol.* (2010); 108(3):998-1006.
[21] Palmieri G., Giardina P., Sannia G. Laccase-mediated Remazol Brillant Blue R (RBBR) decolourization in a fixed-bed bioreactor. *Biotechnol Prog* (2005); 21(5):1436-41.
[22] Giardina P., Cannio R., Martirani L., Marzullo L., Palmieri G. and Sannia G. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus. Applied and Environmental Microbiol.* (1995); 61: 2408–2413.

SUMMARY

In the last decade, biotechnology has been applied in several industrial fields, especially for the evaluation and preservation of environmental quality. The increasingly stringent environmental regulations on hazardous wastes have encouraged the search for innovative solutions for the remediation of contaminated wastewaters. In this field, bioremediation is seen as an attractive solution due to its reputation as a low cost, environmentally friendly and publicly acceptable treatment technology.

This research project - that is part of a broader research project, carried out in collaboration with the University of Turin (Department of Vegetal Biology, Dr. Cristina Varese and financed by the “Compagnia di S. Paolo”) - aims at developing bio-systems for the treatment of industrial coloured wastewaters, based on microbial and enzymatic catalysts. In the present research program two kind of biosystems have been specifically analysed: *Streptomyces spp*. bacteria and laccase isoenzymes produced by the white rot fungus *Pleurotus ostreatus*. Their effectiveness was tested on four textile wastewater models (*Acid Bath*, *Optional Acid*, *Direct*, *Reactive*) and single dyes composing them, by assessing and comparing their performances in terms of decolourisation ability of the treated effluent.

In particular, the decolourization ability of eleven *Streptomyces spp.* strains has been tested towards single dyes and the four wastewater models. Since the analysed strains originate from soil contaminated by gasoil their expanded metabolic capabilities can be considered a promising starting point for the selection of new dye degraders. The best performing *Streptomyces spp*. strains have shown good decolourization abilities (percentage up to 50%), although the process is mainly ascribable to absorption processes instead of degradation ones. However, according to the obtained data, these biomasses may stand out as good candidates for biosorption treatment of industrial wastewaters.

As far as the enzymatic system, decolouration ability of recombinant laccases from *Pleurotus ostreatus* has been analyzed. In order to ascertain the contribution of single laccase isoenzyme to the decolourization process, and to identify the most efficient laccase based biosystem, the decolourization ability of the single *P. ostraetus* laccase isoenzymes POXA1b, POXC and POXA3, heterologously expressed in the yeast *Kluyveromyces lactis*, has been tested towards the four wastewater models. The data indicate laccases preference in decolourizing anthraquinonic type dye and aniline mono-azo dye, characteristics of Acid wastewater models. In particular recombinant POXC laccase displays a slightly higher decolourization ability against *Acid Bath*, reaching up to 22% decolourization after 24h, in comparison with POXA3 and POXA1b. The differences in decolourization efficiency can be ascribed to structural differences of the dyes and to substrate specificity of the laccase isoenzymes.

A parallel aspect of this section has been focused on selecting laccase variants that are better suited to the conditions of industrial wastewaters (high pHs, heterogeneous composition), i.e able to operate on a wider range of substrates, or stable, and especially active, in extreme conditions of pH. For this reason, seven POXA1b variants, previously selected from a collection of mutants on the basis of different criteria (increased activity toward different substrates and higher stability at pHs and temperature), have been tested for their decolourization ability towards the four wastewater models. Selected POXA1b variants show an increased decolourization ability than POXA1b wild-type toward the *Acid Bath* model. As a fact,

for all the mutants, a two-fold increase in decolourization percentage compared to that of POXA1b has been obtained.

Another section of the work has been focused on the understanding of the structure/function relationships of laccases with the aim to characterize molecular determinants of the activity of these enzymes, and to develop and characterize new laccases obtained from rational design using POXA1b *P. ostreatus* isoenzyme. Rational mutants of POXA1b laccase allowed to demonstrate a role of the C-terminal tail of POXA1b in affecting its catalytic and stability properties. Moreover, site-directed mutagenesis experiments allowed to demonstrate that introducing Arg205 mutation, instead of Asp 205, in a highly conserved region perturbs the structural local environment in POXA1b, leading to a large rearrangement of the enzyme structure. Hence, a single substitution in the binding site introduces a local conformational change that not only leads to very different catalytic properties, but can also significantly destabilize the protein.

Finally, in order to improve industrially useful enzymatic biosystems, suitable for decolourization processes of coloured wastewaters, the last session of the project was aimed at producing new *P. ostreatus* dikaryotic strains with improved efficiencies in laccase expression, by classical breeding approach. In particular, starting from two different *P. ostreatus* variants, three laccase higher-producing dikaryotic strains have been obtained by crossing compatible characterized monokaryons. The three selected strains reached expression levels of 100,000 U/L, increasing the trite of parental strains up to four folds. Moreover, a new laccase isoenzyme, POX1, has been produced and identified in these culture conditions.

Introduction

- ***Industrial wastes and bioremediation issues***

1. Environmental biotechnology and industrial waste-waters treatment

Life on our planet is inextricably linked to the presence of water; although water is 'apparently' a renewable resource, only an infinitesimal part of the earth's water reserves (approximately 0.03%) constitutes the resource available for human activities [1]. With the growth of mankind, society, science, technology our world is reaching to high horizons but the cost which we are paying or, we will pay, in the near future is going to be too high. Environmental disorder is among the consequence of this rapid growth. Besides other needs the demand for water has increased tremendously with agricultural, industrial and domestic sectors consuming 70%, 22% and 8% of the available fresh water, respectively, and this has resulted in the generation of large amounts of wastewater [2] containing a number of 'pollutants', contaminants, residues or by-products of industrial activities.

In the last decade, biotechnology has been applied in several industrial fields, especially for the evaluation and preservation of environmental quality. The increasingly stringent environmental regulations on hazardous wastes have encouraged the search for innovative solutions for the remediation of contaminated wastewaters.

In this field, bioremediation is seen as a fascinating solution due to its reputation as a low cost, environmentally friendly and publicly acceptable treatment technology. With the term 'bioremediation' can be defined any process that uses microorganisms or their enzymes to degrade, transform, or reduce the concentration of, hazardous wastes on a contaminated site.

Bioremediation technologies can be broadly classified as *ex situ* and *in situ*. *Ex situ* technologies are those treatments which involve the physical removal of the contaminated material for the treatment process. In contrast, *in situ* techniques involve treatment of the contaminated material in place. Until now, a large number of microorganisms have been isolated and applied to both *in situ* and *ex-situ* bioremediation processes, and the identification of new microbes with novel metabolic potential offers an attractive route to solve environmental problems [3-5].

2. Synthetic dyes and the textile industry

Synthetic dyes

Synthetic dyes are used extensively for textile dyeing, paper printing, colour photography and as additives in petroleum products and in many other industrial sectors. The presence of these dyes in water, even at very low concentrations, is highly visible and undesirable [6, 7]. Colour is the first contaminant to be recognised, and environmental regulations in most of the countries (EU directive 91/271) have made it mandatory to decolourise the dye wastewater prior to discharge [8]. With the growing use of a variety of dyes, pollution by coloured waste waters is becoming increasingly serious. Dyes are generally aromatic organic compounds with complex structures. When describing a dye molecule, nucleophiles are referred to as *auxochromes*, while the aromatic groups are called *chromophores*. Together, the dye molecule is often described as a *chromogen*. The absorption and reflection of visible and UV irradiation is ultimately responsible for the observed colour of the dye [9]. The most important chromophores are azo (–N≡N–), carbonyl (–C=O), nitro ($–NO_2$) and quinoid groups. The most important *auxochromes* are amine ($–NH_3$), carboxyl (–COOH), sulfonate ($–SO_3H$) and hydroxyl (–OH). Dyestuff can be classified according to chemical structures, physical properties and characteristics related to the application process (e.g., inks, disperse, pigments or vat dyes) [10] but they are

generally divided into anionic (including direct, acid and reactive dyes), cationic (basic dyes) and non-ionic (disperse dyes). The *chromophores* differ between these classes: anionic and non-ionic dyes are mostly azo- or anthraquinone groups, whilst reactive dyes are typically azo-based chromophores combined with different types of reactive groups.
Azo dyes are the largest class of commercially produced dyes and have wide spread use in textile industry and dyestuff manufacturing industry. Azo dyes are characterized by the presence of one or more azo linkages and aromatic rings with simple application-technique requirements and high waterfastness. Also reactive dyes are extensively used because of their favourable characteristics of bright colour and low energy consumption during application [11]. The chemical structures of dye molecules are designed to resist fading on exposure to light or chemical attack and they prove to be quite resistant towards microbial degradation; moreover, synthetic dyes are often toxic, carcinogenic and mutagenic to various organisms, being detrimental to human health [12].

Textile industry and dyestuff effluent

The textile industry is one of the main sources of coloured environmental pollution due to an annual production of approximately 800,000 tons of dyes. The dyeing process produces a large amount of intensely coloured wastewater due to the release of a high percentage (up to 40%) of the used dyes. These molecules are produced in an effort to improve human standard of living and fashion but ironically, their unplanned intrusion into the environment can reverse the same standard of living by impacting negatively on the environment [13].
Effluent derived from the textile and dyestuff activities can provoke serious environmental impact in the neighbouring receptor water bodies because of the presence of toxic reactive dyes, chlorolignin residues and dark coloration. Therefore the composition of dyeing effluent varies with the textile produced. Depending upon customer orders, types of manufactured textile materials and production programs, textile wastewaters display a great chemical complexity and variability in terms of quantities and pollution load, type of dyes, pH, and temperature [14]. Modern textile dyes are required to have a high degree of chemical and photolytic stability in order that they maintain their structure and colour [15]. New dyes are continuously studied to find those that resist breakdown caused by time and exposure to sunlight, water, soap, and other parameters such as bleach and perspiration. Moreover they must resist to anti-microbial agents that are frequently added to make textiles, particularly natural fibres such as cotton, resistant to biological degradation [16] and unattractive to be colonized by organisms (e.g. mites, fungi and bacteria).
Besides dyes, textile industry effluents contain a complex mixture of many polluting substances, such as heavy metals associated with dyes or required for the dyeing process, additives, detergent and surfactants which have an adverse impact in terms of amount of suspended solids and COD value of coloured effluents. All these factors render wastewaters from textile industries the most difficult to treat [13].
Nature has demonstrated its capacity to disperse, degrade, absorb or otherwise dispose of unwanted residues in the natural sinks of the atmosphere, waterways, ocean and soil. Up now scientists have been trying to develop a single and economical method for the treatment of dyes in textile wastewaters, but it still remains a big challenge [17].

3. Conventional treatment processes

The main problem in textile effluent treatment is the presence of dyes released into the effluent during dyeing stage. Textile wastewaters treatment may require the removal of over 99% of the colour in samples and for this level of colour removal to be maintained with large volumes of rapidly changing effluents [18]. The frequently high volumetric rate, diversity in composition and recalcitrance of industrial effluents in combination with increasingly stringent legislation, make the search for appropriate treatments technologies an important priority [8].

There are various methods for the treatment of textile wastewater for the removal of dye; these broadly fall into three categories: physical, chemical and biological. These methods are almost expensive and applicable only in a limited number of cases, as reported in table 1 [13]. Many of these treatments allow the removal of contaminants from wastewater, but not their destruction, so dyes are then recovered and need to be disposed or destroyed by incineration. Moreover, some of these treatments require the use of hazardous chemicals compounds (bleach, ozone or peroxide) dangerous for human health and/or for the environment [19].

Table 1: Principal existing and emerging processes for dyes removal (Crini 2006).

	Technology	Advantages	Disadvantages
Conventional treatment processes	Coagulation Flocculation	Simple, economically feasible	High sludge production, handling and disposal problems
	Biodegradation	Economically attractive, publicly acceptable treatment	Slow process, necessary to create an optimal favourable environment, maintenance and nutrition requirements
	Adsorption on activated carbons	The most effective adsorbent, great, capacity, produce a high-quality treated effluent	Ineffective against disperse and vat dyes, the regeneration is expensive and results in loss of the adsorbent, non-destructive process
Established recovery processes	Membrane separations	Removes all dye types, produce a high-quality treated effluent	High pressures, expensive, incapable of treating large volumes
	Ion-exchange	No loss of sorbent on regeneration, effective	Economic constraints, not effective for disperse dyes
	Oxidation	Rapid and efficient process	High energy cost, chemicals required
Emerging removal processes	Advanced oxidation process	No sludge production, little or no consumption of chemicals, efficiency for recalcitrant dyes	Economically unfeasible, formation of by-products, technical constraints
	Selective bioadsorbents	Economically attractive, regeneration is not necessary, high selectivity	Requires chemical modification, non-destructive process
	Biomass	Low operating cost, good efficiency and selectivity	

A wide number of different biotechnological approaches that employ bacteria, fungi or plants to solve the problem of dye-containing wastewaters have recently emerged. Use of microorganisms to solve different pollution problems is defined 'Bioremediation'.

4. Bioremediation for the treatment of industrial wastes: biosorption and biodegradation.

Different techniques have been suggested by many authors to manage environmental pollution source of pollution in an efficient and ecological manner, including the use of microorganisms in combination with physico-chemical processes [20, 21]. The term "bioremediation" describes the process of contaminant degradation in the environment by biological methods using the metabolic potential of microorganisms to degrade a wide variety of organic compounds [22]. The main advantage of bioremediation is its reduced cost compared to conventional techniques. Besides cost-effectiveness, it may be a permanent solution, that can lead to complete mineralization of the pollutant. Bioremediation is a non-invasive technique, leaving the ecosystem intact. Furthermore, it can deal with low concentration of contaminants where the cleanup by physical or chemical methods would not be feasible.

Bioremediation can be achieved using two different principles: bioaccumulation also named biosorption and biodegradation.

Biosorption

As synthetic dyes in wastewater cannot be efficiently decolorized by traditional methods, adsorption of synthetic dyes on inexpensive and efficient solid supports was considered as a simple and economical method for their removal from water and wastewater. Adsorption techniques are widely used to remove certain classes of pollutants from waters, especially those that are not easily biodegradable. Adsorption based methods produce high-quality treated effluents, as they are effective on different types of dyes and provide high and rapid decolourisation [23]. Decolourisation is a result of two mechanisms: adsorption and ion exchange [24], and it is influenced by many physco-chemical factors like pH, temperature, initial dye concentration and the type of the dye present in the solution [25]. Most commercial systems currently use activated carbon as adsorbent because of its excellent adsorption ability. However its industrial application is restricted because of the high cost, the ineffectiveness against disperse and vat dyes and the problem of its regeneration which is expensive and results in loss of the adsorbent. These factors have driven the research interest toward the use of non-conventional low cost adsorbents. The use of microbial biomass (in dead or living form) has emerged as a promising and cost-effective alternative technology [25, 26].

The term 'Biosorption' is used to indicate a number of metabolism-independent processes (physical and chemical adsorption, ion exchange, complexation and chelation) taking place essentially at level of cell wall [11]; it is defined as the process of concentration of pollutants and a prefix 'bio' means that the sorbent is of biological origin, a surface of biological matrix. The process is simple in operation and very similar to conventional adsorption or ion-exchange, except that adsorbent of biological origin is employed. Biosorbents are selective and regenerable and a process is in particular highly effective in the treatment of diluted effluents [27, 28].

Textile dyes vary greatly in their chemistries, and therefore their interactions with micro-organisms depend on the chemistry of a particular dye and the specific

chemistry of the microbial biomass [29]. Use of certain low-cost adsorbents for dye removal has been extensively reviewed by Hai *et al* 2007 [14], Crini 2006 [30] and Crini and Badot, 2010 [31]. Both living and dead biomass can be used to remove hazardous organics, but the use of dead microbial cells in biosorption is more advantageous for wastewater treatment, due to the fact that the organisms are not affected by toxic wastes, by chemical conditions and do not pollute the environment; they do not require a continuous supply of nutrients and, in some cases, they can be regenerated and reused for many cycle [32].
Up now a wide variety of microorganisms including bacteria, yeast and fungi have been used as a cheap source of biosorbent for the purpose of decolourising dye-containing effluents. Moreover, adsorption capacity can be increased by certain physical or chemical pre-treatments like drying, autoclaving, use of organic or inorganic chemicals, which damage the cell surface, bringing to the exposure of potential binding sites for dyes [11].
Finally, at the end of the decolourization process, desorption is an important step in the regeneration of the fungal biomass for further use in new biosorption processes. Biosorbents can be regenerated by treatment with certain chemicals as alkalis, chelating agents etc. Recovery of the biosorbent and dyes makes the treatment process more economical.

Biodegradatrion
The application of microorganisms for the biodegradation of synthetic dyes is an attractive and alternative method for the wastewater treatments. The use of microorganisms for the removal of synthetic dyes from industrial effluents offers considerable advantages. The process is relatively inexpensive; running costs are low and end products of complete mineralization are generally not toxic. As a result of the primary or secondary metabolic activity, a wide variety of microorganisms including bacteria, fungi and plants are capable of decolourizing different pollutants, comprising dyes [33], in different anaerobic or aerobic conditions. The most of dyes are difficult to degrade in anaerobic conditions, but azo-dyes represent a special problem. These dyes, in fact, are transformed by organic and inorganic reactions in aromatic amines, increasing the toxicity of effluents. Under static or anaerobic conditions, bacterial decolorization generally demonstrates good colour removal effects. However, aerobic treatment of azo dyes with bacteria usually achieves low efficiencies because oxygen is a more efficient electron acceptor than azo dyes [34]. Although decolourization under anaerobic conditions generally cannot realize the complete mineralization of azo dyes, aromatic amines, as decolorized products, are usually more susceptible to oxygenase attack. Thus, bacterial mineralization of azo dyes generally takes two steps: anaerobic degradation to the corresponding colorless aromatic amines (occasionally carcinogenic and toxic) and then complete mineralization under aerobic conditions [17].
By far the principal class of microorganisms most efficient in breaking down synthetic dyes are the white-rot fungi (WRF) [10, 35]. These constitute a diverse ecophysiological group comprising mostly basidiomycetous (and, to a lesser extent, litter-decomposing) fungi capable of extensive aerobic lignin depolymerization and mineralization. This property is based on the capacity to produce one or more extracellular lignin-modifying enzymes (LME), which, thanks to their low substrate specificity, are also capable of degrading a wide range of xenobiotics e.g. polycyclic aromatic hydrocarbons, chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives and synthetic dyes [10]. Moreover, due to fungal enzymes

being generally secreted, the substrate diffusion limitation into the cell, observed in bacteria, is not encountered; thus, fungal treatments, are also advantageous in tolerating high concentrations of the toxicants [19]. All these features promote white-rot fungi as good candidates for the treatment of dyes and textile wastewater.

- ***Bacteria, fungi and their enzymatic systems for the treatment of coloured effluents***

5. Dye removal by bacterial treatment

Nowdays several species of gram negative bacterial of different genus as *Aeromonas*, *Escherichia*, *Citrobacter*, *Pseudomonas, Sphingomonas* has been proved to decolourise dye solution or simulated effluents [36]. Among them one of the best degraders is *Aeromonas hydrophila* [37] capable to degrade nine different acid, direct and reactive dyes. Also, gram positive bacteria have been investigated for dye degradation abilities, since 1996. Species from genera *Bacillus, Clostridium, Nocardia, Paenibacillus* and *Streptomyces* have been found to degrade synthetic dyes [36].

The ability of bacteria to metabolize azo dyes has been investigated by a number of research groups. Under aerobic conditions azo dyes are not readily metabolized although Kulla [38], reported the ability of *Pseudomonas* strains to aerobically degrade certain azo dyes. However, the intermediates formed by these degradative steps resulted in disruption of metabolic pathways and the dyes were not actually mineralized. Under anaerobic conditions, such as anoxic sediments, many bacteria gratuitously reduce azo dyes thanks to the activity of unspecific, soluble, cytoplasmic reductases, known as azo reductases. These enzymes are reported to result in the production of colourless aromatic amines which may be toxic, mutagenic, and possibly carcinogenic to animals.

Mixed bacterial cultures from a wide variety of habitats have been shown to decolourise the diazo-linked chromophore of dye molecules [19]. Nigam and Marchant [39] demonstrated that a mixture of dyes were decolourized by anaerobic bacteria in 24–30 h, using free growing cells or in the form of biofilms on various support materials. Ogawa and Yatome [40] also demonstrated the use of bacteria for azo dye biodegradation. These microbial systems have the drawback of requiring a fermentation process, and are therefore unable to cope with larger volumes of textile effluents.

Increasingly literature evidence suggests that additional processes may also be involved in azo dye reduction. It has been reported that many bacteria reduce a variety of sulfonated and non-sulfonated azo dyes under anaerobic conditions without specificity of any significance. In addition many highly charged and high molecular-sized sulfonated and polymeric azo dyes are unlikely to pass the cell membrane. Taken together both pieces of evidence point to the existence of a reducing activity which is not dependent on the intracellular availability of the azo dye [41].

Bacteria capable of dye decolourization, either in pure cultures or in consortia, have been reported [42-44]. It has been demonstrated that synergistic metabolic activities of mixed microbial consortium can lead to complete mineralization of azo dyes [45].

The mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (-N=N-) with the help of azoreductase under anaerobic conditions and resulted into the formation of colorless solutions [46]. For the reduction of azo dyes, reduction to the anion radical occurs by a fast one-electron transfer reaction, followed by a second, slower electron transfer event to produce the stable dianion [47]. Thus

the functional group of azo dye with higher electronic density might be unfavorable to this second electron transfer to form the dianion, leading to low or no capability for decolorization. Due to this reason sulfonated reactive group of azo dyes are normally considered to be more recalcitrant than carboxylated azo dyes. In addition, the rate limiting step during bacterial decolorization of sulfonated azo dyes is the permeation through the bacterial cell membrane [48].
Zissi and Lyberatos (2001) [49] showed that *Bacillus subtilis* could be used to break down p-aminoazobenzene, a specific azo dye. Further research using mesophilic and thermophilic microbes has also shown them to degrade and decolourize dyes [50, 51]. Several actinomycete strains, including *Streptomyces spp*., have been proven to modify effluents which are produced during the chemical bleaching of pulps [52]. Although there is considerable potential for treating these effluents by biological methods, the mechanism of colour removal by these biological system is poorly understood.

6. Dye removal by fungal treatment and their enzymatic system
In the last decade many works highlighted the potentials of several fungal strains to degrade dyes and so potentially applicable for wastewater treatment [53, 54].
Many genera of fungi have been employed for the dye decolourization either in living or dead form [54]; ligninolityc fungi have been found to be the most promising species. Lignin is a particularly hardly degradable natural polymer. The degradation patterns of the lignin polymer in filamentous fungi can be mediated or not by enzymes. The most of the mechanisms involved, in the degradation process, have characteristics of extreme aspecificity that allow them to enlarge the range of substrates to be used, making ligninolytic fungi potentially capable to degrade several xenobiotic compounds [55]. Thus, the attention to bioremediation has been focused on the possibility to apply these mechanisms to solve environmental pollution problems. One of the most important class of microorganisms that is more efficient in breaking down synthetic dyes is that of white rot fungi [35, 10]. WRF, mostly belonging to basidiomycetes, are capable of extensive aerobic lignin depolymerization and mineralization in nature, thanks to the production of one or more extracellular lignin-modifying enzymes (LMEs) [10]. Moreover, white rot fungi do not require preconditioning to particular pollutants because enzyme secretion depends on nutrient limitation, nitrogen or carbon, rather than on the presence of pollutants.
The most of WRF, especially *Pleurotus* species, are robust organisms and are generally more tolerant to high concentrations of polluting chemicals than bacteria [56]. WRF have been reported, on several occasions, as good producers of extracellular ligninolytic enzymes and active strains for textile dye decolourisation [13, 57, 58].

The complex pattern of LMEs produced by white-rot fungi to accomplish lignin degradation comprises several oxidative activities, mainly Manganese- dependent peroxidases (MnP), Lignin-peroxidases (LiP), versatile peroxidases (VP) and Laccases, together with other enzymes (glucose oxidase, cellobiose dehydrogenase (CDH), hydrolases or factors (e.g. the reactive oxygen species (ROS)), which also play an important role in the process. Some white rot fungi produce all three main classes of LMEs, while others produce only one or two of them [55].
LMEs are produced by white-rot fungi during the secondary metabolism since lignin oxidation provides no net energy to the fungus. Synthesis and secretion of these

enzymes is strictly influenced by nutrient levels (mostly C or N limitation), culture conditions (shaking or stationary) and by the presence of different inducers (metals, aromatic compounds and so on). Frequently, ligninolytic enzymes secreted by white-rot fungi have a low substrates specificity, therefore, they can degrade a wide variety of recalcitrant compounds and even complex mixtures of pollutants including dyes. This ability has opened new prospects for the development of biotechnological processes and attracted increasing scientific attention on the use of white-rot fungi and/or their enzymes. Frequently, more than one isoforms of LMEs is expressed by different taxa depending on culture conditions [59]. These features are important in process design and optimization of fungal treatment of colorant-containing effluents.

The most common ligninolytic peroxidases produced by almost all white-rot basidiomycetes and by various litter-decomposing fungi are Manganese peroxidases (MnPs). MnPs are glycoproteins with an iron protoporphyrin IX (heme) prosthetic group and molecular weights between 32 and 62.5 KDa. They are usually secreted in multiple isoforms in carbon and nitrogen limited media by several fungi [60]. MnPs preferentially oxidize Mn^{+2} into Mn^{+3} [61], which is stabilized by chelators such as oxalic acid [60], itself also excreted by the fungi [62, 63]. Chelated Mn^{+3} acts as a highly reactive (up to 1510mV in H_2O) low molecular weight, diffusible redox-mediator. Thus, MnP are able to oxidize and depolymerise their natural substrate, i.e., lignin as well as recalcitrant xenobiotics, such as nitroaminotoluene and textile dyes [64].

Lignin peroxidases are N-glycosylated enzymes with molecular masses between 38 and 47 KDa, which catalyzesthe oxidation of non-phenolic aromatic lignin moieties and similar compounds by one electron abstraction to form reactive radicals. They contain heme in the active site and show a classical peroxidase mechanism. The role of LiPs in ligninolysis could be the further transformation of lignin fragments which are initially released by MnP. LiPs are not essential for the attack on lignin: several highly active WRF and litter-decaying fungi do not produce this enzyme [55, 65]. LiPs have been used to mineralize a variety of recalcitrant aromatic compounds, such as PAHs [66] polychlorinated biphenyls [67] and dyes [68].

A third group of peroxidases is represented by Versatile peroxidases (VP), that can be regarded as hybrid between MnPs and LiPs, since they can oxidize not only Mn^{+2} but also phenolic and nonphenolic aromatic compounds including dyes, in a manganese-independent reactions [69]. VPs have been described in *Pleurotus* and *Bjerkandera* species [70-72].

Another group of extracellular enzymes produced by almost all white-rot basidiomycetes is that of laccases. These enzymes are multicopper-containing enzymes belonging to the group of blue oxidases, which catalyze the oxidation of a variety of aromatic hydrogen donors with the concomitant reduction of oxygen to water. A more detailed description of this class of enzyme is given in the following sections.

Moreover, many other enzymes produced by fungi are involved in lignin degradation. Some of these play an important role in the process, such as several extracellular oxidases (glucose, glyoxal, galactose and aryl alcohol oxidases), that have been shown to play an important role in the lignin degrading process since they provide H_2O_2, an essential cofactor for peroxidases.

Given the random polymer nature of lignin and the bulk of LMEs, direct and specific interactions between lignin (or recalcitrant structural analogs) and LMEs are highly improbable [73]. Instead low-molecular weight and diffusible redox mediators are able to migrate into the lignocellulose complex, providing high redox potential

(>900mV) to degrade lignin. They could be involved in the LME-catalyzed generation of reactive radical moieties from a variety of lignin-like substrates, but also in the formation of reactive oxygen species (ROS) which either directly or indirectly could attack lignin or xenobiotic molecules [74]. Both native and synthetic mediators have been found to promote LMEs activities.
3-hydroxyanthranilic acid (3-HAA) was the first natural mediator for described laccases. This mediator anables a laccase-catalyzed oxidation of non-phenolic lignin model dimers [75]. Another example is the veratryl alcohol (VA), a secondary metabolite of several WRF [76] which after its oxidation to the VA cation radical by LiPs, acts as a mediator for the degradation of lignin, probably by protecting the enzyme against the damaging effect of H_2O_2 [77].

Numerous WRF strains have been successfully used for decolourisation of several synthetic dyes. In many cases, the concerted action of different enzymatic systems, extracellular, wall associated or intracellular [78], is necessary to achieve both efficient dye removal and toxicity reduction of treated wastewaters. Moreover, cells may synthesize several cofactors required to maintain enzyme activity and to promote dye decolourisation, [79] or hydrogen peroxide [80]. On the other hand, application of fungal treatment for dye wastewater treatment is still hindered by the complex and variable composition of real effluents. The harsh conditions provided by the wastewaters (extreme pH values, high salt concentration, presence of chelating agents, precursors, by-products, surfactants and very complex dye composition) can affect fungal growth, extending the time of treatment, or inhibiting the process.
Application of enzyme-based processes would allow a greater independence from the influences of wastewater composition which may affect fungal growth. Exploitation of different enzymatic mixtures produced by each ligninolytic strain appears to be of most interest for practical applications. Several authors opted for the use of crude culture filtrates in dye-treatment, since they offer some distinct advantages: i) their use allows to skip some expensive purification steps; ii) other factors present in the medium may stabilize the main oxidative enzymes or act in a synergistic way with them [80].

7. Enzymes for wastewater treatments: the case of laccases

Laccases, EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase, are N-glycosilated multi copper oxidases belonging to the group of the blue copper proteins [81, 82] which includes, among others, ascorbic acid oxidase and ceruloplasmin [83]. Laccases are able to catalyze direct oxidation of *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, and aryl diamines as well as some inorganic ions [84, 85]. They couple the four single-electron oxidations of the reducing substrate to the four electron reductive cleavage of the dioxygen bond, using four Cu atoms distributed against three sites, defined according to their spectroscopic properties [86]. The catalysis ability of this enzyme family is guaranteed by the presence of these different copper centres in the enzyme molecule; based on spectroscopic analysis, which reflects geometric and electronic features, copper centres are differentiated as Type 1 Cu (T1), or blue copper centre, Type 2 (T2) or normal copper, and Type 3 (T3) or coupled binuclear copper centres [86, 87]. The T1 copper, which is the primary oxidation site, is characterized by a strong absorption around 600 nm, caused by the covalent copper-cysteine bond, which gives rise to the typical bleu colour of the copper oxidase. Two hystidines and one cysteine serve as ligands for type-1 Cu at the T1 center. While usually in type-1 centers, a sulfur from a methionine residue

functions as an additional axial ligand, the fourth ligand for this copper center is not present in fungal laccases, in which uncoordinating Phe or Leu occupies this position [88]. Due to its high redox potential of 500- 800mV, Type 1 copper is the site where substrate oxidation takes place. The T2 copper exhibits only weak absorption in the visible region and 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. Type 2 and Type 3 copper ions form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place Type 2 copper is coordinated by two histidines and Type 3 copper atoms by six histidines. The two copper ions of the T3 site are EPR-silent due to an antiferromagnetic coupling mediated by a bridging hydroxyl ligand. Substrates (phenols and aromatic or aliphatic amines) are oxidized by 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 [87]. Despite the amount of information on laccases as well as on other blue multicopper oxidases, neither the precise electron transfer pathway nor the details of dioxygen reduction in blue multicopper oxidases are fully understood [88]. It was found that the catalytic efficiency of laccases for some reducing substrates depends linearly with the redox potential of the T1 site. Thus, laccases with a high redox potential in the T1 site are of special interest for biotechnological applications.

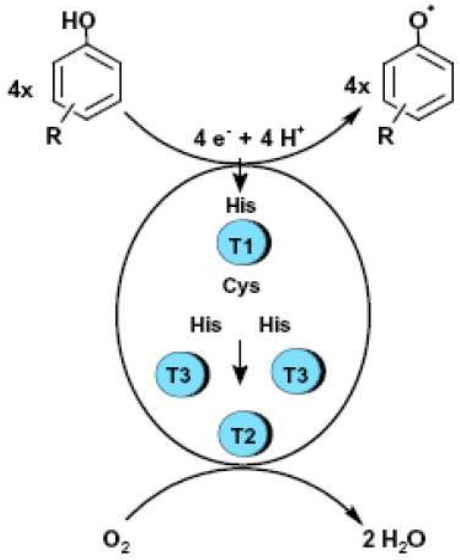

Fig.2 Catalytic cycle of laccase

Kinetic data suggest a mechanism of reaction “two site ping-pong bi bi” type; according to this mechanism the products are released before a new substrate molecule is ligated [90]. Thus, the reaction of the fully reduced enzyme with O_2 proceeds via two sequential two-electron steps, generating the peroxy intermediate (PI) and the native intermediate (NI). The first step is rate determining, while the second, involving the $2e^-$ reductive cleavage of the O–O bond, is faster [83, 91].

Laccase was first described by Yoshida in 1883 when he extracted it from the exudates of the Japanese lacquer tree *Rhus vernicifera*, from which the name laccase was derived [81, 92]. Subsequently, laccases have been discovered from numerous other plants [93]. More recently, findings of laccase activity in prokaryotes [94] and insects [95] have also been reported.

The majority of laccases characterized so far have been derived from fungi, especially from white rot basidiomycetes. In fungi, laccases carry out a variety of physiological roles including morphogenesis, fungal plant-pathogen/host interaction, stress defense, and lignin degradation [81, 96]. Most fungi produce several isoforms of laccases. The majority of white-rot fungi are shown to produce both intracellular and extracellular laccases, but the enzyme is mostly (95%) located outside the cell.

Cellular localization of laccases seems to be associated with their physiological functions.
Multiplicity of laccase genes is common feature in fungi, and the production of several laccase isozymes has been observed in many species. Perry and coworkers, in 1993 [97], described the presence of two laccase genes in the same chromosome of the basidiomycetes. *Agaricus bisporus*, thus reporting the first example of a laccase gene family in fungi. Indeed, several laccase gene family in fungi have been described [98- 100].
Laccase gene families have also been described in *Pleurotus* genera, with four isolated members in *P. sajior-caju* [101], two in *P. eryngii* [102] and seven in *P. ostreatus* [103]. The analysis of the recently released *P. ostreatus* genome has highlighted the presence of previously uncharacterized laccase genes, enriching the panel of laccase genes up to 12 memebers (unpublished data).
Until now, more than 100 fungal laccases have been purified and somehow characterized. Typical fungal laccase has a molecular mass from about 50 to 70 kDa with an acidic isoelectric point around pH 4.0; it can be polymeric, and the enzymatically active form can be a monomer, dimer, trimer or tetramer [83].
Laccases presents a high level of glycosylation that may contribute to the high stability of the enzyme through the covalent link of carbohydrate moieties that can reach from 10 to 45% of the total weight [104, 105]. It was proposed that in addition to the structural role, glycosylation can also participate in the protection of laccase from proteolysis and inactivation by free radicals [106].
Laccases have a lower redox potential (450-800 mV) than that of ligninolytic peroxidases (> 1 V), so it was initially thought that laccases would only be able to oxidize phenolic substrates [107]. However, the range of substrates oxidized by laccases can be increased through a mediator-involved reaction mechanism.
Reports on pH optimum, substrate specificity and other properties of laccases show extreme diversity (Tab.2) [108].

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

Table 2: Properties of fungal laccases: Q25, lower quartile; Q75, upper quartile (Baldrian, 2006).

There is a considerable heterogeneity in the properties of laccases isolated from ascomycetes, especially with respect to molecular weight [108]. The woodrotting species *Phellinus ribis* [109], *Pleurotus pulmonarius* [104] and *Trametes villosa* [100], the mycorrhizal fungus *Cantharellus cibarius* [110] and the ascomycete *Rhizoctonia*

solani [111] 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. Analyses of purified POXA3a and POXA3b laccases from *P. ostreatus* have revealed the presence of a heterodimeric structure with a catalytic subunit (67 kDa) and a small, not catalytic active, subunit (18 or 16 kDa) affecting complex stabily [112, 113].
A number of 3D structures of basidiomycetes laccases have been reported so far. Crystal structures have been solved for the laccases of: *Coprinus cinereus* (CcL1A65) [114], *T. versicolor* (Lcc1 and LacIIIb)[115, 116], *P. cinnabarinus* [117], *M. albomyces* [118] and *R. lignosus* (R1G1V10) [89], *Lentinus tigrinus* (LtL2QT6) [119] and *Trametes torgii* (TtL2HRG) [120].

Because of their high nonspecific oxidation capacities, to the lack of a requirement for cofactors and to the use of readily available oxygen as an electron acceptor, laccases are useful biocatalysts for a wide range of emerging biotechnological applications. As a fact, commercially they find applications in food industry, in which the selective removal of phenol derivatives by laccases is exploited for stabilization and improvement of the quality of different drinks [121]; in pulp and paper industries, in which the treatment of wood pulp with these enzymes, provides milder and cleaner strategies of delignification that are also respectful of the integrity of cellulose [122]. Moreover, amperometric biosensors based on laccases have been described for the analysis of different compounds (chloro-substituted phenols, catecholamines, lignin, tea tannins, and also ascorbic acid) and laccases have also found applications in biofuel cells construction [123].
Dye decolourization remains one of the widely studied applications of laccases in general. In the last years, the use of laccase in the textile industry is growing very fast, since besides to decolourise textile effluents, laccases are being used to bleach textiles and even to synthesise dyes [124]. Related to textile bleaching, in 1996 Novozyme (Novo Nordisk, Denmark) launched a new industrial application of laccase enzyme in denim finishing: DeniLite®, the first industrial laccase and the first bleaching enzyme acting with the help of a mediator.
An emerging field of application is the synthetic chemistry, where laccases have been proposed be applicable for oxidative deprotection [125] and production of complex polymers and medical agents [126].
A very wide variety of bioremediation processes employ laccase in order to protect the environment from damange caused by industrial effluents. Laccases from different sources have been shown to be useful for the degradation of several xenobiotics compounds and of persistent environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs) [127, 128], 2,4,6-trinitrotoluene [129], chlorinated phenolics [130], pesticides [131] and synthetic dyes [132].

8. Overexpression of laccase

Laccase secreted from native sources are usually not suitable for large-scale purposes, mainly due to low production yields and high cost of preparation/purification procedures. The ever-increasing demand for laccases in the industrial sectors requires large quantities of enzyme to be produced, and hence, any attempt to increase the production of enzymes from microbial sources would be of considerable interests.

For the application of fungal enzymatic systems to large scale wastewater treatment, two aims have to be pursued: optimization of their production and improvement of their performances. To make laccases available for industrial applications, methods to reduce costs include fermentation media optimization, novel fermentation methods, and genetic modification for large scale production via eukaryotic recombinant strains. Much research has been done to identify effective methods for mass production of laccase using the above mentioned methods.
The production of laccase can be considerably enhanced by the addition of inducers which includes a wide variety of aromatic and phenolic compounds [104]. The use of inducers for laccase production has been widely reported from fungi [133]. Nevertheless, determination of optimum fermentation media can easily be achieved but cofactors and inducer compounds can cause an undesirable increase in cost during growth at industrial scale.
Another valued approach for enhanced enzyme production is the recombinant protein expression in easily cultivable and handling hosts; it could allow higher productivity in shorter time and reduces the costs of production. The versatility and scaling-up possibilities of the recombinant protein production opened up new commercial opportunities for their industrial uses [134]. In addition, heterologous expression may permit to produce laccase with desired properties; protein engineering can be employed to improve the stability, activity and/or specificity of an enzyme [135, 136], thus tailor made enzymes can be produced to suit the requirement of the users or of the industrial process [137].
Up now efficient expression systems of LME have been proposed [138, 139]. Laccases have been expressed at high yields in many hosts, whilst the heterologous expression of peroxidases seems to be critical due to the more complex post-translational modifications required for correct folding and activity of these enzymes [140].
Alternatively, Homolka *et al.* 1995 [141] and Eichlerová- Voláková & Homolka 1997 [142] in their works showed that besides classical mutagenization, preparation of protoplast derived isolates or basidiospore-derived monokaryotic isolates is an efficient method of reaching higher variation in the production of enzymes involved in lignin degradation, in fungi. As a fact, Eichlerov and Homolka in 1999 [143] in their work obtained laccase and other ligninolytic enzyme higher-producing dikaryons of *Pleurotus ostreatus* after crossing of compatible basidiospore-derived monokaryons selected from the parental basidiospore population. Thus, the classical cross-hybridization is a good alternative to achieve improvements of fungi and to optimise specific traits of the natural strains, also because genetic transformation and mutagenic treatments produce strains not suitable for natural or safe processes. New dikaryotic strains obtained by classical breeding, with improved efficiencies are still considered by the legislation as GRAS and can be good candidates for safe biotechnological exploitation.

- ***Application of Streptomyces spp. bacteria and of the white-rot fungus Pleurotus ostreatus to wastewater treatment: state of art***

9. Case study I: *Streptomyces spp.* and their laccases-like enzymes

In the last decade, research paid particular attention to decolourization ability of *Streptomyces spp.* bacteria.
Streptomyces are Gram-positive bacteria known for their capability of producing large numbers of useful substances. This organism performs secondary metabolism to synthesize many kinds of antibiotics and biologically active substances, which have

important application in medicinal, agricultural and chemical industries [144]. *Streptomyces* also produces a wide variety of useful enzymes such as proteases, nucleases and enzymes that hydrolyze polysaccharides, which benefit the saprophytic life cycle of the organism by catalyzing the degradation of many kinds of organic molecules [145].
Another characteristics feature of this group of bacteria is the ability to perform complex morphological development resembling that of filamentous fungi [146]. Early in the life cycle on solid medium, the organism undergoes vegetative growth as a branching multinucleoide substrate mycelium. In response to environmental and physiological signals, the substrate mycelium then produces aerial hyphae, which finally differentiate into spore chains by septum formation at regular intervals. Involvement of phenoloxidizing enzymes in cellular development and in the morphogenesis of *Streptomyces spp*. was been suggested.
Endo and coworkers identified EpoA, a two-domain laccase, in *Streptomyces griseus* which operates as a trimer [147]. The identified enzyme, EpoA is a secreted laccase-like protein, whose activity is closely linked to cellular differentiation of this organism. It contains the multicopper oxidase signatures that are common to the laccase family in its C-terminal region. Moreover, Endo and his collaborators demonstrated the presence of an intrinsic copper-dependent regulatory mechanism for the onset of morphogenesis and antibiosis in *Streptomyces griseus*. A coincidental event caused by exogenous copper in various *Streptomyces spp.* is melanin production, is catalysed by phenol oxidases, copper-containing enzymes, including tyrosinases and laccases.
The presence of a homologue of EpoA in *S. coelicolor* A3(2), a phylogenetically divergent organism from *S. griseus*, implies wide distribution of the enzyme among *Streptomyces*.
In *Streptomyces cyaneus*, a laccase-type phenol oxidase was found to be produced during growth under solid-substrate fermentation conditions, and it was suggested that this enzyme was involved in the solubilization and mineralization of lignin from wheat straw [148]. Further studies demonstrated that this organism could be used to improve the qualities of pulp after 2 weeks of incubation under solid-substrate fermentation conditions.
In the last decade many works showed the presence of laccase-like enzymes in different *Streptmoyces spp.;* in their work Machczynski *et al.* [149] identified and characterized a small laccase produced by *Streptomyces coelicolor.* This enzyme represents a new family of laccases that possesses only two domains; it is denoted “small laccase” (SLAC) because it lacks a domain and it is thus much smaller than other laccases. Despite the lack of this domain, the enzyme maintains the normal complement of four copper ions and is active against the common range of substrates, preferring to bind those that are negatively charged.
In 2003, for the first time, Arias M.E. [150] and his collaborator described the potential applications of *Streptomyces* laccases in the pulp industry. In particular they purified the extracellular laccase by *S. Cyaneus* CECT 3335, in submerged cultures, and described its application in biobleaching of kraft pulps. In particular in this work they demonstrated that laccase produced by *S. cyaneus* can delignify eucalyptus kraft pulp in the presence of ABTS as a mediator. The results obtained are comparable to those described by other workers who used different laccases produced by ligninolytic fungi and ABTS as the mediator. Also Dubè *et al*. (2008) [151], showed that the combination of *Streptomyces coelicolor* laccase and the mediator acetosyringone was able to rapidly decolourize, to various degrees,

different dyes tested. In 10 min, decolourization was achieved at 94% for acid blue 74, 91% for direct sky blue 6b and 65% for reactive black 5. Furthermore, decolourization was achieved at 21% for reactive blue 19 and at 39% for the direct dye Congo red in 60 min. These results demonstrate the potential use of this laccase in combination with acetosyringone, a natural mediator, for dye decolourization.

10. Case study II: white-rot fungus *Pleurotus ostreatus* and its laccases

Pleurotus ostreatus is a commercially important edible mushroom commonly known as the oyster mushroom. This fungus is industrially produced as human food, and it accounts for nearly a quarter of the world mushroom production [152].
Several different *P. ostreatus* varieties are industrially produced. Commercial varieties *florida* and *ostreatus* differ in size, colour and temperature tolerance. In the world of fungi, *P. ostreatus* has emerged a model system in textile, polycyclic aromatic hydrocarbon (PAH), and pulp and paper mill effluent remediation. This basidiomycete fungus is able to degrade complex compounds such as starch, cellulose, pectin, lignin, lignocelluloses, which are characteristics of textile effluent.
The white-rot fungus *Pleurotus ostreatus* is able to express multiple laccase genes encoding isoenzymes with peculiar properties considering both industrial applications and structure-function relationships. Amount of each specific enzyme produced depends on culture conditions.
Seven laccase gene members have already been isolated in *P.ostreatus*; the existence of a “laccase subfamily” consisting of three members has been postulated, based on sequence similiarity and intron-exon structure [103]. Furthermore, a careful investigation of the recently released genome of this fungus by the DOE- Joint Genome Institute, suggested an even more complex multicopper oxidases (MCOs) family. The analysis of the recently released *P. ostreatus* genome has highlighted the presence of previously uncharacterized laccase genes, enriching the panel of laccase genes up to 12 memebers (unpublished data).
So far, seven isoenzymes secreted by the mycelium have been purified and characterized: POXC [153, 154], POXA1w [155], POXA1b [156], the two strictly related isoenzymes POXA3a and POXA3b [112, 113, 139] and more recently POX3 and POX4 [103]. The latter two enzymes, were not isolated from the *P. ostreatus* broth culture, but have been characterized as recombinant proteins.
Moreover, in the last year, Lettera and coworkers [157], shown for the first time the identification of a laccase from the fruting body of *P.ostreatus,* adding a new piece to the knowledge about *P. ostreatus* laccase multigene family.
POXC is the most abundantly produced in all growth conditions tested so far; POXA1w shows peculiar differences with regard to metal ions content, containing two zinc atoms, one iron atom, and only one copper atom per molecule; POXA1b is the most stable at alkaline pH and shows activity inside the cell and/or on the cell wall [159]; POXA3a and POXA3b are heterodimeric laccases [112].
P. ostreatus was shown to be able to decolorize industrial dyes. Experiments have been performed using a representative antraquinonic dye, RBBR, in solid and liquid culture and the key role played by laccases in this process has been demonstrated. Successively, decolourization experiments with *P. ostreatus* whole cells and crude laccase mixtures have been carried out for biodegradation of model waste water from textile industry [159].
The structural and functional characterization of *P. ostreatus* laccases was also achieved by heterologous expression in two yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae* [160]. Moreover, the availability of a good system for

heterologous expression has allowed the development of new oxidative catalysts through molecular evolution techniques [135, 136].

11. Aim of the thesis

Streptomyces spp., white-rot fungi and their oxidative enzymes represent promising tools for the treatment of industrial effluents due to their outstanding abilities to degrade a wide range of pollutants, including synthetic dyes.

In this context, research has been aimed at exploiting bacterial and WRF potential by developing new biosystems for the treatment of industrial coloured wastewaters. This project is part of a broader research project, carried out in collaboration with the University of Turin (Department of Vegetal Biology, Dr. Cristina Varese and financed by the "Compagnia di S. Paolo") and it aims at developing biosystems for the treatment of industrial coloured wastewaters, based on microbial and enzymatic catalysts.

In order to select biosystems able to decolorize and detoxify efficiently the industrial wastewaters, it has been necessary to standardize the analysis methods, so that the different systems have been analyzed and compared under the same conditions. In particular the different biosystems have been analyzed against four wastewater models (described in the following chapter Model Systems) and in some cases on the single dye which composed them.

Work description has been organized in the following sections:

Section I

- Case study I: *Application of bacterial biosystem for the treatment of synthetic dyes and industrial coloured wastewaters*

Decolouration ability of the bacteria *Streptomyces spp*., will be tested on different single synthetic dyes and on four wastewater models from textile industry.

- Case study II: *Application of enzymatic biosystems for the treatment of industrial coloured wastewaters*

Decolouration ability of native laccases of *P.ostreatus* (POXA1b, POXC and POXA3), produced by heterologous expression in *K. lactis,* will be tested against the four wastewater models. Moreover, selected POXA1b mutants with improved stability to temperature and pH, improved catalytic efficiency), obtained by random mutagenesis [161, 162] and expressed in the heterologous system *S. cerevisiae*, will be tested on the four wastewater models.

Section II

Molecular determinants of peculiar properties of a Pleurotus ostreatus laccase

In order to identify and characterize molecular determinants of peculiar properties POXA1b laccase, mutants of this laccase will be obtained by site directed mutagenesis strategy.

Section III

Improvement of P. ostreatus laccase production yield by classical breeding

In order to improve industrially useful enzymatic biosystems, suitable for decolourization processes of coloured wastewaters, we aimed at obtaining new higher laccases producers *P. ostreatus* strains by classical breeding approach.

New dikaryons will be obtained by crossing monokaryons derived from *P.ostreatus ATCC 2306* and *P.ostreatus D1208* parental strains, selected on their improved laccase productivity.

Analysis of phenol oxidase production profile of the submerged growth of the dykariotic strains and the identification of the laccase isoforms produced by the new dycariotic *P.ostreatus* strains, monitored in liquid cultures will be performed.

12. References

[1] Allegre C., Moulin P., Maisseu M., Charbit F. Treatment and reuse of reactive dyeing effluents. *J. Membr. Sci.* (2006); 269:15-34.

[2] Helmer R. and Hespanhol I. (eds.), E & FN Spon. Water Pollution Control. A Guide to the Use of Water Quality Management Principles. *Water, Air, & Soil Pollution* (1999); 110:435-437.

[3] Dua M., Singh A., Sethunathan N., Johri AK. Biotechnology and bioremediation: successes and limitations. *Appl. Microbiol. Biotechnol.* (2002); 59(2-3):143-52.

[4] Debarati P., Pandey G., Pandey J. and Rakesh KJ. Accessing microbial diversity for bioremediation and environmental restoration. *Trends in Biotech*. (2005); 23:135-142.

[5] Boopathy R. Factors limiting bioremediation technologies. *Biores. Technol. 74.* (2000); 63-67.

[6] Ramakrishna KR., Viraraghavan T. Dye removal using low cost adsorbents. *Water Sci Technol.* (1997); 36:189–96.

[7] Nigam P., Armour G., Banat I.M., Singh D., Marchant R. Physical removal of textile dyes and solid state fermentation of dye-adsorbed agricultural residues. *Bioresour. Technol.* (2000); 72:219–26.

[8] O'Neill C., Hawkes FR., Lourenco ND., Pinheiro HM., Delee W. Colour in textile effluents - sources, measurement, discharge consents and simulation: a review. *J. Chem Technol Biotechnol.* (1999);74:1009–18.

[9] Zollinger H. Colour Chemistry – Synthesis, Properties and Applications of Organic Dyes and Pigments. (1987) VCH, New York.

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

[11] Aksu Z., Tezer S. Application of biosorption for the removal of organic pollutants: a review. *Process Biochem*. (2005); 40:997-1026.

[12] Oliveira D.P., Carneiro P.A., Sakagami M.K., Zanoni M.V.B., Umbuzeiro G.A. Chemical characterization of a dye processing plant effluent - Identification of the mutagenic components**.** *Mutation Research/Genetic Toxicology and Environmental Mutagenesis.* (2007); 626: 135-142.

[13] Fu Y. and Viraraghavan T. Fungal decolourization of wastewaters: a review. *Bioresour. Technol.* (2001); 79:251-262.

[14] Hai F.I., Yamamoto K., Fukushi K. Hybrid treatment system for dye wastewater. *Crit. Rev. Environ. Sci. Technol.* (2007); 37:315-377.

[15] Easton J.R., The dye maker's view. In P. Copper (ed.), *Colour in Dyehouse Effluent*, *Society of Dyers and Colourists*. Oxford (1995) pp 9±21.

[16] Yau L. and Milner P. Preventing biological degradation of textiles. *International Dyer* (1997); 182(2):31.

[17] Dos Santos A.B., Cervantes F.J., Van Lier J.B. Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. *Bioresour. Technol.* (2007); 98:2369-2385.

[18] Hoyle P.J. Colour removal from dyehouse effluent using synthetic organic coagulants, In P. Copper (ed.), *Colour in Dyehouse Effluent*, *Society of Dyers and Colourists*. Oxford. (1995); 171±184.

[19] Knapp J.S., Newby P.S., Reece L.P. Decolorization of dyes by wood-rotting basidiomycete fungi. Enzyme and Microbial Technology. (1995); 17:664-668.
[20] McMullan G., Meehan C., Conneely A., Kirby N., Robinson T., Nigam P.I., Banat M., Marchant R. and Smyth W.F. Microbial decolourisation and degradation of textile dyes. *Appl. Microb. Biotechnol.* (2001); 56:81–87.
[21] Robinson T., McMullan G., Marchant R. and Nigam P. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative**.** *Bioresource Technology.* (2001); 77:247-255.
[22] Beydilli M.I., Pavlostathis S.G., Tincher W.C. Decolourization and toxicity screening of selected reactive azo dyes under methanogenic conditions. *Water Sci Technol.* (1998) 38:225–232.
[23] Choy K.K.H., McKay G. and Porter J.F. Sorption of acid dyes from effluents using activated carbon. *Resources, Conservation and Recycling*. (1999); 27: 57-71.
[24] Slokar Y.M. and Le Marechal A.M. Methods of decolourisation of textile wastewaters. *Dyes Pigm*. (1997); 37:335–356.
[25] Kumar V., Wati L., Nigam P., Banat I.M., Yadav B.S., Singh D. and Marchant R. Decolorization and biodegradation of anaerobically digested sugarcane molasses spent wash effluent from biomethanation plants by white-rot fungi. *Process Biochemistry.* (1998); 33: 83-88.
[26] Tsezos M., and Bell J.P. Comparison of the biosorption and desorption of hazardous organic pollutants by live and dead biomass. *Water Res.* (1989); 23**:** 561–568.
[27] Orhan Y. and Buyukgungor H. The removal of heavy metals by using agricultural wastes. *Water. Sci. Technol.* (1993); 28: 247–255.
[28] Norton L., Baskaran K. and McKenzie T. Biosorption of zinc from aqueous solutions using biosolids. *Adv. Environ. Res*. (2004); 8: 629-635.
[29] Polman J.K. and Breckenridge C.R. Biomass mediated binding and recovery of textile dyes from waste effluents. *Textile Chemist and Colorist*. (1996); 28:31–35.
[30] Crini G., Non-conventional low-cost adsorbents for dye removal: a review. *Bioresource Technol.* (2006); 97:1061–1085.
[31] Crini G. and Badot P.M. Starch-based biosorbents for dyes in textile wastewater treatment. *International Journal of Environmental*. (2010); 12:129-150.
[32] Kapoor A. and Viraraghavan T. Fungal-biosorption: an alternative treatment option for heavy metal bearing wastewater: a review. *Biores. Technol.* (1995); 53: 195–206.
[33] Manu and Chaudhari S. Anaerobic decolorization of simulated textile wastewater containing azo dyes, Bioresource Technology (2002); 82: 225–231.
[34] Stolz A. Basic and applied aspects in the microbial degradation of azo dyes. *Appl Microbiol Biotechnol.* (2001); 56:69–80.
[35] Balan D.S.L. and Monteiro R.T.R. Decolorization of textile indigo dye by ligninolytic fungi. *J Biotechnol.* (2001); 89: 141–145.
[36] Forgacs E., Cserhátia T. and Orosb G. Removal of synthetic dyes from wastewaters: a review. *Environment International.* (2004) ; 30 :953-971.
[37] Chen K.C., Wu J.Y., Liou D.J., Hwang S.C.J. Decolorization of the textile dyes by newly isolated bacterial strains. *Journal of Biotechnology*. (2003) – Elsevier. 101:57-68.
[38] Kulla H.G. Aerobic bacterial degradation of azo dyes. In: Leisinger T, Cook AM, Hutter R, Nuesch J (eds) Microbial degradation of xenobiotics and recalcitrant compounds. *Academic Press, London*. (1981); 387±399.

[39] Nigam, P., Marchant, R. Selection of a substratum for composing biofilm system of a textile-effluent decolourizing bacteria. *Biotech. Lett*. (1995); 17:993±996.
[40] Ogawa T. and Yatome C. Biodegradation of azo dyes in multistage rotating biological contactor immobilization by assimilating bacteria. *Bulletin of Environmental Contamination and Toxicology*. (1990); 44:12–14.
[41] Keck A., Klein J., Kudlich M., Stolz A., Knackmuss H.J., and Mattes R. Reduction of Azo Dyes by Redox Mediators Originating in the Naphthalenesulfonic Acid Degradation Pathway of *Sphingomonas* sp. Strain BN6. Applied and Environmental Microlbiology. (1997); 3684–3690.
[42] Olukanni O.D., Osuntoki A.A. and Gbenle G.O. Decolourization of azo dyes by a strain of *Micrococcus* isolated from a refuse dump soil. Biotechnology. (2009); 8:442-448.
[43] Alia N., Hameedb. A., and Ahmed S. Physicochemical characterization and Bioremediation perspective of textile effluent, dyes and metals by indigenous bacteria. *Journal of Hazardous Materials*. (2009); 164(1):322-328.
[44] Novotný C., Svobodová K., Benada O., Kofroňová O., Heissenberger A., Fuchs W. Potential of combined fungal and bacterial treatment for color removal in textile wastewater. *Bioresour Technol*. (Sep. 2010).
[45] Tony B.D., Goyal D., Khanna S. Decolorization of Direct Red 28 by mixed bacterial culture in an up-flow immobilized bioreactor. *J Ind Microbiol Biotechnol*. (2009); 36(7):955-60.
[46] Chang S.W., La H.J., Lee S.J. Microbial degradation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) contaminated groundwater in Korea. *Water Sci Technol*. (2001); 44(7):165-71.
[47] Zimmermann T., Kulla H.G., Leisinger T. Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur J Biochem.* (1982); 129(1):197-203.
[48] Lourenço N.D., Novais J.M. and Pinheiro H.M. . Kinetic Studies of Reactive Azo Dye Decolorization in Anaerobic/aerobic Sequencing Batch Reactors. *Biotechnology Letters*. (2006); 28:733-739.
[49] Zissi U., Lyberatos G. Partial degradation of p-aminoazobenzene by a defined mixed culture of *Bacillus subtilis* and *Stenotrophomonas maltophilia*. *Biotechnol Bioeng*. (2001); 72(1):49-54.
[50] Robinson T., Chandran B., Nigam P. Studies on the production of enzymes by white-rot fungi for the decolorisation of textile dyes. *Enzyme Microb. Technol.* (2001); 29:575– 579.
[51] Meehan C., Banat I. M., , McMullan G., Nigam P., Smyth F. and Marchant R. Decolorization of Remazol Black-B using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3. *Environment International.* (2000); 26:75-79.
[52] Winter B., Fiechter A., and Zimmermann W. Degradation of organochlorine compounds in spelt sulfite bleach plant effluents by actinomycetes. *Appl. Environ. Microbiol*. (1991); 57:2858-2863.
[53] Brar S.K., Verma M., Surampalli R.Y., Misra K., Tyagi R.D., Meunier N. and Blais J.F. Bioremediation of hazardous wastes — a review. *Practice periodical of hazardous, toxic and radioactive waste management.* (2006); 59–72.
[54] Prachi K., Anushree M. Fungal dye decolourization: Recent advances and future potential. *Environment International.* (2009); 35:127–141.
[55] Hatakka A. Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS Microbiology Reviews.* (1994); 13:125-135.

[56] Levin L., Papinutti L, Forchiassin F. Evaluation of *Argentinean* white rot fungi for their ability to produce lignin modifying enzymes and decolorize industrial dyes. *Biores Technol* (2004). 94:169-176.
[57] Nilsson I., Moller A., Mattiasson B., Rubindamayugi M.S.T., Welander U. Decolorization of synthetic and real textile wastewater by the use of white-rot fungi. *Enzyme Microb Technol.* (2006); 38:94-100.
[58] Zhao X.H. & Hardin, I.R. Biodegradation mechanisms in fungal decolorization of dyes. In L. E. Pawly, *Leading Edge Environmental Biodegradation Research*, (2007); New York: Nova Science Publishers.
[59] Urzula U., Larrondo L.F., Lobos S., Larraın J., Vicuna R. Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Ceriporiopsis subvermispora*. *FEBS Lett.* (1995); 371:132-6.
[60] Wariishi H., Valli K., Gold M.H. Manganese (II) oxidation by manganese peroxidase from the basidiomycetes *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. *J. Biol. Chem.* (1992); 267: 23688-95.
[61] Glenn J.K., Akileswaran L. and Gold M.H. Mn(II) oxidation is the principle function of the extracellular Mn-peroixidase from *PlutncroclHiete chrysosporium*. *Arch Biochcm. Biophys.* (1986); 251(2): 688-696.
[62] Galkin S., Vares T., Kalsi M., and Hatakka A. Production of organic acids by different white-rot fungi as detected using capillary zone electrophoresis. *Biotechnol. Technol.* (1998); 12: 267-271.
[63] Kuan I.C., Jhonson K.A., Tien M. Kinetic analysis of manganese peroxidase. The reaction with manganese complexes. *J. Biol. Chem.*(1993); 268: 20064-20070.
[64] Heinfling A., Martinez M.J., Martinez A.T., Bergbauer M., Szewzyk U. Transformation of industrial dyes by manganese peroxidises from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. *Appl. Environ. MIcrobiol.* (1998); 64: 2788-2793.
[65] Nerud F. and Mišurcová Z. Distribution of ligninolytic enzymes in selected white-rot fungi. *Folia Microbiologica*. (1996); 41: 264-266.
[66] Günther T., Sack U., Hofrichter M. and Laetz M. Oxidation of PAH and PAH-derivatives by fungal and plant oxidoreductases. *Journal of Basic Microbiology.* (1998); 38: 113–122.
[67] Krcmár P., Kubátová A., Votruba J., Erbanová P., Novotný C. and Sasek V. Degradation of polychlorinated biphenyls by extracellular enzymes of *Phanerochaete chrysosporium* produced in a perforated plate. *World Journal of Microbiology and Biotechnology.* (1999); 15: 269-276.
[68] Chivukula M., Spadaro J.T. and Renganathan V. Lignin peroxidase-catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxides. *Biochemistry* . (1995); 35: 7765–7772.
[69] Shrivastava R., Christian V., Vyas B.R.M. Enzymatic decolorization of sulfonphthale in dyes. *Enzyme Microb Technol.* (2005); 36:333-337.
[70] Rodakiewicz-Nowak J., Jarosz-Wilkołazka A., Luterek J. Catalytic activity of versatile peroxidase from *Bjerkandera fumosa* in aqueous solutions of water-miscible organic solvents. *Appl. Catal. A Gen.* (2006); 308:56-61.
[71] Heinfling A., Martinez M.J., Bergbauer M., Szewzyk U. and Martinez A.T. A study on reducing substrates of manganese oxidizing peroxidises from *Pleurotus eryngii* and *Bjerkandera adusta. FEBS letter.* (1998); 428:141-146.
[72] Honda Y., Watanabe T., Watanabe T. Exclusive overexpression and structure-function analysis of a versatile peroxidase from white-rot fungus, *Pleurotus ostreatus*. *Sustainable Humanosphere.* (2006); 2:2-6.

[73] Evans B.R., Margalit R., Woodward J. Veratryl alcohol oxidase activity of a chemically modified cellulase protein. *Arch Biochem. Biophys.* (1994); 312(2):459-66.
[74] Van Aken B., Agathos S.N. Implication of manganese (III), oxalate, and oxygen in the degradation of nitroaromatic compounds by manganese peroxidase (MnP). *Appl Microbiol Biotechnol*. (2002); 58(3):345-51.
[75] Eggert C, Temp U, Dean JF, Eriksson KE. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett.* (1996); 391(1-2):144-8.
[76] De Jong E., Field J.A., de Bont J.A.M. Aryl alcohols in the physiology of ligninolytic fungi. *FEMS Microbiol. Rev.* (1994);13:153- 88.
[77] Akhtar M, Blanchette RA, Kirk T.K. Fungal delignification and biomechanical pulping of wood. *Advances in Biochemical Engineering/Biotechnology*. Berlin; *Springer*. (1997).
[78] Cha C.J., Doerge D.R., Cerniglia C.E. Biotransformation of malachite green by the fungus *Cunninghamella elegans*. *Appl Environ Microbiol*. (2001); 67:4358-60.
[79] Koduri RS, Tien M. Kinetic analysis of lignin peroxidase: explanation for the mediation phenomenon by veratryl alcohol. *Biochemistry*. (1994); 33:4225-30.
[80] Trupkin S., Levin L., Forchiassin F., Viale A. Optimization of culture medium for ligninolytic enzyme production and synthetic dye decolorization using response surface methodology. *J Ind. Microbiol. Biotechnol.* (2003); 30:682-69046.
[81] Thurston F. The structure and function of fungal laccases. *Microbiology.* (1994); 140:19–26.
[82] Xu F. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition. *Biochemistry* (1996); 35: 7608-7614.
[83] Giardina P., Faraco V., Pezzella C., Piscitelli A., Vanhulle S. and Sannia G. Laccases: a never-ending story . *Cell Mol Life Sci*. (2010); 67(3):369-85.
[84] Sakurai T. Anaerobic reactions of *Rhus vernicifera* laccase and its type-2 copper-depleted derivatives with hexacyanoferrate(II). *Biochem J.* (1992); 284:681–685.
[85] Schlosser D., Hofer C. Laccase-catalyzed oxidation of Mn2+ in the presence of natural Mn3+ chelators as a novel source of extracellular H_2O_2 production and its impact on manganese peroxidase. *Appl Environ Microbiol.* (2002); 68:3514–3521.
[86] Solomon E.I., Sundaram U.M., Machonkin T.E. Multicopper oxidases and oxygenases. *Chem Rev.* (1996); 96:2563–2605.
[87] Messerschmidt A. and Huber R. The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. *Eur. J. Biochem.* (1990); 187:341- 352.
[88] Sakurai T. and Kataoka K. Structure and function of type I copper in multicopper oxidases. *Cell.Mol.Life.Sci.* (2007); 64:2642-56.
[89] Garavaglia S., Cambria M.T., Miglio M., Ragusa S., Lacobazzi V., Palmieri F., D'Ambrosio C., Scaloni A. & Rizzi M. 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* .(2004); 342:1519–1531.
[90] Petersen L.C., Degn H. Steady-state kinetics of laccase from *Rhus vernicifera. Biochim Biophys Acta* (1978); 526:85-92.
[91] Solomon E.I., Augustine A.J., Yoon J. O_2 reduction to H_2O by the multicopper oxidases. *Dalton Trans.* (2008); 30:3921–3932.
[92] Reinhammar, B. In "Copper Proteins and Copper Enzymes". (Lontie, R., ed.), (1984) 3:1-35, CRC Press, Boca Raton, FL.

[93] Ranocha P., McDougall G., Hawkins S., Sterjiades R., Borderies G., Stewart D., Cabanes-Macheteau M., Boudet A.M., Goffner D. Biochemical characterization, molecular cloning and expression of laccases - a divergent gene family - in poplar. *Eur. J Biochem.* (1999); 259(1-2):485-95.
[94] Sharma P., Goel R., Capalaash N. Bacterial laccases. *World J Microbiol Biotechnol*. (2007); 23:823-832.
[95] Kramer K.J., Kanost M.R., Hopkins T.L., Jiang H., Zhu Y.C., Xu R., Kerwin J.L., and Turecek F. Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron* (2001); 57:385-392.
[96] Gianfreda L., Xu F., Bollang J.M. Laccases: a useful group of oxidoreductive enzymes. *Bioremediat J*. (1999); 3:1-26.
[97] Perry C.R., Matcham S.E., Wood D.A., Thurston C.F. The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus biporus*. *J Gen Microbiol.* (1993); 139:171-178.
[98] Kilaru S., Hoegger P.J. and Kües U. The laccase multi-gene family *Coprinopsis cinerea* has seventeen different members that divide into two distinct subfamilies. *Curr. Genet.* (2006); 50: 45-60.
[99] Xiao Y.Z. , Hong Y.Z. , Li J.F., Hang J., Tong P.G., Fang W. and Zhou C.Z. Cloning of novel laccase isozyme genes from *Trametes sp.* AH28-2 and analyses of their differential expression. *Appl Microbiol and Biotechnol.* (2006); 71:493-501.
[100] Yaver D.S., Xu F., Golightly E.J., Brown K.M., Brown S.H., Rey M.W., Schneider P., Halkier T., Mondorf K., Dalboge H. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. *Appl. Environ. Microbiol*. (1996); 62(3):834-41.
[101] Soden D.M., Dobson A.D.W. Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. *Microbiology.* (2001); 147: 1755-1763.
[102] Rodriguez E.,. Ruiz-Due˜nas F.J., Kooistra R., Ramb A., Mart´ınez A.T., Mart´ınez M.J. Isolation of two laccase genes from the white-rot fungus *Pleurotus eryngii* and heterologous expression of the pel3 encoded protein. *Journal of Biotechnology*. (2008); 134: 9–19.
[103] Pezzella C., Autore F., Giardina P., Piscitelli A., Sannia G. and Faraco V. The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. *Curr. Genet.* (2009); 55: 45-57.
[104] De Souza C.G.M., Peralta R.M. Purification and characterization of the main laccase produced by the white-rot fungus *Pleurotus pulmonarius* on wheat bran solid state medium. *J Basic Microbiol.* (2003); 43: 278-286.
[105] Shleev S.V., Morozova O.V., Nikitina O.V., Gorshina E.S., Rusinova T.V., Serezhenkov V.A., Burbaev D.S., Gazaryan I.G., Yaropolov A.I. Comparison of physico-chemical characteristics of four laccases from different basidiomycetes. *Biochimie*. (2004); 86:693-703.
[106] Yoshitake A., Katayama Y., Nakamura M., Iimura Y., Kawai S., Morohoshi N. N-Linked carbohydrate chains protect laccase-III from proteolysis in *Coriolus versicolor*. *J Gen. Microbio.* (1993);139:179-85.
[107] Kersten P.J. Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. *Proc Natl Acad Sci* U S A. (1990);87(8):2936-40.
[108] Baldrian P. Fungal laccases occurrence and properties. *FEMS Microbiol Rev* (2006); 30:215-242.

[109] Min K.L., Kim Y.H., Kim Y.W., Jung H.S., Hah Y.C. Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. *Arch Biochem. Biophys*. (2001); 392(2):279-86.
[110] Ng T.B., Wang H.X. A homodimeric laccase with unique characteristics from the yellow mushroom *Cantharellus cibarius*. *Biochem Biophys Res Commun*. (2004); 313(1):37-41.
[111] Wahleithner J.A., Xu F., Brown K.M., Brown S.H., Golightly E.J., Halkier T., Kauppinen S., Pederson A., Schneider P. The identification and characterization of four laccases from the plant pathogenic fungus *Rhizoctonia solani*. *Curr Genet.* (1996); 29(4):395-403.
[112] 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); 33:220–230.
[113] Giardina P., Autore F., Faraco V., Festa G., Palmieri G., Piscitelli A., Sannia G. Structural characterization of heterodimeric laccases from *Pleurotus ostreatus*. *Appl Microbiol Biotechnol*. (2007); 75: 1293-1300.
[114] Ducros V., Brzozowski A.M., Wilson K.S., Brown S.H., Ostergaard P., Schneider P. Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 A resolution. *Nat. Struct. Biol.* (1998); 5:310-6.
[115] Bertrand T., Jolivalt C., Briozzo P., Caminade E., Joly N., Madzak C. Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. *Biochemistry* (2002);41:7325-33.
[116] Pionetek K., Antorini M., Choinowski T. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *J Biol. Chem*. (2002); 277:37663–37669.
[117] Antorini M., Herpoël-Gimbert I., Choinowski T., Sigoillot J.C., Asther M., Winterhalter K., Piontek K. Purification, crystallisation and X-ray diffraction study of fully functional laccases from two ligninolytic fungi. *Biochim Biophys Acta*. (2002); 1594(1):109-14.
[118] Hakulinen N., Kiiskinen L.L., Kruus K., Saloheimo M., Paananen A., Koivula A., Rouvinen J. Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. *Nat Struct Biol* (2002); 9:601–605.
[119] Ferraroni M., Myasoedova N.M., Schmatchenko V., Leontievsky A.A., Golovleva L.A., Scozzafava A., Briganti F. Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. *BMC Struct Biol*. (2007); 7: 60-72.
[120] Matera I., Gullotto A., Tilli S., Ferraroni M., Scozzafava A., Briganti F. Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complete with p-toluate. *Inorg Chim Acte*. (2008); 361:4129-4137.
[121] Minussi R., Pastore G.M., Duran N. Potential applications of laccase in food industry. *Trends Food Sci Technol.* (2002); 13:205-216.
[122] Bajapai P. Application of enzymes in the pulp and paper industry. *Biotechnol Prog*. (1999); 15:147-157.
[123] Amir L., Tam T.K., Pita M., Meijler M.M., Alfonta L., Katz E. Biofuel cell controlled by enzyme logic systems. *J Am Chem Soc.* (2009); 131:826-832.
[124] Abadulla E., Tzanov T., Costa S., Robra K.H., Cavaco-Paulo A., Gubitz G.M. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. *Appl Environ Microbiol*. (2000); 66:3357-3362.

[125] Semenov AN, Lomonosova IV, Berezin V, Titov I. Peroxidase and laccase as catalysts for removal of phenylhydrazide protecting group under mild conditions. *Biotechnol Bioeng* (1993); 42:1137-41.
[126] Nicotra S., Cramarossa M.R., Mucci A., Pagnoni U.M., Riva S., Forti L. Biotransformation of resveratrol: synthesis of trans-dehydrodimers catalyzed by laccases from *Myceliophtora thermophyla* and from *Trametes pubescens*. *Tetrahedron* (2004); 60:595-600.
[127] Pozdnyakova N.N., Rodakiewicz-Nowak J. & Turkovskaya O.V. Catalytic properties of yellow laccase from Pleurotus ostreatus D1*. J Mol Catal B.* (2004); 30:19–24.
[128] Li X., Lin X., Zhang J., Wu Y., Yin R., Feng Y., Wang Y. Degradation of polycyclic aromatic hydrocarbons by crude extracts from spent mushroom substrate and its possible mechanisms. *Current Microbiol*. (2010); 60(5):336-342.
[129] Duran N. and Esposito E. Potential applications of oxidative enzymes and phenoloxidase like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental*. (2000); 28(2):83–99.
[130] Bollag JM, Chu HL, Rao MA, Gianfreda L. Enzymatic oxidative transformation of chlorophenol mixtures. *J Environ Qual*. (2003); 32(1):63-9.
[131] Torres E., Bustos-Jaimes I. & Le Borgne S. Potential use of oxidative enzymes for the detoxification of organic pollutants. *Applied Catal B-Environ*.(2003); 46:1-15.
[132] Palmieri G, Giardina P, Sannia G. Laccase-mediated Remazol Brilliant Blue R decolorization in a fixed-bed bioreactor. *Biotechnol Prog*. (2005); 21(5):1436-41.
[133] Minussi R.C., Pastore G.M., Durán N. Laccase induction in fungi and laccase/N-OH mediator systems applied in paper mill effluent. *Bioresour Technol*. (2007); 98(1):158-64.
[134] Ferrer-Miralles N., Domingo-Espin J., Corchero J.L., Vazquez E., Villaverde A. Microbial factories for recombinant pharmaceuticals. Microb Cell Fact. (2009); 24:8-17.
[135] Miele A., Giardina P., Sannia G., Faraco V. Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation. *J Appl Microbiol.* (2010); 108(3):998-1006.
[136] Autore F., Del Vecchio C., Fraternali F., Giardina P., Sannia G., Faraco V. Molecular determinants of peculiar properties of a *Pleurotus ostreatus* laccase: analysis by site directed mutagenesis. *Enzyme and Microbial Technology.* (2009); 45:507-513.
[137] Alcalde M., Bulter T., Zumarraga M., Garcia-Arellano H., Mencia M., Plou F.J., Ballesteros A. Screening mutant libraries of fungal laccases in the presence of organic solvents. *J Biomol Screen*. (2005); 10:624-31.
[138] Kaushik P., Malik A. Fungal dye decolourization: Recent advances and future potential. *Environ. Int.* (2009); 35(1):127-41.
[139] Faraco V., Ercole C., Festa G., Giardina P., Piscitelli A., Sannia G. Heterologous expression of heterodimeric laccases from *Pleurotus ostreatus* in *Kluyveromyces lactis*. *Appl Microbiol Biotechnol* . (2008); 77:1329–1335.
[140] Conesa A., Punt P.J., Van den Hondel CAMJJ. Fungal peroxidases: molecular aspects and applications. *J. Biotechnol*. (2002); 93:143-158.
[141] Homolka, L., Volakova, I. & Nerud, F. Variability of enzymatic activities in lignolytic fungi *Pleurotus ostreatus* and *Letinus tigrinus* after protoplasting and UV-mutagenization. *Biotechnology Techniques* . (1995); 9:157–162.

[142] Eichlerová-Voláková I. and Homolka L.. Variability of ligninolytic enzyme activities in basidiospore isolates of the fungus *Pleurotus ostreatus* in comparison with that of protoplast-derived isolates*. Folia Microbiologia*. (1997); 42(6):583-588.
[143] Eichlerová I, Homolka L. Preparation and crossing of basidiospore-derived monokaryons--a useful tool for obtaining laccase and other ligninolytic enzyme higher-producing dikaryotic strains of *Pleurotus ostreatus*. Antonie Van Leeuwenhoek. (1999); 75(4):321-7.
[144] Miyadoh S. Research on antibiotic screening in Japan over the last decade: a producing microorganism approach. *Acinomyce-tologica* (1993); 9: 100-106.
[145] Williams S.T., Goodfellow M., Alderson G., Wellington E.M.H., Sneath5 and M. J. Sackin P.H.A. Numerical Classification of *Streptomyces* and Related Genera. *Journal of General Microbiology* . (1983); 129:1743-1813.
[146] Chater K.F. Developmental decisions during sporulation in the aerial mycelium in *Streptomyces*. *Prokaryotic Development,* (2000).
[147] Endo K., Hosono K., Beppu T. and Ueda K. A novel extracytoplasmic phenol oxidase of *Streptomyces*: its possible involvement in the onset of morphogenesis *Microbiology.* (2002); 148:1767-1776.
[148] Berrocal M.M., RodrõÂguez J., Ball A.S., PeÂrez-Leblic M.I., Arias M.E. Solubilisation and mineralisation of [^{14}C] lignocellulose from wheat straw by *Streptomyces cyaneus* CECT 3335 during growth in solid-state fermentation. *Appl Microbiol Biotechnol.* (1997); 48: 379±384.
[149] Machczynski M.C., Vijgenboom E., Samyn B., Canters G.W. Characterization of SLAC*:* a small laccase from *Streptomyces coelicolor* with unprecedented activity*. Protein Sci.* (2004); 13(9):2388-97*.*
[150] Arias M.E., Arenas M., Rodríguez J., Soliveri J., Ball A.S. and Hernández M. Kraft Pulp Biobleaching and Mediated Oxidation of a Nonphenolic Substrate by Laccase from *Streptomyces cyaneus* CECT3335**.** *Applied and Environmental Microbiology*. (2003); 69:1953-1958.
[151] Dubé E., Shareck F., Hurtubise Y., Beauregard M. and Daneault C. Decolourization of recalcitrant dyes with a laccase from *Streptomyces coelicolor* under alkaline conditions. *J of Industrial Microbiology & Biotechnol*. (2008); 35:1123-1129.
[152] Chang R. Functional properties of edible mushrooms. *Nutr. Rev.* (1996); 54:91-93.
[153] Palmieri G., Giardina P., Marzullo L., Desiderio B., Nitti G., Cannio R. and Sannia G. Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Microbiol. Biotechnol*. (1993); 39:632-636.
[154] Giardina P., Aurilia V., Cannio R., Marzullo L., Amoresano A., Siciliano R., Pucci P., Sannia G. The gene, protein and glycan structures of laccase from *Pleurotus ostreatus. Eur J Biochem* (1996); 235:508–515.
[155] Palmieri G., Giardina P., Bianco C., Scaloni A., Capasso A. & Sannia G. A novel white laccase from *Pleurotus ostreatus*. *J Biol Chem* (1997); 272:31301-31307.
[156] Giardina P., Palmieri G., Scaloni A., Fontanella B., Faraco V., Cennamo G. and Sannia G. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. *Biochem. J.* (1999); 341:655-663.
[157] Lettera V., Piscitelli A., Leo G., Birolo L., Pezzella C., Sannia G. Identification of a new member of *P.ostreatus* laccase family from mature fruiting body. *Fungal Biology.* (2010), doi:10.1016/j.funbio.2010.06.004.

[158] Palmieri G., Giardina P., Bianco C., Fontanella B., and Sannia G. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus. Appl. Environ. Microbiol.* (2000); 66:920-924.
[159] Faraco V., Pezzella C., Miele A., Giardina P., Sannia G. Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochete chrysosporium* and *Pleurotus ostreatus* and their enzymes. *Biodegradation*. (2009); 20(2): 209-20.
[160] Piscitelli A., Giardina P., Mazzoni C., Sannia G. Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* (2005); 69:428–439.
[161] Festa G., Autore F., Fraternali F., Giardina P., Sannia G. Development of new laccases by directed evolution: Functional and computational analyses. *Proteins.* (2008); 72 (1): 25–34.
[162] Miele A., Giardina P., Notomista E., Piscitelli A., Sannia G., Faraco V. A semi-rational approach to engineering laccase enzymes. *Mol Biotechnol.* (2010); 46(2):149-56.

Waste model systems

In the last decades there has been an increasing concern on the aquatic environment pollution. Coloured textile effluents contribute enormously to water deterioration and their treatments (decolourisation and detoxification) is subject of discussion and regulation in many countries. Textile effluents are one of the most difficult-to-treat wastewaters on account of their considerable amount of suspended solids and of their high chemical oxygen demand (COD) due to the massive presence of weakly biodegradable and often toxic substances such as additives, detergents, surfactants and dyes. They exhibit large fluctuations in terms of quantities and pollution load, pH and temperature depending upon customer orders, types of manufactured textile materials and production schedules [1, 2]. Given to the great variety of fibres, dyes, process aids and finishing products in use, dyed wastewaters display a great chemical complexity and diversity which are not adequately treated in conventional wastewater treatments and therefore dyes and their derivates accumulate in the environment [1, 3, 4]. The presence of even trace concentrations of dyes in effluent is highly visible and undesirable.
Considerable efforts have been made by many researchers to find appropriate treatment systems in order to remove pollutants and impurities of wastewaters from different industries, in particular, textile industry [5]. Most of these works used commercially available dyes as model pollutants [6].
Nevertheless, real industrial effluents usually include mixtures of several dyes, and only limited data are now available on mixed dye degradation. The degradation of a mixture of reactive dyes, simulating a real textile effluent, has been successfully formulated and tested in the framework of the SOPHIED EU project ("Novel Sustainable Bioprocesses for European Colour Industries" -FP6-NMP2-CT-2004-505899), indicating the possibility of implementing this technique for the treatment of textile-dyeing wastewaters.

Dyes and preparation of simulated wastewaters

In order to identify new oxidative biosystems – microbial and enzymatic- for the treatment of industrial wastewaters containing synthetic dyes, applicable on industrial scale it is necessary to validate the degradative performances of the analyzed biosystems in conditions simulating that of real wastes.
Four simulated wastewaters, designed to mimic effluents produced during cotton, wool and leather textile dyeing processes, have been prepared using mixed industrial dyes at high concentrations. These simulated wastewaters, previously developed by the industrial partners of the EC FP6 Project SOPHIED (NMP2-CT- 2004-505899), were used under the permission of the SOPHIED Consortium. The industrial dyes used in these experiments were selected because they are representative of different structures of commercially important dye types, and are widely applied in textile industry. They are commercial products purchased from Town End (Leeds, UK) plc., containing in addition to dye molecules, which constitute 30–90% of the total weight other organic molecules as additives. The single dyes have been dissolved in de-ionized water at 500ppm. The chemical–physical properties of the tested dyes are listed in table 1.

Table 1: Dyes used in the study, their acronym, CI name, chemical class.

Acronym	*CI name*	*Chemical Class*	*λ max*
ABu62	Acid blue 62	Acid	595/637
AR266	Acid red 266	Acid	307/500
AY49	Acid Yellow 49	Acid	402
Abk194	Acid Black 194	Acid	524/607
AR195	Acid Red 195	Acid	492/607
Abk210	Acid Black 210	Acid	411
DrBu71	Direct blue 71	Direct	587
DrR80	Direct red 80	Direct	527
DrY106	Direct Yellow 106	Direct	407
RBk5	Reactive black 5	Reactive	608
Rbu222	Reactive blue 222	Reactive	540
RR195	Reactive red 195	Reactive	607
RY145	Reactive Yellow 145	Reactive	419

In addition to the dyes, these simulated wastewater models mimic than industrial effluents ones also for the presence of different salts, often in high concentrations, and for the pH values: the first wastewater (W1) contained a mix of 3 acid dyes with a pH of 5; the second wastewater (W2) contained a mix of 4 reactive dyes with a pH of 10; the third wastewater (W3) contained a mix of 3 direct dyes with a pH of 9 and the fourth (W4) contained a mix of 3 acid dyes with a pH 5.
To simulate industrial processing the operating conditions followed in table 2 have been considered. All the mimicked wastewaters were sterilised by tindalisation (three 1h cycles at 60 ℃ with 24h interval between cycles at room temperature) before using.

Table 2: Composition of the wastewater models.

***Acid Bath for wool* (W1)**							
Dye	Molecular weight	[Dye] (g/l)	Salt	[Salt] (g/l)	pH	Base or Acid	Conc. (gl^{-1})
ABu 62	422	0.10	Na_2SO_4	2	5	Acido Acetico	2
AY 49	414						
AR 266	444.5						
***Reactive Dye for cotton* (W2)**							
Dye	Molecular weight	[Dye] (g/l)	Salt	[Salt] (g/l)	pH	Base or Acid	Conc. (gl^{-1})
RBu 222	1322	1.25	Na_2SO_4	70	10	NaOH	2
RR 195	1135.5						
RY 145	957.5					Na_2CO_3	20
RBk 5	947						

***Direct Dye for cotton* (W3)**							
Dye	Molecular weight	[Dye] (g/l)	Salt	[Salt] (g/l)	pH	Base or Acid	Conc. (gl^{-1})
DrBu 71	1029	1.00	NaCl	5	9	Na_2CO_3	2
DrR 80	1388						
DrY 106	1338						
***Optional Acid for leather* (W4)**							
Dye	Molecular weight	[Dye] (g/l)	Salt	[Salt] (g/l)	pH	Base or Acid	Conc. (gl^{-1})
ABk 210	905	0,10	Na_2SO_4	0	5	Formic Acid	2
ABk 194	485						
AY 194							

References

[1] Alinsafi A., da Motta M., Le Bonte` S., Pons M.N., Benhammou A. Effect of variability on the treatment of textile dyeing wastewater by activated sludge. *Dyes Pigments.*(2006); 69:31–39.

[2] Hai F.I., Yamamoto K., Fukushi K. Hybrid treatment system for dye wastewater. Crit*. Rev. Environ. Sci. Technol.* (2007); 37:315–377.

[3] Vandevivere, P.C., Bianchi, R., Verstraere, W. Treatment and reuse of wastewater from the textile wet-processing industry: review of emerging technologies. *J. Chem. Technol. Biotechnol*. (1998); 72:289–302.

[4] Fu, Y., Viraraghavan, T. Dye biosorption sites in *Aspergillus niger*. *Bioresour. Technol*. (2002); 82:139–145.

[5] Arami M., Limaee N.Y., Mahmoodi N.M., Tabrizi N.S. Removal of dyes from coloured textile wastewater by orange peel adsorbent: equilibrium and kinetic studies. *Journal of Colloid and Interface Science* (2005); 28:371-376.

[6] Pereira L., Coelho A.V, Viegas C., Ganachaud C., Iacazio G., Tron T., Robalo M.P., O. Martins L. On the Mechanism of Biotransformation of the Anthraquinonic Dye Acid Blue 62 by Laccases. *Advanced Synthesis & Catalysis. (2009);*351(11-12):1857–1865.

Section I

Application of new biosystems for the treatment of synthetic dyes and industrial coloured wastewaters

Chapter I

Decolourization ability of *Streptomyces spp.* bacteria

1. Introduction

Nowadays, an increasing number of microorganisms has been characterized for the ability to decolorize, a wide range of natural or synthetic dyes. Recent studies reported the degradation capability exhibited by some fungi and bacteria toward aliphatic and / or aromatic compounds, ascribing this capacity to the production of oxidative enzymes [1-4]. These micro-organisms and their isolated enzymes have therefore emerged as good candidates for the treatment of wastewater pollutants.

In the wide panorama of species capable of degrading aromatic compounds, a substantial portion is occupied by white-rot fungi thanks to their ability to produce a complex array of extracellular oxidative enzymes, including lignin peroxidases, manganese peroxidases and, mainly, laccases. These enzymes, due to their low substrate specificity and structural similarity of the dyes with their natural substrates, are able to degrade a wide range of synthetic dyes, avoiding the formation of toxic aromatic intermediates [5-7]. While many eukaryotic phenol oxidases have been identified and studied, only few examples of phenol oxidases of prokaryotic origin have been reported so far.

Only few examples of the presence of laccase-like proteins have been reported in bacteria, such as *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Bacillus subtilis and Streptomyces griseus* [8-12]. A number of roles for laccases in bacterial systems have been suggested, such as their involvement in melanin production and spore coat resistance or their contribution in morphogenesis and sporulation processes [11, 14]. Recent studies have revealed the presence of novel extracytoplasmatic phenol oxidases in some species of *Streptomyces* too [13-15]. The finding of oxidative activities in actinomyces strains has suggested that similar enzymes are widespread among this group of bacteria.

Recently, a *Streptomyces* laccase that decolorizes an indigoid and an azo-type dye has been described, opening up the possibility to use these bacterial systems for new purposes [16,17]. Moreover in their work, Dubè *et al*. (2008) [18], have demonstrated the potential use of *Streptomyces coelicolor* laccase for decolourization of different classes of dyes with and without addition of acetosyringone, a natural mediator.

However, the suitability of *Streptomyces spp.* and their enzymatic systems to wastewater treatment still needs to be assessed.

Streptomycetes are Gram-positive soil-living bacteria, belonging to the *Actinomycetales* order. They are known for their “earthy” smell that derived from the production of a volatile metabolite, the geosmina.

These organisms perform secondary metabolism to synthesize a wide variety of antibiotics and biologically active substances, which have important applications in medicinal, agricultural and chemical industries [19]. These organisms also produce a wide variety of useful enzymes such as proteases, nucleases and enzymes that hydrolyze polysaccharides, which benefit the saprophytic life cycle of the organism by catalyzing the degradation of many kinds of organic molecules [20].

Another characteristic feature of this group of bacteria is the ability to perform complex morphological development resembling that of filamentous fungi [21-23]. Early in the life cycle on solid medium, *Streptomyces* undergo vegetative growth as a branching, multinucleate substrate mycelium. In response to nutritional limitation and various environmental and physiological signals, the mycelium produce aerial hyphae, which finally differentiate into spore chains via septum formation at regular

intervals. It is known that initial regulatory steps for the morphological and physiological development of this organism are controlled by common regulatory elements [22,23]. On solid medium a *Streptomyces* colony, often coloured, shows a definite organization of differentiated cells forming real "tissues".
Three different portions can be distinguished in a colony (Fig.1):

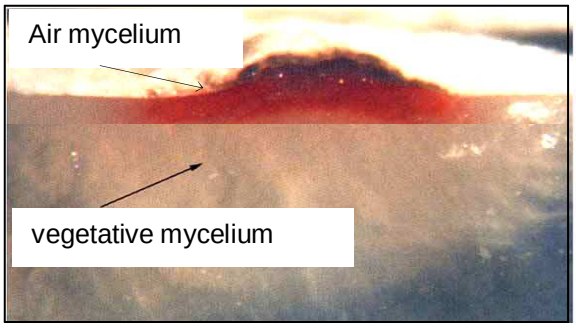

Figure 1: Cross section of a *Streptomyces coelicolor* colony (Chater 2001).

- vegetative mycelium, formed by hyphae that penetrate into the substrate and give a translucent appearance to the crop;
- air mycelium, recognizable as a matte surface layer;
- the spores, formed by the distal part of the aerial hyphae, forming a greyish layer on the colony.

The morphological differentiation begins after 2-3 days of growth at 28/30°C, with the development of air mycelium from new branches upwards of vegetative mycelium. The air mycelium, setting from the apex toward the base, generates long chains of spores. The switch from vegetative mycelium to air mycelium is preceded by a slowdown in growth rate and by the synthesis of macromolecules; during this switch, the secondary metabolism begins [24]. The complex structure and the functional organization of the *Streptomyces* colony turns to the adaptive response of microorganism to environmental changes. The presence of a vegetative mycelium allows the use of soil components through the secretion of extracellular hydrolytic enzymes, that solubilises biopolymers with high molecular weight [25].
Streptomyces are versatile organisms, using various molecules as nutritional font such as polysaccharides, proteins, lipids and other substrates which are then effectively reserved in the vegetative mycelium.

In the following section the decolourization ability of eleven *Streptomyces spp.* strains, isolated from soil contaminated by gasoil, has been tested towards single dyes and the four wastewater models. This research activity is a part of a broader project funded by *Compagnia di San Paolo* and done in collaboration with the University of Turin.
Since *Streptomyces* originate from contaminated soils, these strains have evolved the ability to metabolize a wide range of aliphatic and aromatic substances by using them as sources of carbon and energy. These expanded metabolic capabilities may be a promising starting point for the selection of new dye degraders.

2. Materials and Methods

Substrates and chemicals

Unless otherwise specified, all substrate and chemicals were purchased from Sigma-Aldrich.

Microorganism and its maintenance

The eleven unclassified *Streptomyces spp.* (*Strep* TO1 - *Strep* TO11) used in this study are aerobic bacteria isolated from soil contaminated by gasoil (kindly supplied by Dott. G.C. Varese - Department of Plant Biology of Turin University).

The culture was grown and maintained on starch casein agar slants (containing (gl^{-1}): starch (Difco), 10; casein (Sigma), 0,3; KH_2PO_4, 2; $CaCO_3$, 0,02; NaCl, 2; $NaNO_3$, 2; $FeSO_4$, ,0,01; agar (Difco), 15).

Medium and culture conditions for decolourization analysis

One-week old fully grown slants were used for culture preparation. Two plugs (8mm) of mycelial agar were aseptically transferred in Erlenmeyer flasks (100 mL) containing 20 mL of YMPG (Yeast and Malt-extract, Peptone, Glucose), with the following composition (gl^{-1}): yeast extract (Difco), 2; meat extract (Difco), 2; bacto peptone, 4; NaCl, 5; $MgSO_4$, 2; glucose, 10; pH 7.2.

Incubation was carried out, in the dark, at 30℃ on a rotary shaker at 125 rpm for a period of 5 days. After the cultivation, mycelia were collected by centrifugation at 5000 rpm, 4 °C for 5 min and used for decolourizat ion analysis.

The obtained biomasses were used to inoculate each wastewater model (added with 2 mL YMPG (20 mL final volume) and YMPG containing the single dyes (500ppm final concentration) in a final volume of 20 mL.

Inactive biomasses

After incubation biomasses were collected by centrifugation at 5000 rpm, 4 ℃ for 5 min, rinsed several times with distilled sterilised water to remove residual medium and then inactivated by two cycles of autoclaving, at 121 ℃ for 30 min. They were then collected in sterile conditions and utilized for the decolourization analysis.

Dyes and wastewaters preparation

See previous section of Model Systems.

Dye decolourization tests on solid medium

Cells were inoculated with sterile toothpicks to form a confluent lawn (Fig.2), on YMPG solid medium added with single dyes (500 ppm final concentration). Plates were incubated for 2-5 days at 30 ℃. Decolourizati on ability was evaluated according to the diameter of the decolorized zone on the solid medium and its degree of decolourization on the 7th day of incubation. As these characteristics were difficult to evaluate quantitatively, the following marks were used: - = no decolorized zone; + = zone diameter smaller than that of the mycelial colony; ++ = zone diameter roughly equal in size with the mycelial colony; +++ = zone diameter substantially greater than that of the mycelial colony. The control colour is the plates without colony streaked.

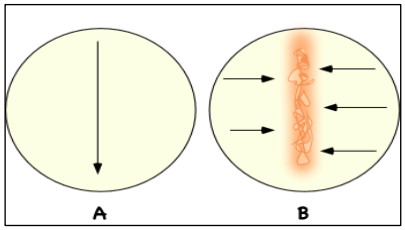

Figure 2: Strake of *Streptomyces* colonies on solid medium obtained by sterile toothpicks. A) strake direction; B) colony grown.

Dye decolourization tests in liquid culture

Decolourization was determined by monitoring the absorbance decrease at the maximum wavelength in the visible spectrum of each dye and was expressed in terms of percentage decolourization.

Performances of bacterial cells in model wastewater decolourization were evaluated by recording light absorption spectra between 280 and 800 nm at different times

(every 24 h), compared with the corresponding spectra of un-inoculated controls. Decolourization was measured as the extent of decrease of spectrum area recorded between 380 and 740 nm with respect to a control sample. All spectra were recorded after 1:100 dilution of the sample in water.
All the experiments were carried out in triplicates, and the mean values were taken. The standard deviation for the experiments was less than ±5%.

Enzyme assays

Laccase activity was assayed using 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [26]. The assay mixture contained 2 mM ABTS and 0.1 M Sodium Citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (e = 36,000 M-1 cm-1). The reaction mixture (3 mL) contained 1mL of cultured filtrate. One unit of enzyme was defined as 1µM of ABTS oxidized per minute. To calculate enzyme activity an absorption coefficient of 3.6. x 10^4 M^{-1} cm^{-1} was used.

3. Results and discussion

I° Screening on solid medium toward single dyes

In the first screening, eleven *Streptomyces* strains have been analyzed for their decolourization ability on solid medium supplemented with 500 ppm of each dye composing the wastewater models. Six better performed strains (*Strep* TO- 3, 5, 6, 7, 8 and 9) have been selected on the basis of their decolourization ability toward more dyes and their decolouration rate.
The six selected strains decolourized the entire surface of the plate or provided a clear decolourization halo after few days of incubation. Some selected decolourized plates are reported in the Fig. 3 as example.

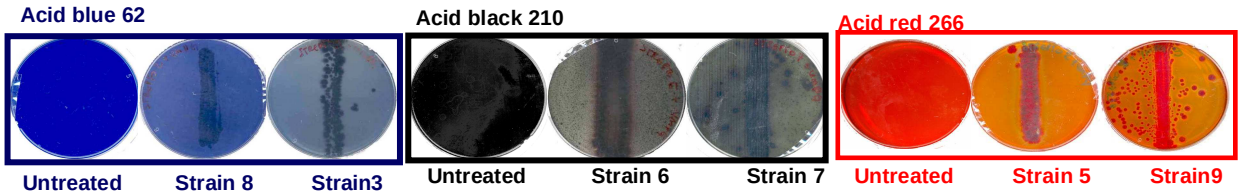

Figure 3: Examples of decolourisation halo on solid media obtained with some strains towards some acid dyes.

Model wastewater decolourization in liquid cultures

The strains selected in the first screening have been tested for their decolourization ability on the four wastewater models. The analysis was performed carrying out liquid cultures in the presence of the four Sophied models, described in the previous section Model Systems.
Performances of strains in model wastewater decolourization have been evaluated by recording light absorption spectra between 280 and 800 nm at different times (every 24 h), compared with the corresponding spectra of un-inoculated controls. Decolourization was measured as the extent of decrease of spectrum area recorded between 380 and 740 nm with respect to a control sample and expressed as decolourization percentage (tab 1 a, b, c, d).
The six analyzed strains caused an effective decolourization of the four wastewater models reaching up 50% decolourization, with a maximum of decolourization after 7 or14 days. However, the strain showed different specificity, depending both by the type of dye and by the different composition (pH and salt concentration) of the analyzed wastewater models [27].

a)

Tabel 1: a) % decolourization of *Acid Bath* (W1) model; b) % decolourization of *Optional Acid* (W2) model; c) % decolourization of *Direct* (W3) model; d) % decolourization of *Reactive* (W4) model.

Strain	1 th day	7 th day	14 th day	21 th day
Strep TO3	4.7 ± 1.2	32.1 ± 2.3	45.3 ± 4.2	17.1 ± 7.7
Strep TO5	36.5 ± 5.4	31.4 ± 1.5	43.9 ± 4.5	31.4 ± 3.6
Strep TO6	37.9 ± 6.2	30.7 ± 2.3	42.8 ± 6.6	36.9 ± 2.2
Strep TO7	29.2 ± 1.3	31.5 ± 1.8	44.7 ± 3.5	34.3 ± 3.5
Strep TO8	26.7 ± 4.3	25.2 ± 3.3	45.7 ± 5.2	28.2 ± 1.2
Strep TO9	32.6 ± 2.3	22.2 ± 2.8	35.8 ± 4.3	21.6 ± 1.5

b)

Strain	1 th day	7 th day	14 th day	21 th day
***Strep* TO3**	66.2 ± 5.5	66.3 ± 6.2	64.3 ± 5.8	67.8 ± 7.2
***Strep* TO5**	61.5 ± 2.2	74.2 ± 8.6	63.1 ± 1.5	65.1 ± 2.5
***Strep* TO6**	73.5 ± 3.3	68.1 ± 4.6	74.3 ± 2.2	76.7 ± 4.5
***Strep* TO7**	30.4 ± 5.3	44.7 ± 3.2	39.0± 4.6	33.5 ± 3.7
***Strep* TO8**	57.9 ± 2.2	64.3 ± 2.6	59.5 ± 5.2	61.8 ± 2.1
***Strep* TO9**	58.8 ± 4.3	67.1 ± 5.3	63.1 ± 2.4	62.6 ± 3.1

c)

Strain	1 th day	7 th day	14 th day	21 th day
***Strep* TO3**	63.0 ± 5.2	63.1 ± 3.5	64.9 ± 4.3	0
***Strep* TO5**	49.6 ± 5.5	35.3 ± 6.5	37.4 ± 6.6	0
***Strep* TO6**	58.1 ± 5.5	53.4 ± 7.5	63.4 ± 3.4	0
***Strep* TO7**	67.1 ± 5.5	42.1 ± 5.5	62.6 ± 8.1	0
***Strep* TO8**	52.6 ± 5.5	50.8 ± 3.6	26.3 ± 7.2	0
***Strep* TO9**	23.2 ± 5.5	19.7 ± 4.2	19.2 ± 6.6	0

d)

Strain	1 th day	7 th day	14 th day	21 th day
***Strep* TO3**	84.2 ± 8.5	85.0 ± 3.8	87.9 ± 5.2	84.0 ± 7.1
***Strep* TO5**	86.4 ± 6.2	83.8 ± 4.5	83.5 ± 4.4	82.9 ± 5.2
***Strep* TO6**	83.4 ± 4.3	85.9 ± 2.5	84.2± 6.7	83.5 ± 6.1
***Strep* TO7**	81.1 ± 2.9	82.6 ± 7.1	78.5 ± 3.8	82.3 ± 4.1
***Strep* TO8**	82.7 ± 1.9	83.0 ± 3.7	87.1 ± 9.1	82.5 ± 6.1
***Strep* TO9**	80.1 ± 6.3	80.4 ± 5.5	81.6 ± 6.2	78.9± 7.6

In general, the obtained decolourization percentage decrease during the last days of treatment, especially for Direct waste; probably this phenomenon has been caused

by cellular desorption. Moreover, the six analyzed strains shown a grater decolourization percentage (up 80%), toward Reactive waste; it is particularly interesting because this waste is recalcitrant to treatments with other microorganisms utilized until now [28].

Decolourization analysis with inactive biomasses

Spectra analysis of treated samples revealed in most of the cases, an uniform reduction of all peaks of the absorption spectra. The selective abatement of a specific absorption peak has been observed only in few cases. Moreover, bacterial biomasses recovered during wastewater treatment, turned out to be coloured. These findings have led to the hypothesis that the observed decolouration may be the sum of two processes: biodegradation and biosorption. Thus, in order to evaluate the effect of biosorption on the whole process, decolourization experiments have been performed with inactivated biomasses of the six selected strain.

Biomasses used for biosorption experiments have been inactivated by a cycle of pasteurization followed by two cycles of autoclaving. As control, the same experiment was performed with the corresponding not inactivated biomasses.

At regular intervals, samples of 1mL have been collected and the absorption spectra of supernatants have been recorded and analyzed.

Experiments have been performed in duplicate and data reported in the spectra correspond to their averages. The six selected strains provided similar results against all the tested wastewaters: absorption spectra of samples treated with active (Fig. 4 a) or inactive biomasses (Fig 4 b) exhibited comparable profiles. Moreover both treatments produce the same decolourization percentages on all the analysed wastewaters and with all the tested strains. The absorption spectra and the decolourization percentages, obtained with active and inactive biomasses of strain 7 against the *Reactive* wastewater model, are reported in figure 4 and table 2 respectively, as example.

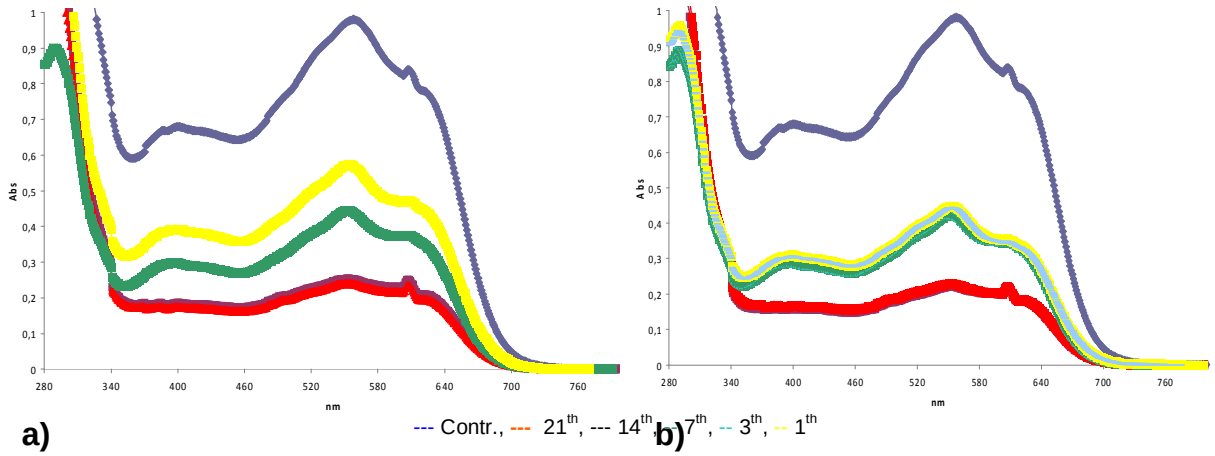

Figure 4: Absorption spectrum of *Reactive* (W4) wastewater treated with *Strep*TO7: a) active biomass; b) inactive biomass.

Days	% decol. Active biomass	% decol. Inactive biomass
1°	74 ± 3	76 ± 2
3°	75 ± 2	77± 1
7°	57 ± 7	56± 4
14°	54 ± 2	57± 5
21°	58 ± 1	58± 2

Table 2: % of decolourization obtained with active and inactive biomass of *Strep*TO7 against *Raective* waste.

Thus, the decolourization process can be ascribed to mainly biosorption phenomena. In addition, no decolourization could be achieved when the wastewaters were treated with supernatants derived by *Streptomyces* sp. cultures, confirming that no oxidative activities secreted in the extracellular medium take part to the decolourization process.

The selected *Streptomyces spp*. strains have shown good absorbent properties, reaching decolourization percentage up to 50%, also when they were been inactivated. These biomasses may thus prove to be potential good candidates for dye biosorption from industrial wastewaters. Innovative technologies, such as biosorption, are needed as alternatives to conventional methods to find inexpensive ways of removing dyes from large volumes of effluents.
Moreover, in agreement with Aksu (2005) [29] and as partly stated in the Introduction, dead biomasses are preferable because they do not require a constant input of nutrients and are not affected by the toxicity of dyes and other wastes. They can also be regenerated and reused in many cycles, as already developed for fungal biomass, and are both safe and environment-friendly [27].

4. References:

[1] Forgacs E., Cserhátia T. and Orosb G. Removal of synthetic dyes from wastewaters: a review. *Environment International.* (2004) ; 30 :953-971.
[2] Chen K.C., Wu J.Y., Liou D.J., Hwang S.C.J. Decolorization of the textile dyes by newly isolated bacterial strains. *Journal of Biotechnology*. (2003) – Elsevier. 101:57-68.
[3] Brar S.K., Verma M., Surampalli R.Y., Misra K., Tyagi R.D., Meunier N. and Blais J.F. Bioremediation of hazardous wastes — a review. *Practice periodical of hazardous, toxic and radioactive waste management.* (2006); 59–72.
[4] Prachi K., Anushree M. Fungal dye decolourization: Recent advances and future potential. *Environment International*. (2009); 35:127–141.
[5] Fu Y. and Viraraghavan T. Fungal decolourization of wastewaters: a review. *Bioresour. Technol*. (2001); 79:251-262.
[6] Nilsson I., Moller A., Mattiasson B., Rubindamayugi M.S.T., Welander U. Decolorization of synthetic and real textile wastewater by the use of white-rot fungi. *Enzyme Microb Technol.* (2006); 38:94-100.
[7] Zhao X.H. & Hardin, I.R. Biodegradation mechanisms in fungal decolorization of dyes. In L. E. Pawly, *Leading Edge Environmental Biodegradation Research*, (2007); New York: Nova Science Publishers.
[8] Sanchez-Amat, Lucas-Elio A., Fernandez E., Garcia-Borron J.C., and Solano F. Molecular cloning and functional characterization of a unique multipotent

polyphenol oxidase from Marinomonas mediterranea. *Biochim. Biophys Acta*. (2001); 1547: 104-116.
[9] Call, H-P., and I. Mücke. History, overview and applications of mediated ligninolytic systems,especially laccase-mediator-systems (Lignozym-process). *J.Biotechnol*. (1997); 53:163-202.
[10] Givaudan A.A., Effosse D., Faure P., Potier M.L., Bouillant and Bally R.. Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. *FEMS Microbiol*. (1993); 108:205-210.
[11] Hullo M.F., Moszer I., Danchin A., and Martín-Verstraete I. CotA of *Bacillus subtilis* is a copper-dependent laccase. *J. Bacteriol*. (2001). 183**:**5426-5430.
[12] Solano F., Lucas-Elío P., López-Serrano D., Fernández E., and Sánchez-Amat A. Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. *FEMS Microbiol. Lett*. (2001); 204**:**175-181.
[13] Machczynski M.C., Vijgenboom E., Samyn B., Canters G.W. Characterization of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. *Protein Sci.* (2004);13(9):2388-97.
[14] Endo K., Hosono K., Beppu T., Ueda K. A novel extracytoplasmic phenol oxidase of *Streptomyces*: its possible involvement in the onset of morphogenesis. *Microbiology*. (2002); 148(6):1767-76.
[15] Suzuki T., Endo K., Ito M., Tsujibo H., Miyamoto K., Inamori Y. A thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. *Biosci Biotechnol Biochem.* (2003); 67(10):2167-75.
[16] Dubé E., Shareck F., Hurtubise Y., Daneault C., Beauregard M. Homologous cloning, expression, and characterisation of a laccase from Streptomyces coelicolor and enzymatic decolourisation of an indigo dye. *Appl Microbiol Biotechnol.* (2008); 79:597-603.
[17] Molina-Guijarro J.M., Pérez J., Muñoz-Dorado J., Guillén F., Moya R., Hernández M., Arias M.E. Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from *Streptomyces ipomoea*. *Int Microbiol*. (2009);12(1):13-21.
[18] Dubé E., Shareck F., Hurtubise Y., Beauregard M., Daneault C. Decolourization of recalcitrant dyes with a laccase from *Streptomyces coelicolor* under alkaline conditions. *J Ind Microbiol Biotechnol*. (2008); 35(10):1123-9.
[19] Miyadoh S. Research on antibiotic screening in Japan over the last decade: a producing microorganism approach. *Actinomycetologica.* (1993); 7:100-106.
[20] Williams S.T., Goodfellow M., Alderson G., Wellington E.M.H., Sneath P.H.A. and Sackin M.J. Numerical classification of *Streptomyces* and related genera. *Journal of General Microbiology.* (1983); 129:1743-1813.
[21] Chater, K.F. Sporulation in *Streptomyces .* Regulation of prokaryotic development. Structural and functional analysis of bacterial sporulation and germination. (1989); *I. Smith et al., eds.* 277-299, *American Society for Microbiology*, Washington, DC.
[22] Ueda K., Tomaru Y., Endoh K., Beppu T. Stimulatory effect of copper on antibiotic production and morphological differentiation in *Streptomyces tanashiensis. J Antibiot.* (1997); 50(8):693-5.
[23] Ueda K., Endo K., Takano H., Nishimoto M., Kido Y., Tomaru Y., Matsuda K. and Beppu T. Carbon-source-dependent transcriptional control involved in the initiation of cellular differentiation in *Streptomyces griseus. Antonie Van Leeuwenhoek.* (2000); 78:263-268.

[24] Brasa A.F, Mendez C., Diaz L.A., Manzanal M.B. and Hardisson C. Glycogen and trehalose accumulation during colony development in *Streptomyces antibioticus. Journal of General Microbiology.* (1986); 132:1319-1326.
[25] Chater K.F. Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex?. *Current Opinion in Microbiology.* (2001); 4: 667-673.
[26] Giardina P., Aurilia V., Cannio R., Marzullo L., Amoresano A., Siciliano R., Pucci P., Sannia G. The gene, protein, and glycan structures of laccase from *Pleurotus ostreatus*. *Eur J Biochem* (1996); 235:508–515.
[27] Prigione V., Varese G.C., Casieri L., Marchisio V.F. Biosorption of simulated dyed effluents by inactivated fungal biomasses. *Bioresource Technology.* (2008); 99:3559–3567.
[28] Faraco V., Pezzella C., Miele A., Giardina P. and Sannia G. Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. *Biodegradation*. (2009); 20:209–220.
[29] Aksu, Z. Application of biosorption for the removal of organic pollutants: a review. *Process Biochem*. (2005); 40:997–1026.

Chapter II

Decolouration ability of recombinant laccases from *Pleurotus ostreatus*

1. Introduction

Biotechnological approaches were proven to be potentially effective in treatment of dye- contaminated effluent [1-6]. The white rot fungi are, so far, the most efficient microorganisms in degrading synthetic dyes.

This WRF's property is due to the production of extracellular lignin-modifying enzymes (LMEs), such as manganese peroxidases (MnP), E.C. 1.11.1.13, [7] lignin peroxidases (LiP), E.C. 1.11.1.14 and laccases (Lac), E.C. 1.10.3.2, [8] that are directly involved in lignin degradation. LMEs play significant roles in dye degradation by WRF: thanks to the structural similarity of the most commercially relevant dyes to lignin (sub)structures and due to the low substrate specificity of these enzymes, LMEs are able to degrade a wide range of xenobiotic compounds [9-11] including dyes [12-16].

In recent years many studies have demonstrated that several white-rot fungi are capable of oxidizing various types of synthetic dyes, such as azo, anthraquinone based, metal, triphenyl methane, and phthalocyanine [17-19]. Most of these studies have been performed using fungi in batch or semicontinuous operations [20, 21]. Process efficiency can be greatly enhanced using continuous decolorizing systems; this goal, however, is not easily achievable, the main reasons being the increase of the sludge volume due to the excessive fungal growth in the bioreactor and the length of the process.

Direct use of enzymes involved in the decolourization process represent an attractive alternative approach. The application of enzyme-based processes would allow a greater independence from the influences of wastewater composition which may affect fungal growth. Moreover, by decoupling the process of fungal growth from wastewater treatment, it's possible to overcome some of the problems related with whole cell based processes, such as the disposal of spent biomass and the need for sterile conditions. Several authors opted for the use of crude culture filtrates in dye-treatment, since they offer some distinct advantages: I) their use allows to skip some expensive purification steps; II) other factors present in the medium may stabilize the main oxidative enzymes or act in a synergistic way with them [22].

The physiology of LME production by WRF for ligninolysis or recalcitrant pollutant degradation has been extensively studied. Sometimes it's difficult to find a correlation between dye decolourisation and the activity of a specific class of LME (MnP, LiP or Lac). In most cases, the capability of decolorizing the dyes is due to the activities of LiP [23] and MnP [24]. However, laccase-mediated dye decolourization has been shown by many others [25]. Laccase-based decolourization treatments are potentially advantageous to bioremediation technologists since the enzyme is produced in large amounts and is often produced constitutively or requires less stringent induction conditions then either LiP or MnP. Given their versatility and broad substrate specificity, laccases, as a family of copper-containing oxidases catalyzing a variety of oxidations, could become among the most important biocatalysts in fungal biotechnology. Because of this, their biochemical properties and molecular evolution are considerable interesting and have been summarized in several reviews [26-28]. Their biotechnological applications range from delignification of lignocellulosics [29,30], purification of coloured wastewaters [31], textile dyes decolourization [32,33] and the transformation and inactivation of toxic environmental pollutants [34].

Laccase alone has a limited effect on textile dyestuff degradation owing to its specificity for phenolic compounds. However, increasing the range of substrates of laccase is possible by adding redox mediators to the reaction medium, first described by Bourbonnais and Paice [35], thereby oxidizing non-phenolic compounds. This

property acquired by the enzyme provides an advantage for biotechnological applications [36].
In vitro decolourisation using purified LME was widely described, e.g., using Lac from *Pyricularia oryzae* [37], *P. ostreatus* [38], *T. versicolor* [39] *Pycnoporus cinnabarinus* [40], LiP from *P. chrysosporium* [41], *T.versicolor* [42], and MnP from *B. adusta* [43], *P. chrysosporium* [44], *T. versicolor* [45].
However, the exploitation of different enzymatic mixtures produced by each ligninolytic strain appears to be of most interest for practical applications.
Several recent studies have been focused on dye degradation by laccases. Most of these works used commercially available dyes as model pollutants [46]. Laccase potential in the decolourization of recalcitrant azo dyes, such as those commonly used in the leather industry, has been assessed [47]. A crude mixture preparation from *P.ostreatus* broth has been shown to be able to decolorize the Remazol Brillant Blue R (RBBR) antraquinonic dye [48] achieving a maximum of 70% decolourization. Moreover the same preparation can be re-used several times when immobilized in copper alginate beads [49]. Nevertheless, real industrial effluents usually include mixtures of several dyes, and only limited data are now available on mixed dye degradation. Recently, an extracellular laccase mixture from *P. ostreatus*, composed of different laccase isoenzymes, has been proved capable to decolorize and detoxify the Acid wastewater model [50]. As a fact, the extracellular enzyme mixture decolorized acid dye wastewater model up to 30% after only 1 h incubation with 1 U/ml.

In order to ascertain the contribution of each laccase isoenzyme to the decolourization process, and to identify the most efficient laccase based biosystem, this part of the work has been focused on testing the decolourization ability of the single *P. ostraetus* laccase isoenzyme towards the four wastewater models. In particular POXA1b, POXC and POXA3 laccases have been heterologous expressed in the yeast *Kluyveromyces lactis* [51] and analyzed for their decolourisation ability.

Moreover, the availability of recombinant expression systems for laccase isoenzymes has allowed their engineering with the aim of improving several enzymatic features for specific industrial needs. Directed evolution techniques have been used to generate a collection of 3300 laccase variants by random mutagenesis of POXA1b laccase and expressed as recombinant proteins in the yeast *Saccharomyces cerevisiae* [52, 53]. The selected POXA1b variants showed improved activity toward phenolic and non-phenolic substrates, exhibited stability higher than that of POXA1b and improved catalytic efficiency [53].
In order to use the phenol oxidases for the decolourization of industrial wastewaters, it is essential that they operate on a wider range of substrates and they are stable, and especially active in extreme conditions of pH, normally found in the industrial wastewaters. As a fact, many POXA1b variants generated by random mutagenesis, represent good candidates for industrial applications at alkaline pHs, such as bioremediation of textile wastewaters.
For these reasons, starting from the collection of mutants previously selected on the basis of various screening criteria (greater activity towards substrates such as 2,6 dimethxyphenol (DMP) and acid 2,2 '-Azin-bis(3-etilbenzotiazolin-6 -sulfonic acid) (ABTS), increased activity at different pHs), seven POXA1b variants, whose properties best suited wastewater conditions, have been chosen to be tested for their decolourization ability towards the four wastewater models.

2. Materials and methods

Strains and culture conditions

The *Saccharomyces cerevisiae* strain used for heterologous expression was W303-1A (MATade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100). The plasmid used for *S. cerevisiae* expression was B-pSAL4 [51] carrying URA3 gene for auxotrophic selection and the cDNA encoding each laccase isoforms.

S. cerevisiae was grown on a selective medium [6.7 g L^{-1} yeast nitrogen base without amino acids and ammonium sulfate, 5 g L^{-1} casamino acids, 30 mg L^{-1} adenine, 40 mg L^{-1} tryptophan, 50 mM succinate buffer (pH 5.3), 20 g L^{-1} glucose].

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.

The *Kluyveromyces lactis* strain used for heterologous expression was CMK5 (a thr lys pgi1 adh3 adh1:URA3 adh2:URA3). The plasmid used for *K. lactis* expression was pYG132 [51], containing an ethanol inducible 1,200-nucleotides extended KlADH4 promoter and the *S. cerevisiae* phosphoglycecerate kinase terminator.

K. lactis was grown on YPG medium [Yeast extract (Difco) 10 g L^{-1}, Bacto tryptone (Difco) 40 g L^{-1}, Glucose 20 g L^{-1}] supplemented with 100 µg ml^{-1} of geneticin G418, 0.1 mM $CuSO_4$ and 0,5% ethanol (daily added). The cultures were conducted at 28°C on a rotary shaker (150 rpm).

At the end of growth, on the day of maximum enzyme production, the supernatant of each culture was recovered by centrifugation (10 'at 5000 rpm at 4 °C) and concentrated by filtration on Amicon membranes (cut-off 30kDa). The crude enzyme obtained was further exploited for dye decolourization studies.

Laccase activity assay

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.

Dyes and wastewaters preparation

See previous section of Model Systems.

Dyes decolourization tests

Batch decolourization experiments have been performed incubating crude preparation of laccase containing different enzyme amounts (0.1 U, 1U and 3U) in the 4 wastewater models (1 mL final volume of reaction).

Performances of selected laccases in model wastewater decolourization were evaluated by recording light absorption spectra between 280 and 800 nm at different times (10min, 20min, 1h, 2h, 3h e 24h), and comparing them with the corresponding spectra of controls (the waste incubated with the supernatant of yeast cultures transformed with the expression vector without the cDNA encoding the laccase, and processed as mutant). Decolourization was measured as the extent of decrease of spectrum area recorded between 380 and 740 nm with respect to a control sample. All spectra were recorded after 1:100 dilution of the sample in milliQ water.

All experiments were carried out in duplicates, and the mean values were taken. The standard deviation for the experiments was less than ±5%.

Selected POXA1b mutants

The activity and stability properties of the seven POXA1b variants selected for this study are summarized in the following tables (tab. 1), in comparison with POXA1b wild-type:

A)

$t_{1/2}$ (days)								
pH	**1M9B**	**3L7H**	**3M7C**	**2L4A**	**R4**	**1H6C**	**4M10G**	**POXA1b (*wt*)**
3	3.54	2.8	1.01	2.3	2.3	3	3	5
5	5.83	21	4	26.2	12.3	35	25	10
7	10.2	6.9	7	10	45	39	25	10
10	17.7	11	34.4	31	61	63	52	30
$t_{1/2}$ (hours) 60°C								
7	2.2	5.7	5.2	5.8	6.5	7.2	6	4.5

B)

ABTS				
Laccases	**K_M (mM)**	**Specific Activity (U/mg)**	**Optimum pH**	**pH range**
POXA1b wt	0.08 ± 0.01	183 ±1	3	2-5
1M9B	0.07±0.02	230 ± 1	3.5	3-4.6
3L7H	0.16±0.01	328 ± 1	3	2.5-3.5
3M7C	0.11±0.01	420 ± 2	2	n.d
2L4A	0.24±0.01	400 ± 2	3	3-4.2
R4	0.13± 0.01	420 ± 2	3	2-4.5
4M10G	0.11± 0.01	700 ± 1	2	2-4
1H6C	0.23± 0.01	750 ± 2	2	2-5

C)

DMP				
Laccases	**K_M (mM)**	**Specific Activity (U/mg)**	**Optimum pH**	**pH range**
POXA1b wt	0.54 ± 0.02	187 ± 1	5.5	3.5-5.5
1M9B	0.39±0.01	148±1	4.6	4-5
3L7H	0.41±0.01	240±1	5.4	4.5-5.5
3M7C	0.31±0.01	295±2	4.5	n.d
2L4A	0.38±0.01	231±2	5.4	4.5-5.5
R4	0.15±0.01	280± 2	5.6	4.2-5.8
4M10G	0.15±0.02	313± 2	5.0	4-6
1H6C	0.13±0.01	270± 1	5.5	4-5.8

Table 1: Characteristics of the selected POXA1b variants in comparison with wild type enzyme. A) half-life at different pHs (at 21°C) and at 60°C; B) catalytic parameters on ABTS; C) catalytic parameters on DMP.

3. Results and discussion

Decolourization experiments have been performed by incubating 0.1 U, 1U and 3U of crude recombinant laccase in the four wastewater models (1mL final volume); each test has been run in duplicate and the data presented (in the table and in the spectra) corresponding to their averages.

The decolourization ability of POXC, POXA1b and POXA3 laccases, heterologous expressed in the yeast *Kluyveromyces lactis,* have been analyzed and results are shown in table 2.

% decolourization (time)				
		POXC *K .lactis*	POXA3 *K. lactis*	POXA1b *K. lactis*
Acid bath	0,1U	13 (24h)	7 (24h)	5 (2h)
	1U	22 (24h)	10 (24h)	9 (2h)
	3U	22 (24h)	11 (24h)	11 (3h)
Optional Acid	0,1U	8 (24h)	0	0
	1U	7 (24h)	10 (24h)	5 (24h)
	3U	10 (24h)	13 (24h)	6 (2h)

Table 2: % decolourization obtained with native laccases.

Results indicate that recombinant POXC displays a slightly higher decolourization ability against Acid Bath, reaching up to 22% decolourization after 24h, in comparison with POXA3 and POXA1b. The differences in decolourization efficiency can be ascribed to structural differences of the dyes and to substrate specificity of the laccase isoenzymes. An increase in enzyme concentration does not correspond to a proportional increase in the extent of decolourization (tab 3).

On the other hand, as far as Optional Acid wastewater treatment, no significant differences among isoenzymes' behaviour have been detected.

No significant decolourization has been obtained towards the others analyzed wastewaters, such as Direct and Reactive models. The different dyes structure, the extreme conditions of pH and salt concentration of these wastewater model could prevent efficient functioning of laccases in decolourization.

In the same way, the decolourization ability of the selected POXA1b variants, heterologous expressed in the yeast *S.cerevisiae,* has been analyzed and results are shown in table 3.

% decolourization (hours)									
		1M9B	3M7C	R4	1H6C	3L7H	2L4A	4M10G	POXA1b
Acid bath	0,1U	3 (3h)	6 (3h)	6 (24h)	15(24h)	0	0	0	9 (3h)
	1U	18 (3h)	30 (2h)	31 (24h)	30(24h)	30(24h)	15 (3h)	30 (24h)	12 (2h)
	3U	31 (24h)	38 (3h)	24 (24h)	24(24h)	30(24h)	20 (3h)	28 (24h)	13 (3h)

Table 3: Maximum percentage of decolourization of wastewater models, obtained with the analyzed enzymes.

As example, one of the most significant absorption spectrum, obtained after the treatment, is shown below.

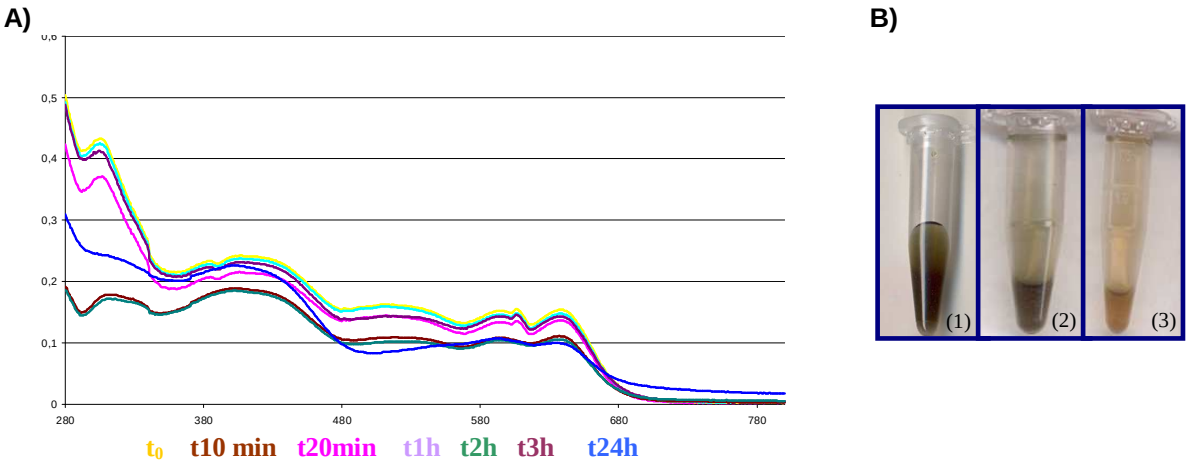

Figure 1: A) absorption spectrum of the Acid Bath wastewater model treated with 3U of 3M7C mutant B) Acid Bath wastewater treated with different concentrations of enzyme (1-3U) 3M7C: (1) control, (2) 1U, (3) 3U.

Selected POXA1b variants show an increased decolourization ability than POXA1b wild-type toward the Acid Bath model. As a fact, for all the mutants, a two-fold increase in decolourization percentage respect to that of POXA1b has been obtained. In particular the mutant 3M7C shows a decolourization percentage of 38% after 3 hours of incubation with 3U (Fig.1 A, B). The obtained decolourization corresponds to a reduction of the absorption of the main peaks of the individual dyes composing the Acid waste (Fig 1 A).
No significant decolourization has been obtained towards the others analyzed wastewaters, such as Optional Acid, Reactive and Direct models.
According to Faraco *et al.* [50], the data emphasize the preference shown by the laccase to decolorize azo dyes and anthraquinone type, which composing the Acid bath wastewater. Furthermore, the decolourization percentage obtained with single enzymes is comparable to that obtained with a mixture of laccase isoenzymes from *P. ostreatus* [14], approximately 40%.
In conclusion, since the analyzed isoenzymes show different specificities, even against the same wastewater, could prepare a mixtures of isoenzymes tested (wild-type and mutated), 'ad hoc' for the treatment of coloured effluents from the textile industry. This would exploit the synergic effect between the several enzymes, already observed in fungal laccase mixture[21].

4. References:

[1] Willmott N., Guthrie J., Nelson G. The biotechnology approach to colour removal from textile effluent. *J Soc Dyers Colour.* (1998); 114:38–41.
[2] Vaithanomsat P., Apiwatanapiwat W., Petchoy O. and Chedchant J. Production of ligninolytic enzymes by white-rot fungus datronia sp. KAPI0039 and their application for reactive dye removal. *International Journal of Chemical Engineering.* (2010).
[3] Robinson T., McMullan G., Marchant R., Nigam P. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresour Technol.* (2001); 77:247–255.

[4] Borchert M., Libra J.A. Decolourization of reactive dyes by the white rot fungus Trametes versicolor in sequencing batch reactors. *Biotechnol Bioeng.* (2001); 75:313–321.
[5] Baldrian P. and Snajdr J. Production of ligninolytic enzymes by litter-decomposing fungi and their ability to decolorize synthetic dyes. *Enzyme and Microbial Technology*. (2006); 39(5):1023–1029.
[6] Novotný C., Svobodová K., Benada O., Kofroňová O., Heissenberger A., Fuchs W. Potential of combined fungal and bacterial treatment for color removal in textile wastewater. *Bioresour Technol*. (2010).
[7] Glenn J.K., Akileswaran L., Gold M.H. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. *Arch Biochem Biophys.* (1986); 251:688–696.
[8] Edens W.A., Goins T.Q., Dooley D., Henson J.M. Purification and characterization of secreted laccase of Gaeumannomyces graminis var tritici. *Appl Environ Microbiol* (1999); 65:3071–3074.
[9] Christian V., Shrivastava R., Shukla D., Modi H.A., Vyas B.R. Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: enzymology and mechanisms involved. *Indian J Exp Biol*. (2005); 43(4):301-12.
[10] Pointing S.B. Feasibility of bioremediation by white-rot fungi. *Appl Microbiol Biotechnol*. (2001); 57:20–33.
[11] Scheibner K., Hofrichter M., Fritsche W. Mineralization of 2-amino-4, 6-dinitrotoluene by manganese peroxidase of the white-rot fungus *Nematoloma frowardii. Biotechnol Lett.* (1997); 19:835–839.
[12] Glenn J.K., Gold M.H. Decolourization of several polymeric dyes by the lignin-degrading basidiomycetes *Phanerochaete chrysosporium*. *Appl Environ Microbiol*.(1983); 45:1741–1747.
[13] Pasti-Grigsby M.B., Paszczynski A., Gosczynski S., Crawford D.L., Crawford R.L. Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces sp*. and *Phanerochaete chrysosporium*. *Appl Environ Microbiol*. (1992); 58:3605–3613.
[14] Paszczynski A., Pasti-Grisgby M.B., Gosczynski S., Crawford R.L., Crawford D.L. Mineralization of sulfonated azo dyes and sulfanilic acid by *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*. *Appl Environ Microbiol.* (1992); 58:3598–3604.
[15] Spadaro J.T., Gold M.H., Renganathan V. Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. *Appl Environ Microbiol*. (1992); 58:2397–2401.
[16] McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P et al Mini-review: microbial decolourisation and degradation of textile dyes. *Appl Microbiol Biotechnol.* (2001); 56:81–87.
[17] Libra, J. A.; Borchert, M.; Banit, S. Competition strategies for the decolorization of a textile-reactive dye with the whiterot fungi *Trametes versicolor* under nonsterile conditions. *Biotechnol. Bioeng*. (2003); 82, 736-44.
[18] Novotny C., Rawal B., Bhatt M., Patel M., Sasek V., Molitoris H. P. Capacity of Irpex lacteus and *Pleurotus ostreatus* for decolorization of chemically different dyes. *J. Biotechnol.* (2001); 89,113-122.
[19] Blanquez P., Casas N., Font X., Gabarrell X., Sarra M., Caminal G., Vicent T. Mechanism of textile metal dye biotransformation by *Trametes versicolor*. *Water Res*. (2004); 38, 2166-2172.

[20] Borchert M., Libra J.A. Decolorization of reactive dyes by the white rot fungus *Trametes versicolor* in sequencing batch reactors. *Biotechnol. Bioeng.* (2001); 75, 313-321.
[21] Rodriguez Couto S., Sanroman M.A., Hofer D., Gubitz G.M. Stainless steel sponge: a novel carrier for the immobilisation of the white-rot fungus *Trametes hirsuta* for decolourization of textile dyes. *Bioresour. Technol.* (2004); 95, 67- 72.
[22] Trupkin S., Levin L., Forchiassin F., Viale A. Optimization of a culture medium for ligninolytic enzyme production and synthetic dye decolorization using response surface methodology. *J Ind Microbiol Biotechnol.* (2003); 30:682-690.
[23] Young L., Jian Y. Ligninase-catalysed decolourization of synthetic dyes. *Water Res.* (1997); 31:1187-93.
[24] Heinfling A., Martinez M.J., Martínez A.T., Bergbauer M., and Szewzyk U. Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. *Applied and Environmental Microbiology.* (1998); 64(8):2788-2793.
[25] Pointing S.B, and Vrijmoed L.L.P. Decolorization of azo and triphenylmethane dyes by *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase. *World J MIcrobiol Biotechnol*. (2000); 16:317-8.
[26] Hildén K, Hakala TK, Lundell T. Thermotolerant and thermostable laccases. *Biotechnol Lett*. (2009);31(8):1117-28.
[27] Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G. Laccases: a never-ending story. *Cell Mol Life Sci*. (2010);67(3):369-85.
[28] Kunamneni A, Plou FJ, Ballesteros A, Alcalde M. Laccases and their applications: a patent review. *Recent Pat Biotechnol*. 2008;2(1):10-24.
[29] Elegir G., Daina S., Zoia L., Bestetti G., Orlandi M. Laccase mediator system: oxidation of recalcitrant lignin model structures present in residual kraft lignin. *Enzyme Microb Technol.* (2005); 37:340-346.
[30] Widsten P., Kandelbauer A. Laccase applications in the forest products industry: A review. *Enzyme and Microbial Technology. (*2008); 42(4):293-307.
[31] Duran N., Esposito E. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewaters and soil treatment: a review. *Enzyme Microb Technol*. 31:907-931.
[32] Wesenberg D., Kyriakides I., Agathos S.N. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances.* (2003); 22:161–187.
[33] Husian Q. Potential Applications of the Oxidoreductive Enzymes in the Decolorization and Detoxification of Textile and Other Synthetic Dyes from Polluted Water: A Review. *Crit Rev Biotechnol.* (2006); 26(4):201-221.
[34] Gianfreda L., Iamarino G., Scelza R. and Rao M.A. Oxidative catalysts for the transformation of phenolic pollutants: a brief review. *Biocatal Biotransformation*. (2006); 24(3):177-187.
[35] Bourbonnais R. and Paice M.G. Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation *FEBS.* 267(1); 99-102.
[36] Morozova O.V., Shumakovich G.P., Shleev S.V., Yaropolov Y.I. Laccase-mediator systems and their applications: a review. *Applied Biochemistry and Microbiology*. (2007); 43(5):523-535.
[37] Chivukula M, Renganathan V. Phenolic Azo Dye Oxidation by Laccase from *Pyricularia oryzae*. *Appl Environ Microbiol*. (1995); 61:4374-4377.

[38] Palmieri G, Cennamo G and Sannia G. Remazol Brilliant Blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. *Enz. Microb. Technol*. (2005); 36:17-24.
[39] Wesenberg D, Kyriakides I, Agathos SN. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol Adv*.(2003); 22:161-87.
[40] Schleiphake K, Mainwaring DE, Lonergan GT, Jones IK, Baker WL. Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnoporus cinnabarinus*. *Enzyme Microb Technol* (2000); 27:100-7
[41] Schleiphake K, Mainwaring DE, Lonergan GT, Jones IK, Baker WL. Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnoporus cinnabarinus*. *Enzyme Microb Technol* (2000); 27:100-7.
[42] Christian V, Shrivastava R, Shukla D, Modi H, Rajiv B, Vyas M. Mediator role of veratryl alcohol in the lignin peroxidase-catalyzed oxidative decolorization of Remazol Brilliant Blue R. *Enzyme Microb Technol* (2005); 36:426-431
[43] Heinfling-Weidtmann A, Reemtsma T, Storm T, Szewzyk U. Sulfophthalimide as major metabolite formed from sulfonated phthalocyanine dyes by the white-rot fungus *Bjerkandera adusta*. *FEMS Microbiol Lett* (2001); 203:179-83
[44] Pazarlioglu NK, Urek RO, Ergun F. Biodecolourization of Direct Blue 15 by immobilized *Phanerochaete chrysosporium*. *Process Biochem* (2005); 40:1923-9
[45] Champagne PP, Ramsay JA. Contribution of manganese peroxidase and laccase to dye decolouration by *Trametes versicolor*. *Appl Microbiol Biotechnol* (2005); 69:276 85
[46] Pereira L., Coelho A.V., Viegas C.A., Santos M.M., Robalo M.P., Martins L.O. Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase. *J Biotechnol*. (2009); 139:68–77.
[47] Couto S.R. Decolouration of industrial azo dyes by crude laccase from *Trametes hirsuta*. *J Hazard Mater*. (2007); 148:768–770.
[48] Palmieri G., Cennamo G., Sannia G. Remazol Brilliant Blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. *Enzyme Microb Technol*. (2005); 36:17–24.
[49] Palmieri G., Giardina P., Sannia G. Laccase-mediated Remazol Brilliant Blue R decolorization in a fixed-bed bioreactor. *Biotechnol Prog*. (2005); 21:1436–1441.
[50] Faraco V, Pezzella C, Miele A, Giardina P, Sannia G. Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. *Biodegradation*. (2009); 20(2):209-20.
[51] Piscitelli A., Giardina P., Mazzoni C., Sannia G. Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol.* (2005); 69:428–439.
[52] Festa G., Autore F., Fraternali F., Giardina P., Sannia G. Development of new laccases by directed evolution: Functional and computational analyses. *Proteins.* (2008); 72 (1): 25–34.
[53] Miele A., Giardina P., Sannia G., Faraco V. Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation. *J Appl Microbiol.* (2010); 108(3):998-1006.

Section II

Molecular determinants of peculiar properties of POXA1b *Pleurotus ostreatus* laccase: analysis by site-directed mutagenesis

Introduction

The growing use of industrial enzymes is dependent on constant innovation to improve performances and reduce cost. The advent of modern biotechnology, particularly large-scale fermentation and recombinant DNA technology, has not only made enzymes economically feasible, but in many cases they have been used to create enzymes that perform under unnatural conditions, such as in organic solvents [1].

The development of innovative bio-based processes using laccases need deeper knowledge of the structure/function relationships of the native enzymes and the investigation of novel and improved enzymes that are better suited for industrial purposes. Evolutionary design approaches have dedicated considerable attention in modifying native proteins. The generation of stable enzymes with improved and/or novel catalytic activities is a fascinating topic of modern protein biochemistry. This goal is relevant for basic research purposes as well as for enzymes applications in biotechnology. In addition, the search of new biocatalysts can have a major impact on the applications of enzymes in industrial processes. Thanks to enzyme engineering, for instance, it can force enzymatic reactions to proceed in a desired direction, enhance their selectivity and their stability.

In order to optimize and redesign enzymatic properties is possible to apply two different strategies. They are generally known as *directed evolution* and *rational design*.

Directed evolution, requires the availability of the gene (or genes) of interest, but does not require a detailed knowledge of structure and function relationships of the coded protein. Starting with a collection of related but diverse genes (e.g. naturally occurring homologs, a single mutated parental gene or any combination thereof), mutants are screened for an improved property. Selected genes are recombined to create random chimeras in which mutations are 'shuffled' between different enzyme mutants. Screening is then used to identify those mutants with the optimal combination of beneficial mutations [2-4].

The second strategy is *rational design*, which is the planned redesign of the protein sequence by site-directed mutagenesis. Rational design of proteins is driven by the hypotheses of researchers and computer models based on available crystal structures of the protein of interest. In this case, the design of a new protein requires the knowledge of both structure and sequence as well as of the mechanism of action of the enzyme. Selected residues are targeted for site-directed mutagenesis and after expression and purification the properties of the new enzymes are assessed by comparison with those of the native protein. Further residues may be targeted in further rounds of site-directed mutagenesis [5-7].

Laccases are intensely studied for their potential uses in industrial processes. They generally work under mild conditions: room temperature and atmospheric pressure, with water as solvent [8-11]. For their industrial use, the current challenge is to obtain both enhanced expression levels and improved laccase activity with desirable physicochemical characters such as a higher redox potential, optimal activity at neutral or alkaline pH, and thermostability [12].

Mutagenesis (rational or random) is often used to generate laccase variants. In their pioneering work, Xu *et al*. [13] have reported significant changes in pH optimum, K_M and k_{cat} for triply mutated fungal laccases. Replacement of the aspartic acid D206 by alanine in a *Trametes versicolor* laccase resulted in a threefold increase in k_{cat} [14].

The aim of this part of my thesis was been to model and to characterize molecular determinants in the mechanism of functioning of POXA1b laccase, heterologous expressed in the yeast *S. cerevisiae*.

References:

[1] Cherry J.R. and Fidantsef A.L. Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology* . (2003); 14(4):438-443.

[2] Zhao H., Chockalingam K., Chen Z. Directed evolution of enzymes and pathways for industrial biocatalysis. *Curr Opin Biotechnol.* (2002); 13:104-110.

[3] Festa G., Autore F., Fraternali F., Giardina P., & Sannia G. Development of new laccases by directed evolution: functional and computational analyses. *Proteins*, (2007); 72:25–34.

[4] Miele A., Giardina P., Notomista E., Piscitelli A., Sannia G., Faraco V. A semi-rational approach to engineering laccase enzymes. *Mol Biotechnol.* (2010); 46:149–156.

[5] Mabrouk S.B., Aghajari N., Ali M.B., Messaoud E.B., Juy M., Haser R., Bejar S. Enhancement of the thermostability of the maltogenic amylase MAUS149 by Gly312Ala and Lys436Arg substitutions. *Bioresour Technol*. (2010)

[6] Sayut D.J., Sun L. Creating designer laccases. *Chem Biol*. (2010); 17(9):918-20.

[7]Estell D.A., Graycar T.P., and Wells J.A. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. *The Journal of Biological Chemistry*. (1985); 11:6518-6521.

[8] Faber K. Biotransformations in organic chemistry. (2004) Springer, Berlin.

[9] Riva S. Laccases: blue enzymes for green chemistry. *Trends Biotechnol.* (2006); 24:219–226

[10] Couto R., & Herrera T.J. Industrial and biotechnological applications of laccases: a review. *Biotechnol Adv.* (2006); 24:500–513.

[11] Giardina P., Faraco V., Pezzella C., Piscitelli A., Vanhulle S. and Sannia G. Laccases: a never-ending story . *Cell Mol Life Sci*. (2010); 67(3):369-85.

[12] Zumarraga M., Camarero S., Shleev S., Martı´nez-Arias A., Ballesteros A., Plou F.J. & Alcalde M. Altering the laccase functionality by in vivo assembly of mutant libraries with different mutational spectra. *Proteins* (2008); 71:250–260.

[13] Xu F., Berka R.M., Wahleithner J.A., Nelson B.A., Shuster J.R., Brown S.H., Palmer A.E. & Solomon E.I. Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. *Biochem J*. (1998); 334:63–70.

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

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Molecular determinants of peculiar properties of a *Pleurotus ostreatus* laccase: Analysis by site-directed mutagenesis

Flavia Autore[a], Claudia Del Vecchio[b], Franca Fraternali[a,*], Paola Giardina[b], Giovanni Sannia[b], Vincenza Faraco[b,*]

[a] Randall Division of Cell and Molecular Biophysics, New Hunt's House, King's College, SE1 1UL, London, UK
[b] Department of Organic Chemistry and Biochemistry, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, via Cintia, 4, 80126 Naples, Italy

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ABSTRACT

A comparison of laccase sequences highlighted the presence of a C-terminal extension of sixteen amino acids in POXA1b laccase – that represents the most thermostable isoenzyme among *Pleurotus ostreatus* laccases and exhibits a notable stability at alkaline pH ($t_{1/2}$ at pH 10 = 30 days) – whereas this tail is missing in the other analysed laccases from basidiomycetes. Site-directed mutagenesis experiments allowed us to demonstrate a role of the C-terminal tail of POXA1b in affecting its catalytic and stability properties. The truncated mutants lose the high stability at pH 10, while they show an increased stability at pH 5. The effect of substituting the residue Asp205 of POXA1b with an arginine was also analysed in the mutant POXA1bD205R. Following the mutation POXA1bD205R, a remarkable worsening of catalytic properties along with a decrease of substrate affinity and of enzyme stability were found. It was demonstrated that introducing Arg205 mutation in a highly conserved region perturbs the structural local environment in POXA1b, leading to a large rearrangement of the enzyme structure. Hence, a single substitution in the binding site introduces a local conformational change that not only leads to very different catalytic properties, but can also significantly destabilize the protein.

1. Introduction

Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are blue multicopper oxidases able to oxidize a variety of phenolic compounds, as well as diamines and aromatic amines, with concomitant reduction of molecular oxygen to water [1,2]. Besides their natural substrates, laccases are also able to oxidize non-phenolic structures having higher redox potentials, in the presence of small molecules acting as redox mediators [3]. The surprisingly wide variety of substrates oxidized by laccases and the use of oxygen as electron acceptor make very interesting the study of these enzymes for industrial applications, including textile dye bleaching, pulp bleaching, detergents, and enzymatic conversion of chemical intermediates and soil bioremediation [4]. For such reasons, many efforts are being focused on molecular characterization of fungal laccases.

Laccases contain four copper ions distributed into three sites that, according to their spectroscopic properties, are classified as Type 1 Cu (T1), or blue copper centre, Type 2 (T2) or normal copper centres, and Type 3 (T3) or coupled binuclear copper centres [1]. The mononuclear centre T1, blue due to a maximum absorbance at around 600 nm, is the primary acceptor of electrons from the reducing substrate. The trinuclear cluster T2/T3 is composed of a binding site T2 for one copper atom with weak absorbance in the visible spectrum and a binding site T3 for two coupled copper atoms characterized by an absorbance at around 330 nm. The trinuclear cluster acts in binding and in reducing the molecular oxygen upon receipt of four electrons forwarded from the mononuclear centre T1 [5,6].

Current structural information on laccases is based on the crystal structures of native laccases [6–11] and of complexes between laccases and their reducing substrates [12,13].

The laccase family of the basidiomycete fungus *Pleurotus ostreatus* represents a variegated group of enzymes [14], having relevant roles in biotechnological applications of this fungus [15–18]. Within *P. ostreatus* laccase family, POXA1b isoenzyme exhibits notable stability at alkaline pHs ($t_{1/2}$ at pH 9 = 30 days) [19], that represents an useful property for bioremediation of industrial effluents – such as textile wastewaters and wastewaters produced by chemical plants in manufacturing organic molecules of commercial interest – characterized by strongly alkaline pH values.

In this paper, sequence and 3D model structure analyses and site-directed mutagenesis experiments on POXA1b laccase isoenzyme are reported, leading us to increase our knowledge on molecular determinants of the properties of this enzyme.

* Corresponding authors. Tel.: +39 081 674315; fax: +39 081 674313.
E-mail addresses: vfaraco@unina.it (F. Fraternali), franca.fraternali@kcl.ac.uk (V. Faraco).

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Table 1
List of the primers used in amplification experiments.

Primer	Nucleotide sequences
Fwpoxa1b	**TGTTGCAGATCTTGTCGG**
Revpoxa1bΔ4	*GAATTCCTCGAGTCA***CGCAGGCAGACG**
Revpoxa1bΔ16	*GAATTCCTCGAGTCA***GAGTTTCGATGGG**
Reva1b(dr)	**TTGATAGTTGGA**TCT**GCAGGAAGT**
Fwa1b(dr)	**ACTTCCTGC**AGA**TCCAACTATCAA**

Nucleotides in bold are complementary to the laccase cDNA sequences. Nucleotides in italics are recognized by restriction enzymes. Underlined nucleotides correspond to the mutant codon for Arg residue in cDNA coding for the mutant POXA1bD205R.

2. Materials and methods

2.1. Strains, media and plasmids

The *Escherichia coli* strain Top 10 (*F-mcrA D* (*mrrhsdRMS- mcrBC*) *f80lacZDM15 DlacX74 deoR recA1araD139 D* (*ara-leu*) *7697 galU galK rpsL* (*StrR*) *end A1* (*nupG*) was used in all DNA manipulations. Bacterial cells were grown in Luria–Bertani (LB) medium (in g l^{-1}: 10 bacto tryptone, 10 NaCl, 5 yeast extract), supplemented, when required, with 100 μg ml^{-1} of ampicillin; bacterial cultures were incubated at 37 °C.

The *Saccharomyces cerevisiae* strain W303-1A (MAT ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100) was used for heterologous expression of the laccases, by means of the plasmid B-pSAL4, carrying URA3 gene for auxotrophic selection [20]. Transformed yeast was grown on a selective medium (SD) [6.7 g l^{-1} yeast nitrogen base without amino acids and ammonium sulfate, 5 g l^{-1} casamino acids, 30 mg l^{-1} adenine, 40 mg l^{-1} tryptophan, 50 mM succinate buffer (pH 5.3), 20 g l^{-1} glucose].

2.2. Plasmid design and site-directed mutagenesis

To obtain the mutated cDNAs *poxa1bΔ4* and *poxa1bΔ16*, *poxa1b* cDNA (GenBank accession number AJ005018) was used as template in PCR experiments and Fwpoxa1b oligonucleotide as upstream primer. Different downstream primers, containing restriction sites for *EcoRI* and *XhoI*, were used to remove the last twelve and forty-eight nucleotides thus obtaining the two truncated cDNAs (Table 1). To construct the site-directed mutant cDNA *poxa1bD205R*, PCR was performed using the primers Reva1b(dr) and Fwa1b(dr) shown in Table 1. The cDNA resulting from point mutagenesis after digestion with *SmaI* and *BglII* restriction enzymes was cloned in B-pSAL4 expression vector by using homologous recombination of *S. cerevisiae*. Yeast transformation was performed by using the lithium acetate protocol [20]. The cells were spread on selective medium supplemented with 0.6 mM $CuSO_4$ and 0.2 mM ABTS and the plates incubated upside down for 4 days at 28 °C.

2.3. DNA sequencing

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

2.4. Protein purification

Preparative cultures of transformed yeasts were performed in 1 L flasks containing 200 ml of SD medium. Culture media were harvested at day 6, corresponding to the optimal laccase production, cells were sedimented by centrifugation at 1600 × *g* at 4 °C for 15 min. Secreted proteins were filtered through Whatmann sheet. The sample was concentrated with Amicon stirred cells with ultrafiltration membranes with cut-off 30,000 NMWLC (Millipore, Billerica, MA). Then, most secreted proteins were precipitated by the addition of $(NH4)_2SO_4$ up to 100% saturation at 4 °C and centrifuged at 10,000 × *g* for 40 min. The supernatant from ammonium sulphate precipitation was loaded onto a 26/60 Superdex 75 prep grade (GE healthcare Bio-Sciences, AB, Uppsala, Sweden), the column equilibrated with 0.15 M NaCl 50 mM Na–phosphate buffer pH7. The active fractions were pooled and concentrated on an Amicon PM-30 membrane.

Protein concentrations were determined using Bradford method and the commercial reactive of BioRad, with BSA (bovine serum albumin) as standard.

2.5. Laccase activity assays

Culture aliquots (1 ml) were collected daily and cells were pelleted by centrifugation (12,000 × *g* for 2 min 4 °C). Laccase activity in the culture supernatants was assayed at room temperature, monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), and syringaldazine (SGZ) as substrates, spectrophotometrically. In the assay of laccase activity towards ABTS. the assay mixture contained 2 mM ABTS. 0.1 M Na–citrate buffer pH 3.0; oxidation of ABTS was followed measuring absorbance increase at 420 nm (ε_{420} 36,000 M^{-1} cm^{-1}). Laccase activity towards DMP was assayed in a mixture containing 1 mM DMP and the McIlvaine's citrate–phosphate buffer adjusted to pH 5.3. Oxidation product formation was detected following absorbance increase at 477 nm (ε_{477} = 14.800 M^{-1} cm^{-1}). Laccase activity towards SGZ was assayed in a mixture containing 0.1 mM SGZ and 0.2 M Na–phosphate buffer pH 6.0. Oxidation of SGZ was followed monitoring absorbance increase at 526 nm (ε_{526} = 65,000 M^{-1} cm^{-1}). Enzymatic activity was expressed in IU. Phenol oxidase activity as a function of pH was measured at room temperature using citrate–phosphate buffer adjusted to different pH levels in the range 3.0–6.2. pH stability was measured using citrate–phosphate buffer adjusted at pH 3, 5 and 7.0, and Tris–HCl buffer adjusted at pH 10. Thermo-stability was determined incubating all enzymes at 60 °C at neutral pH and then cooling them down to room temperature to measure the residual activity towards ABTS.

2.6. Construction of enzyme–substrate complexes and molecular dynamics simulations

The *in silico* 3D model of the mutant POXA1bD205R was generated using the SWISS-MODEL web server [21] starting from the previously reported model of POXA1b 3D structure [22].

To obtain a reasonable starting structure of the complexes between the analysed laccases and DMP, the crystal structure of *Trametes versicolor* laccase III (Protein Data Bank [PDB] code 1KYA) complexed with an arylamide [12] was chosen as template. This choice is supported by the high sequence identity (60%) shared by the analysed proteins. The backbone and the common residues of laccase models were superimposed onto those of *T. versicolor* laccase. The DMP was also superimposed onto the 2,5-xylidine molecule giving rise to the positioning of the substrate within the modelled proteins.

Simulations on the *in silico* generated mutants POXA1bΔ4, POXA1bΔ16 and POXA1bD205R were performed with the GROMACS package [23] using the GROMOS96 force field [24]. The systems were neutralized and were solvated in a box of SPC water (about 17,000 water molecules) [25], in a solution of 50 mM NaCl. Simulations were carried out at a constant temperature of 300 K. The Berendsen algorithm was applied for temperature and pressure coupling [26]. After a first steeper descent energy minimization with positional restraints on the solute, the LINCS algorithm was used to constrain the bonds [27] and to carry out an initial 200 ps simulation with the positions of the solute atoms restrained by a force constant of 3000 kJ /mol nm^2 to let the water diffuse around the molecule and to equilibrate. The particle mesh Ewald method (PME) [28] was used for the calculation of electrostatic contribution to non-bonded interactions (grid spacing of 0.12 nm). The systems were run for 8 ns each and the last ns of the simulations was used to perform the energy decomposition analysis. GROMACS package and self-written programs have been used for the analysis of the data. Images were produced with visual molecular dynamics (VMD 1.8.5.) [29].

3. Results and discussion

3.1. Design of site-directed POXA1b mutants

3.1.1. C-terminal POXA1b truncated mutants

A comparison of protein sequences between *P. ostreatus* laccases and laccases with known 3D structure was performed by means of the PRALINE program [30]. The presence of a more protruding C-terminal tail of sixteen amino acids (residues 518–533 in the sequence having the GenBank accession number AJ005018) was thus shown in POXA1b [19], compared to the other analysed laccases from basidiomycetes (Fig. 1).

On the other hand, a C-terminal tail (of 13–14 amino acids) was found in deduced amino-acidic sequences of laccases from the ascomycetes *Podospora anserina* [31], *Neurospora crassa* [32], *Melanocarpus albomyces* [33] and *Myceliophthora thermophila* [34,35]. This tail is generally cleaved by proteolysis in the active form of the enzyme at a conserved cleavage site. Analysis of the 3D structure of the laccase from the fungus *M. albomyces* had previously shown this C-terminal extension as a plug obstructing the T2/T3 channel, thus leading to the hypothesis that its cleavage is required to favour the entrance of the oxygen molecule into the T2/T3 channel and the following exit of water molecules [9]. Zumarraga et al. [36] recently reported that C-terminus of the mature form of *M. thermophila* laccase ends in a 4 residues plug (556DSGL559) that blocks the access of oxygen and water to the trinuclear T2/T3 copper cluster. This C-terminal plug is followed by the above mentioned C-terminal tail of 14 residues that is processed in the mature protein. The closure of this entrance affects the function of *Ascomycete laccases* and it is thought that a yet unknown interaction regulates the conforma-

```
TvL1KYA     IPDVASANPV PQAWSDLCPT YDARDPSDQ. .......... .....
TvL1GYC     VADVKAANPV PKAWSDLCPI YDGLSEANQ. .......... .....
POXC        VTSISA..P. PAAWDDLCPI YNALSDNDKG ......GIVP S....
POXA1b      VNQTAAANPV PEAWNNLCPI YNSSNPSKLL MGTNAIGRLP APLKA
R1G1V10     IPNIPIANAI SPAWDDLCPK YNANNPDS.. ......GLA. .....
CcL1A65     MANTVDANNP PVEWAQLCEI YDDLPPEA.. ..TSIQ.TVV .....
POXA3       VNEGEQAQIV TQDWRTLCPA YDGLAPEFQ. .......... .....
```

Fig. 1. Comparison of the C-terminal sequences from the *P. ostreatus* laccases POXC, POXA1b and POXA3, with the corresponding sequences from laccases with known three-dimensional structure: TvL1KYA, *T. versicolor* laccase III (PDB code 1KYA [12]), TvL1GYC, *T. versicolor* laccase I (PDB code 1GYC [8]), R1G1V10, *Rigidoporus lignosus* laccase (PDB code 1V10 [10]), CcL1A65, *Coprinus cinereus* laccase Lac-Cc (PDB code 1A65 [7]). Multiple sequence alignment was performed using the PRALINE program [32]. Deleted residues in C-terminal truncated mutants of POXA1b are highlighted in bold as well as residues that Gelo-Pujic et al. [37] had previously deleted in the *Basidiomycete laccase* TvL1GYC.

tional changes that eventually allow the transit of oxygen to the T2/T3 site.

Whether this feature is also present in laccases from basidiomycetes, which have different C-terminal residues, is not yet known. Gelo-Pujic et al. [37] reported that the redox potential of the *Basidiomycete laccase* TvL1GYC – *T. versicolor* laccase I (PDB code 1GYC) [8] – changes when its C-terminus is truncated of eleven amino acids, thus suggesting that C-terminal amino acids can also affect the function of fungal laccases from basidiomycetes. To check if the presence of a more protruding C-terminal tail in POXA1b in comparison to the other basidiomycete laccases is related to a role of this protein region in affecting the catalytic or stability properties of POXA1b laccase, C-terminal truncated mutants missing four (529PLKA533) and 16 residues (518LMGTNAIGRL-PAPLKA533) and named POXA1bΔ4 and POXA1bΔ16, respectively, were designed. The mutant POXA1bΔ4 was designed taking into account both the results obtained by Zumarraga et al. (2008) and our previous results on the enzyme POXA1b purified from *P. ostreatus* showing the presence of differently C-terminal processed isoforms lacking the four amino acids at the C-terminus [19].

3.1.2. POXA1bD205R mutant

The sequence alignment showed that the residue Asp205 of POXA1b – well conserved in fungal laccases from basidiomycetes – is unusually substituted by an Arg residue (Arg211) in the *P. ostreatus* laccase isoenzyme POXA3 [38,39]. In the crystal structure of TvL1KYA – *T. versicolor* laccase III (Protein Data Bank [PDB] code 1KYA) [12] complexed with 2,5-xylidine – , the amino group of 2,5-xylidine is hydrogen bonded to a terminal oxygen of Asp206 side chain. Enzyme–substrate docking experiments were performed with the phenolic substrate DMP for POXA1b and POXA3 3D models, using the crystal structure of TvL1KYA to predict the structure of the complexes enzyme–substrate (Fig. 2). The residues Asp205 in POXA1b and Arg211 in POXA3 were shown to be involved in the interaction with the reducing substrate, corresponding to the residue Asp206 of TvL1KYA laccase. This Asp residue would be involved in the electrostatic interaction with the ligand with the negative charge of its side chain stabilizing the cation intermediate formed during the oxidation of the phenolic substrate [12]. In order to evaluate the importance of the residue Asp205 in POXA1b and the effect of its substitution with a positively charged residue in the binding cavity of the enzyme, the POXA1b site-directed mutant POXA1bD205R was designed.

3.2. Recombinant expression of wild-type and mutated forms of POXA1b laccase

The heterologous recombinant expression of the wild-type POXA1b laccase and of its mutated forms POXA1bΔ4, POXA1bΔ16 and POXA1bD205R was performed in *S. cerevisiae*, by means of the recombinant expression system previously developed for the *P. ostreatus* laccase isoenzymes [20]. The mutated cDNAs, obtained by site-directed mutagenesis PCR experiments, and the wild-type *poxa1b* cDNA were cloned in pSAL4 vector under the control of the strong copper inducible promoter *CUP1*. The cDNAs were successfully expressed in *S. cerevisiae* and all the mutated proteins secreted in active forms. Production of recombinant laccases was firstly tested by growing transformants on solid medium supplemented with ABTS that was oxidized into a green radical cation by the excreted laccase activity. Time courses of yeast growth and laccase activity production were then analysed in liquid cultures. Growth curves of yeasts expressing mutated and wild-type laccases did not differ significantly (data not shown). Analysis of activity production showed that the specific activity production of POXA1bD205R was similar to that of POXA1b, whereas a slightly higher specific activity production was detected for the mutants POXA1bΔ4 and POXA1bΔ16 (data not shown). Then, POXA1b C-terminus is not essential for secretion of an active recombinant enzyme. On the contrary, Bulter et al. [34] found a dramatic loss in activity of a C-terminal truncated version of *M. thermophila* laccase expressed in *S. cerevisiae*, thus deducing that the C-terminus is essential for early post-translational processing steps.

The recombinant wild-type and mutated forms of POXA1b were partially purified from yeast cultures collected after 6 days, when maximum production of the enzymatic activity was achieved. Similar amounts of total proteins were obtained in all cases.

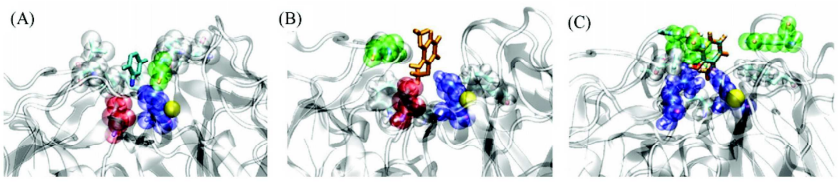

Fig. 2. Close-up views of the reducing substrate binding cavity of TvL1KYA – *T. versicolor* laccase III (PDB code 1KYA [12]) – (A), POXA1b (B) and POXA3 (C) laccases. The T1 copper ion and the residues in close contact with the substrate (DMP or 2,5-xylidine, in stick rendering) are highlighted in van der Waals representation.

Table 2
Catalytic parameters of recombinant wild-type and mutated POXA1b laccases.

Laccase	K_M (mM)	Specific activity (Umg^{-1})	Specific activity/K_M (Umg^{-1} mM^{-1})
ABTS			
Wild-type	0.07 ± 0.01	162 ± 2	2314
POXA1bΔ4	0.11 ± 0.01	100 ± 3	909
POXA1bΔ16	0.11 ⊥ 0.02	39 ⊥ 1	354
POXA1b D205R	0.32 ± 0.06	39 ± 1	122
DMP			
Wild-type	0.57 ± 0.01	155 ± 3	271
POXA1bΔ4	0.43 ± 0.04	189 ± 2	439
POXA1bΔ16	0.43 ± 0.05	129 ± 2	322
POXA1b D205R	1.26 ± 0.07	3 ± 0.1	2.4
SGZ			
Wild-type	0.04 ± 0.01	15 ± 1	375
POXA1bΔ4	0.03 ± 0.01	17 ± 1	566
POXA1bΔ16	0.03 ± 0.01	12 ± 1	400
POXA1b D205R	N.D[a]	–	–

[a] Too low to be determined.

3.3. Characterization of recombinant wild-type and mutated laccases

3.3.1. Catalytic parameters determination

Catalytic parameters of the site-directed mutants POXA1bD205R, POXA1bΔ4 and POXA1bΔ16 expressed in *S. cerevisiae* were determined for the non-phenolic substrate ABTS and for the two phenolic substrates SGZ and DMP, and compared with those of the wild-type POXA1b expressed in the same host. Since recombinant proteins have been only partially purified, specific activities values ($U mg^{-1}$) were estimated using the total protein concentration in the analysed enzyme solution. In Table 2, values of specific activity to K_M ratio ($U mg^{-1} mM^{-1}$) are reported for the new mutated enzymes and for the wild-type POXA1b. It is worth noting that the truncation of four amino acids has a positive effect on this ratio towards DMP and SGZ, but not towards ABTS. Taking into account these opposite effects, it can be ruled out that the four a.a. POXA1b C-terminal tail acts as a plug that blocks the access of oxygen and water to the trinuclear T2/T3 copper cluster, as in the *M. thermophila* laccase. On the other hand, truncation of POXA1b C-terminal tail could affect binding/interaction of the enzyme with the reducing substrate and the different sizes of the analysed substrates could be responsible for the different observed effects. Moreover, truncation of a more extended C-terminal region does not have any positive effect on catalytic properties towards all the analysed substrates.

As far as the mutant POXA1bD205R is concerned, a remarkable worsening of catalytic properties was observed towards all substrates. K_M values of POXA1bD205R towards ABTS and DMP are respectively 4- and 2-fold those of the wild-type enzyme. It was not possible to determine catalytic properties of this mutant towards SGZ, probably due to the too low affinity displayed by POXA1bD205R towards this substrate. The decrease of substrate affinity following the mutation POXA1bD205R can be ascribed to the repulsion that the presence of a positively charged residue (Arg) in the binding site, instead of a negative one (Asp) [12], generates in the formation of the cation intermediate enzyme–substrate, during the oxidation of reducing substrate.

3.3.2. Effect of pH and temperature on laccases activity and stability

The effect of pH on the activity of the recombinant laccases – wild-type POXA1b and its mutants POXA1bD205R, POXA1bΔ4, POXA1bΔ16 – towards the phenolic substrate DMP was studied in the pH range 3.0–6.4. All mutants exhibit a bell-shaped profile of the activity as a function of pH and they do not show any significant difference with respect to the wild-type POXA1b [22] (data not shown). On the contrary, a significant shift (from 3.4 to 4.8) of the maximum activity towards higher pH had been shown for *T. versicolor* laccase following the mutation D206N [40].

Xu [41] postulated that the bell-shaped activity-pH profile typically shown for the fungal laccases with phenolic substrates is a consequence of two opposite effects. The ascending part of the curve at acidic pH is generated by the redox potential difference between the reducing substrate and the type 1 copper of laccase and is favoured, for a phenolic substrate, by higher pH. The descending part is generated by the binding of a hydroxide anion to the type 2/type 3 coppers of laccase, which inhibits the activity at higher pH, combined with a decrease in the oxygen reduction potential with increase in pH. POXA1b can be considered a peculiar laccase for its ability to retain maximum activity towards DMP in a very broad pH range up to pH 5 [22]. At higher pH the inhibition of laccase activity takes place by hydroxide binding at the type 2/type 3 site and this interaction is not affected by the mutation at the position

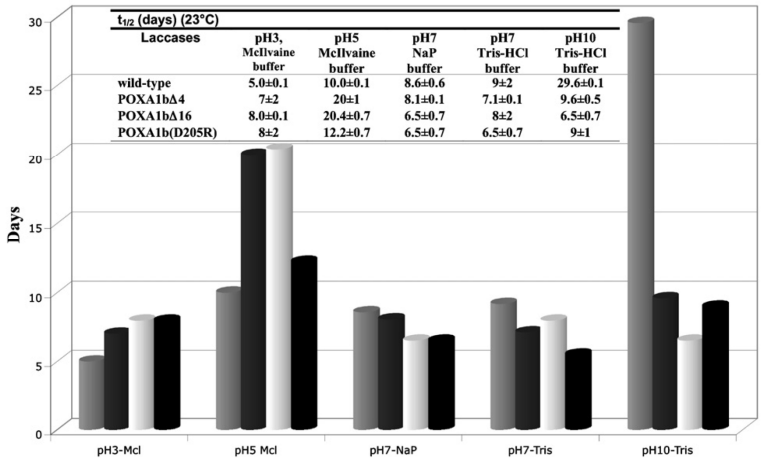

Fig. 3. Half-life of the wild-type POXA1b (▒) and the mutated laccases POXA1bΔ4 (■), POXA1bΔ16 (□) and POXA1bD205R (■) at different pH values.

205, because of its distance from the mutation site. Therefore, the absence of effects by the mutation D205R on the pH-activity profile of POXA1b with DMP could be explained with the predominance of the inhibiting effect on T2/T3 site at pH values higher than 5.

Analysis of the optimal reaction temperature for ABTS oxidation showed that the truncated mutants display a maximum of activity at the same temperature of the wild-type protein, 60 °C, whereas the substitution mutant exhibits the maximum activity at 50 °C (data not shown).

When enzyme stability at different pHs was analysed, an atypical behaviour of the truncated mutants was demonstrated at pH 5 (Fig. 3), both these mutants showing $t_{1/2}$ values more than 3-fold higher with respect to that observed for POXA1b. Moreover, at pH 10 all the analysed mutants drastically lose the high stability characteristic of the wild-type protein (Fig. 3). Taking into account the presence of a lysine residue (Lys532) in the C-terminal tail, the effects of the deletions could be due to electrostatic intra-molecular interactions involving its side chain, dependent on the pH.

On the other hand, all the new mutant enzymes showed similar thermo-stability at 60 °C with respect to the wild-type recombinant POXA1b [22] (data not shown).

Table 3
Energy decomposition analysis of MD simulations.

Energy (Kj mol^{-1})	$E_{initial}$	E_{final}
POXA1b		
Protein–protein (El)	−38626	−38819
Protein–protein (LJ)	−18286	−18822
Protein–protein (total)	−56912	−57641
Protein–solvent (total)	−32272	−32101
POXA1bΔ4		
Protein–protein (El)	−33928	−34170
Protein–protein (LJ)	−17561	−18116
Protein–protein (total)	−51488	−52337
Protein–solvent (total)	−24391	−23498
POXA1bΔ16		
Protein–protein (El)	−33900	−33944
Protein–protein (LJ)	−17280	−17584
Protein–protein (total)	−51180	−51528
Protein–solvent (total)	−23521	−23268
POXA1bD205R		
Protein–protein (El)	−34207	−34240
Protein–protein (LJ)	−17934	−18170
Protein–protein (total)	−52140	−52411
Protein–solvent (total)	−23289	−23552

3.3.3. *Molecular dynamics simulations*

Molecular dynamics simulations were performed to investigate the conformational changes due to the mutations. The data obtained from the analysis of the simulations of the mutated enzymes were compared to those previously obtained for the wild-type POXA1b [22]. The root mean square deviation (RMSD) from the initial structure of the three MD simulations shows that the RMSD values increase during the first 500 ps, and then fluctuate around an average value of 2.5 Å. Analysis of the inter- and intra-molecular energies showed that, under the simulated conditions, all the systems converge to similar energy contributions (Table 3).

To characterize the dynamical behaviour of the proteins, the global motion of the systems was decomposed into the main components to which an eigenvector and an eigenvalue are associated. This approach (Principal Component Analysis, PCA) is useful for the identification and the extraction of the motions that mostly contribute to the dynamical behaviour of the protein. In particular, the displayed motion of the proteins was analysed in terms of atoms that move in a correlated manner, assessing the covariance plots relative to each system (Fig. 4). POXA1bD205R shows a highly correlated motion around the mutated position that also induced a rearrangement of the regions around the catalytic binding site.

A different behaviour of POXA1bD205R has also been observed by analysing the radius of gyration on the analysed proteins during the simulation time. For the truncated mutants and POXA1b a constant value of 21.7 Å is observed during the entire simulation time, as shown in Fig. 5. On the other hand, POXA1bD205R shows an inverse behaviour, as its radius of gyration increases of ∼2 Å during the simulation reaching a constant value of 22.7 Å (Fig. 5).

Hence, molecular dynamics simulations showed that the presence of Arg205 in POXA1bD205R leads to a rearrangement of the structure and in particular to a highly correlated motion of the region close to the mutated residue. The considerable flexibility of

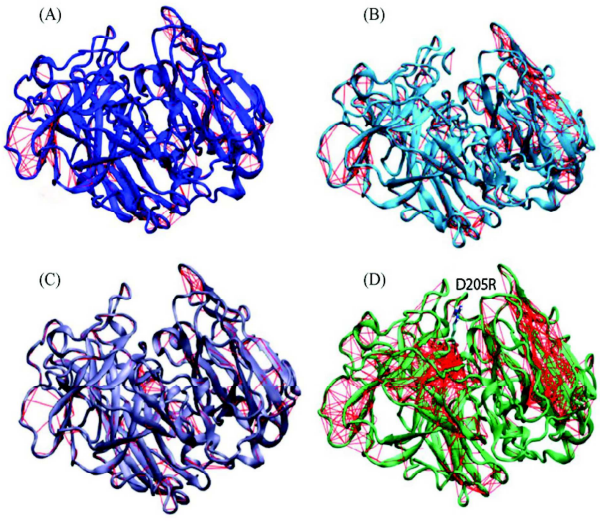

Fig. 4. Covariance lines plots. The lines are drawn between each atom that has a per atom normalized covariance greater than 70% correlation: POXA1b laccase (A), POXA1bΔ4 (B), POXA1bΔ16 (C) and POXA1bD205R (D).

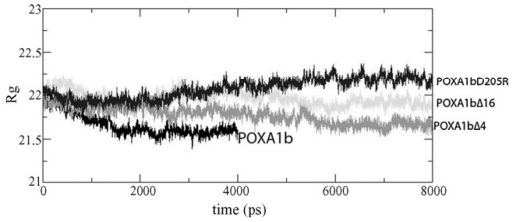

Fig. 5. Radius of gyration (*Rg*) of C-alfa atoms during the simulated time.

POXA1bD205R is due to the fact that the mutation has been introduced in a highly conserved region, thus perturbing the structural local environment and inducing large rearrangement motions. This local rearrangement could explain both the lower activity and the lower stability of POXA1bD205R.

On the other hand, the behaviour of the truncated mutants is comparable to the one exhibited by the wild-type enzyme, being POXA1b, POXA1bΔ4 and POXA1bΔ6 more rigid than POXA1bD205R.

4. Conclusions

The C-terminal tail of *P. ostreatus* POXA1b laccase was shown to affect both catalytic performance and stability properties of the enzyme. It was demonstrated that the truncation of four C-terminal amino acids in POXA1b laccase [19] has a positive effect on the specific activity to K_M ratio towards the phenolic substrates DMP and SGZ, but not towards ABTS. Therefore, it can be ruled out that the four a.a. POXA1b C-terminal tail acts as a plug that blocks the access of oxygen and water to the trinuclear T2/T3 copper cluster, whereas the C-terminal tail could influence binding/interaction with reducing substrate, according to the different sizes of the analysed substrates.

The C-terminal tail of POXA1b affects its stability properties, since the truncated mutants drastically lose the high stability of the wild-type protein at pH 10, while showing a much higher stability at pH 5. The last four C-terminal amino acids seem therefore to be the minimum molecular determinants of the POXA1b stability properties. These observations suggest the possibility to use this amino-acidic stretch as a C-terminal tag to modulate stability properties of other laccases. Moreover, the C-terminal POXA1b truncated mutant lacking 4 residues could represent a more appropriate biocatalyst than the wild-type enzyme for application in bio-processes requiring acidic pHs, such as xenobiotics transformation [42,43], organic synthesis and enzymatic polymerization [44,45].

As the most remarkable effect following D205R mutation in POXA1b, a significant worsening of catalytic properties was observed along with a decrease of stability. Molecular dynamics simulations allowed us to demonstrate that introducing Arg205 mutation in a highly conserved region perturbs the structural local environment in POXA1bD205R, leading to a large rearrangement of the structure. Hence, these studies show that a single substitution in the binding site introduces a local conformational change that not only leads to very different catalytic properties, but can also significantly destabilize the protein.

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References

[1] Thurston F. The structure and function of fungal laccases. Microbiology 1994;140:19–26.
[2] Ullah MA, Bedford CT, Evans CS. Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. Appl Microbiol Biotechnol 2000;53:230–4.
[3] Bourbonnais R, Paice MG. Oxidation of non-phenolic substrates—an expanded role for laccase in lignin biodegradation. FEBS Lett 1990:267:99–102.
[4] Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 2002;60:551–65.
[5] Solomon EI, Sundaram UM, Machonkin TE. Multicopper oxidases and oxygenases. Chem Rev 1996;96:2563–605.
[6] Ferraroni M, Myasoedova NM, Schmatchenko V, Leontievsky AA, Golovleva LA, Scozzafava A, et al. Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. BMC Struct Biol 2007;7:60, doi:10.1186/1472-6807-7-60.
[7] Ducros V, Brzozowski AM, Wilson KS, Brown SH, Østergaard P, Schneider P, et al. Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. Nat Struct Biol 1998;5:310–6.
[8] Piontek K, Antorini M, Choinowski T. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. J Biol Chem 2002;277:37663–9.
[9] Hakulinen N, Kiiskinen LL, Kruus K, Saloheimo M, Paananen A, Koivula A, et al. Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. Nat Struct Biol 2002;9:601–5.
[10] Garavaglia S, Cambria MT, Miglio M, Ragusa S, Iacobazzi V, Palmieri F, et al. 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 2004;342:1519–31.
[11] Matera I, Gullotto A, Tilli S, Ferraroni M, Scozzafava A, Briganti F. Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complexed with p-toluate. Inorg Chim Acta 2008;361(14–15):4129–37.
[12] Bertrand T, Jolivalt C, Briozzo P, Caminade E, Joly N, Madzak C, et al. Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. Biochemistry 2002;41:7325–33.
[13] Enguita FJ, Marcal D, Martins LO, Grenha R, Henriques AO, Lindley PF, et al. Substrate and dioxygen binding to the endospore coat laccase from *Bacillus subtilis*. J Biol Chem 2004;279:23472–6.
[14] Pezzella C, Autore F, Giardina P, Piscitelli A, Sannia G, Faraco V. The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. Curr Genet 2008;55(1):45–57.
[15] Palmieri G, Cennamo G, Sannia G. Remazol brilliant blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. Enz Microb Technol 2005;36:17–24.
[16] Palmieri G, Giardina P, Sannia G. Laccase-mediated Remazol brilliant blue R decolourization in a fixed-bed bioreactor. Biotechnol Prog 2005;21:1436–41.
[17] Faraco V, Pezzella C, Miele A, Giardina P, Sannia G. Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. Biodegradation 2009;20(2):209–20.
[18] Faraco V, Pezzella C, Giardina P, Piscitelli A, Vanhulle S, Sannia G. Decolourization of textile dyes by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus*. J Chem Technol Biotechnol 2009;84:414–9.
[19] 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;341:655–63.
[20] Piscitelli A, Giardina P, Mazzoni C, Sannia G. Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2005;69:428–39.
[21] Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 2003;31:3381–5.
[22] Festa G, Autore F, Fraternali F, Giardina P, Sannia G. Development of new laccases by directed evolution: functional and computational analyses. Proteins 2007;72:25–34.
[23] Berendsen HJC, van der Spoel D, van Drunen R. GROMACS: a message-passing parallel molecular dynamics implementation. Comput Phys Commun 1995;91:43–56.
[24] Daura X, Mark AE, van Gunsteren WF. Parametrization of aliphatic CHn united atoms of GROMOS96 force field. J Comput Chem 1998;19:535–47.
[25] Berendsen HJC, Postma JPM, van Gunsteren WF, Hermans J, Pullman B. Interaction models for water in relation to protein hydration. Intermol Forces 1981:331–42.
[26] Berendsen HJC, Postma JPM, van Gunsteren WF, Dinola A, Haak JR. Molecular dynamics with coupling to an external bath. J Chem Phys 1984;81:3684–90.
[27] Hess B, Bekker H, Berendsen HJC, Fraaije J. LINCS: a linear constraint solver for molecular simulations. J Comput Chem 1997;18:1463–72.
[28] Darden T, Perera L, Li L, Pedersen L. New tricks for modelers from the crystallography toolkit: the particle mesh Ewald algorithm and its use in nucleic acid simulations. Struct Fold Des 1999;7.

[29] Humphrey W, Dalke A, Schulten K. VMD—visual molecular dynamics. J Mol Graph 1996;14:33–8.
[30] Simossis VA, Heringa J. PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. Nucleic Acids Res 2005;33:289–94.
[31] Fernandez Larrea J, Stahl U. Isolation and characterization of a laccase gene from *Podospora anserina*. Mol Gen Genet 1996;252:539–51.
[32] Germann UA, Muller G, Hunziker PE, Lerch K. Characterization of 2 allelic forms of *Neurospora crassa* laccase amino-terminal and carboxyl-terminal processing of a precursor. J Biol Chem 1988;263:885–96.
[33] Kiiskinen LL, Saloheimo M. Molecular cloning and expression in *Saccharomyces cerevisiae* of a laccase gene from the ascomycete *Melanocarpus albomyces*. Appl Environ Microb 2004;70:137–44.
[34] Bulter T, Alcalde M, Sieber V, Meinhold P, Schlachtbauer C, Arnold FH. Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. Appl Environ Microb 2003;69:987–95.
[35] Zumárraga M, Camarero S, Shleev S, Martínez-Arias A, Ballesteros A, Plou FJ, et al. Altering the laccase functionality by in vivo assembly of mutant libraries with different mutational spectra. Proteins 2008;71(1):250–60.
[36] Zumarraga M, Vaz Domínguez C, Camarero S, Shleev S, Polaina J, Martínez-Arias A, et al. Combinatorial Saturation Mutagenesis of the *Myceliophthora thermophila* Laccase T2 mutant: the connection between the C-terminal plug and the conserved 509vsg511tripeptide. Comb Chem High Throughput Screen 2008;11:807–16.
[37] Gelo-Pujic M, Kim HH, Butlin NG, Palmore GT. Electrochemical studies of a truncated laccase produced in *Pichia pastoris*. Appl Environ Microbiol 1999;65:5515–21.
[38] Giardina P, Autore F, Faraco V, Festa G, Palmieri G, Piscitelli A, et al. Structural characterization of heterodimeric laccases from *Pleurotus ostreatus*. Appl Microbiol Biotechnol 2007;75:1293–300.
[39] Faraco V, Ercole C, Festa G, Giardina P, Piscitelli A, Sannia G. Heterologous expression of heterodimeric laccases from *Pleurotus ostreatus* in *Kluyveromyces lactis*. Appl Microbiol Biotechnol 2008;77:1329–35.
[40] Madzak C, Mimmi MC, Caminade E, Brault A, Baumberger S, Briozzo P, et al. Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. Protein Eng Des Sel 2006;19:77–84.
[41] Xu F. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. J Biol Chem 1997;272:924–8.
[42] Ceylan H, Kubilay S, Aktas N, Sahiner N. An approach for prediction of optimum reaction conditions for laccase-catalyzed bio-transformation of 1-naphthol by response surface methodology (RSM). Bioresour Technol 2008;99:2025–31.
[43] Zhang J, Liu X, Xu Z, Chen H, Yang Y. Degradation of chlorophenols catalyzed by laccase. Int Biodeter Biodegr 2008;61:351–6.
[44] Aktaş N, Tanyolaç A. Reaction conditions for laccase catalyzed polymerization of catechol. Bioresour Technol 2003;87(3):209–14.
[45] Kunamneni A, Camarero S, García-Burgos C, Plou FJ, Ballesteros A, Alcalde M. Engineering and applications of fungal laccases for organic synthesis. Microb Cell Factories 2008;7:32, doi:10.1186/1475-2859-7-32.

Section III

Improvement of *P. ostreatus* laccase production yield by classical breeding

Introduction

In nature, lignin is metabolized by several groups of organisms, among which white-rot fungi belonging to Basidiomycetes have been widely recognized as the most efficient lignin degrader. This capacity is assumed to result from the activities of numerous enzymes, among these phenol oxidase (laccase) and peroxidases are some of the most intensively studied groups of lignin–modifying extracellular enzymes [1]. The most of the mechanisms involved, in the degradation process, have characteristics of extreme aspecificity that allow them to enlarge the range of substrates to be used, making ligninolytic fungi potentially capable to degrade several xenobiotic compounds [2].

As a widespread and heterogeneous class of oxidases ligninolytic enzymes and the organisms producing them are widely considered promising tools for the development of biotechnological process aimed at the biodelignification, oxidative enzyme production and environmental bioremediation [3, 4].

Large amounts of enzymes are required for fundamental studies on fungal enzymatic systems proprieties and especially for their practical use. As far, for their application on large scale wastewater treatments, two aims have to be pursued: improvement of their performances and optimization of their production. Nevertheless, LMEs secreted from wild-type fungal organisms may not be suitable for commercial purpose mainly because of low yields or high cost of preparation procedures. Reducing the costs of LME production by optimising the fermentation parameters is the basic research for industrial applications. Numerous reports have been published recently on the improvements of the production of these enzymes, such as discovery of new fungal strains, modification of growth conditions, use of inducers, and use of cheaper growth substrates such as agricultural and food wastes.

With the constantly growing popularity of the edible *P. ostreatus* mushroom, as well as the significantly increased knowledge of the relevant laccase families [5], there is a great incentive for obtaining strains, derived by this fungus, improved for phenoloxidases production at large-scale.

Several methods have been used for strain improvement in *Pleurotus spp*. including selection, hybridization and gene transformation [6-9]. Based on current legislation (European Directive 2001/18/CE), genetic transformation and mutagenic treatments produce strains not suitable for natural or safe processes. In order to achieve improvements of this species, the optimization of a specific traits of natural strains by classical breeding is required.

Chaudhary et al. [10] developed single spore isolates from *P. djamor*, *P. florida*, *P. citrinopileatus* and *Hypsizygus ulmarius*. The hybrids showed improved mycelial growth rate compared to that of the parent. In another work Sawashe and Sawant [11] developed hybrid cultures which required a significantly shorter period for spawn run as compared to the parents species.

In order to improve industrially useful enzymatic biosystems, suitable for decolourization processes of coloured wastewaters, this part of my thesis is aimed at obtaining new higher laccases producers P*. ostreatus* strains by classical breeding approach.

In particular starting from two different *P. ostreatus* variants, three laccase higher producing dikaryotic strains have been obtained through crossing compatible characterized monokaryons. The three selected strains reached expression levels of 100,000 U/L, increasing the trite of parental strains up to four folds. New dikaryotic strains with improved efficiencies are still considered by the legislation as GRAS and

can be good candidates for their safe biotechnological exploitation. Finally, this work allowed production of a new isoenzyme, the laccase POX1, which cDNA was previously isolated [12], but the corresponding native protein had not been yet identified.

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References

[1] Cullen D. and Kersten P. Fungal enzymes for lignocellulose degradation. *In: Kinghorn JR and Turner G (eds) Applied molecular genetics of filamentous fungi.* New York: Chapman & Hall (1992) Chapter 4.

[2] Hatakka A. Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS Microbiology Reviews.* (1994); 13:125-135.

[3] Arora D.S. and Sharma R.K. Ligninolytic fungal laccases and their biotechnological applications. *Appl Biochem Biotechnol* (2010); 160(6):1760-88.

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

[5] Giardina P., Faraco V., Pezzella C., Piscitelli A., Vanhulle S. and Sannia G. Laccases: a never-ending story . *Cell Mol Life Sci*. (2010); 67(3):369-85.

[6] Irie T., Honda Y., Watanabe T. and Kuwahara M. Efficient transformation of filamentous fungus *Pleurotus ostreatus* using single -strand carrier DNA. *Appl Microbiol Biotechnol* (2001); 55:563-565.

[7] Irie T., Honda Y., Hirano T., Sato T., Enei H., Watanabe T. and Kuwahara M. Stable transformation of *Pleurotus ostreatus* to hygromycin B resistance using Lentinus edodes GPD expression signals. *Appl Microbiol Biotechnol* (2001); 56(5-6):707-709.

[8] Kothe E. Mating-type genes for basidiomycete strain improvement in mushroom farming. *Appl Microbiol Biotechnol* (2001); 56(5 -6):602-12.

[9] Kaur J., Sodhi H.S. and Kapoor S. Breeding of *Pleurotus florida* (oyster mushroom) for phenotypic pigmentation and high yield potential. *Journal of the Science of Food and Agriculture* (2008); 88:2676-2681.

[10] Chaudhary A., Dwivedi R.R. and Singh R.P. Evolution and evaluation of hybrids of oyster mushroom, in Proceedings of an International Conference on Mushroom Biology and Biotechnology, NRCM, Solan, India. Mushroom Society of India Publication, Solan, H.P., India, (2007):51.

[11] Sawashe S.G. and Sawant D.M. Studies on evaluation of new strains of *Pleurotus spp.* obtained through hybridization. In Proceedings of an International Conference on Mushroom Biology and Biotechnology, NRCM, Solan, India,. Mushroom Society of India Publication, Solan, H.P., India, (2007):52.

[12] Giardina P., Cannio R., Martirani L., Marzullo L., Palmieri G. and Sannia G. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus. Applied and Environmental Microbiol.* (1995); 61: 2408–2413.

PAPER IN PREPARATION

PLEUROTUS OSTREATUS CLASSICAL BREEDING: AN USEFUL TOOL TO IMPROVE AND INCREASE LACCASE PRODUCTION IN DIKARYOTIC STRAINS.

Del Vecchio C., Lettera V., Leo G., Pezzella C., Piscitelli A., Birolo L. and Sannia G.

Department of Organic Chemistry and Biochemistry, University of Naples Federico II
Complesso Universitario Monte S. Angelo - Via Cinthia 4, IT-80126 Napoli

Abstract
White-rot basidiomycetes, the most common wood-rotting organisms, are characterized by their ability to produce extracellular oxidative enzymes, which are mainly involved in the degradation of lignin. A non specific reaction mechanism characterizes lignin-modifying enzymes allowing them to be also able to degrade a wide range of environmental pollutants, such as polycyclic aromatic hydrocarbons, chlorophenols, and aromatic dyes. Due to their broad substrate range, these oxidative enzymes are being increasingly evaluated for a variety of biotechnological applications. In particular, in the last decades the physiological role of laccases and their use in several industrial fields has been deeply investigated. However, large amounts of enzyme are required both for fundamental studies and for their practical application at industrial scale. Nevertheless, laccases secreted from wild-type fungal organisms may not be suitable for commercial purposes mainly because of low yields of production and high costs of preparation procedures.
In the present work the white rot fungus *Pleurotus ostreatus* has been used as organism for classical breeding experiments. The genome of this basidiomycete has been recently sequenced and several genes coding for laccase isoenzymes have been annotated. Besides recombinant heterologous expression, classical breeding is an useful alternative method to OGM either to improve ligninolytic enzymes production and to increase isoenzymatic variability. In particular, starting from two different *P. ostreatus* variants, three laccase higher-producing dikaryotic strains have been obtained through crossing compatible characterized monokaryons. The three selected strains reached expression levels of 100,000 U/L, increasing the trite of parental strains up to four folds. Furthermore, this work allowed production of a new isoenzyme, the laccase POX1, whose cDNA was previously isolated, but the corresponding native protein was not identified yet.

Introduction:

White-rot fungi (WRF) have been widely recognized as the most efficient lignin degraders [1]. This ability results from the production of various isoforms of extracellular ligninolytic enzymes including manganese peroxidases, lignin peroxidases and laccases [2, 3]. The latter are involved in a variety of physiological roles including morphogenesis, fungal plant pathogen/host interactions, stress defence and lignin degradation [3-5]. Furthermore, due to their broad substrate range, laccase enzymes can play a role not only in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various xenobiotic compounds [6]. Thus, WRF and their enzymes are widely considered to have

potential for industrial purposes. As a fact, laccases offer industrially useful advantages of great interest for biotechnological applications such as biodegradation of environmental pollutants (e.g. like textile dye or explosives), bioconversion of lignin and detoxification of agricultural by-products, including olive mill wastes or coffee pulp [7].

Laccases secreted from native sources are usually not suitable for large scale applications, mainly due to low production yields and high cost of preparation and purification procedures. To successfully utilize laccases for biotechnological purposes, production of large quantities of enzymes at low cost is required. Only few examples of industrial uses of laccases currently exist and most of them utilize genetically modified tailored laccase, produced by recombinant expression [8]. Therefore, any attempt to increase the production of enzymes from microbial sources could be of considerable industrial interest.

The constantly growing popularity of the edible fungus *P. ostreatus*, as well as the significantly increased knowledge of its ligninolytic enzymatic system, render worthwhile spending efforts for obtaining strains, derived from this mushroom, improved for phenoloxidases production at large-scale.

So far, eight members of the *P. ostreatus* laccase multigene family have been isolated and sequenced [9, 10] and up to 12 members of this family have been identified in the released *P. ostreatus* genome databank. However, the redundancy of laccase genes raises the question about their respective functions *in vivo*, and this question is even more pertinent since cDNAs or proteins for some of these genes have not been found yet [9].

Several methods have been carried out for strain improvement in *Pleurotus* including selection, hybridization and gene transformation [11-14]. However, based on current legislation (European Directive 2001/18/CE), genetic transformation and mutagenic treatments produce strains not suitable for “natural or safe processes”. Therefore, the construction of genetically modified organisms can not be chosen to improve the addressed quality of the fungus, and breeding should be based on classical genetic approaches. This technique is based on the mating of two monokaryotic compatible strains, whose hyphae are able to fuse and give rise to a dikaryotic mycelium in which the two parental nuclei remain independent [13, 14]. Production of tmonokaryotic strain, germinating from uninucleate basidiospores, is achieved when the fungus enters into the reproductive phase triggering basidiocarp formation: during basidia formation, karyogamy takes place immediately before the onset of the meiosis, giving rise to four uninucleate basidiospores. At this stage, genetic recombination occur, although some reports have also suggested the occurrence of parasexual somatic recombination in higher basidiomycetes [15]. As a fact, dykariotic strains derived from single spore germinating monokaryons could result in improved specific multifactorial traits like mycelium growth, colony fitness and protein expression or secretion.

Chaudhary *et al.* [16] developed single spore isolates from the white-rot fungi *Pleurotus djamor, P. ostreatus var. florida, Pleurotus citrinopileatus* and *Hypsizygus ulmarius*. The hybrids showed improved mycelial growth rate compared to that of the parental strains. In another work, Sawashe and Sawant [17] developed hybrid cultures which required a significantly shorter period for spawn run as compared to the parent species. In the frame of improvement of quantity and variability of enzymatic expression, selection of new hybrid strains for laccase production could be viewed as a solution to make the entire process cost effective. Till now only few data in literature have been reported to improve laccase production by classical breeding

in *P. ostreatus* [18]. To the best of our knowledge, increasing of extracellular laccase activities have ever been not correlated to the production of phenoloxidase isoenzymes, previously unexpressed.
In the present work, improvement of different strains of *P. ostreatus* obtained by classical breeding and screening for their laccase productivity has been performed. The obtained dikaryotic strains, in according to European legislation, are considered generally recognized as safe (GRAS) and therefore they could be good candidates for their use in bioproduction as natural strains. We have performed dikaryotization through crossing of characterized basidiospore-derived monokaryons of two *P. ostreatus* variants in order to increase the trite and the expressed pattern of laccase isoenzymes.

Materials and methods

Substrates and chemicals

Unless otherwise specified, all substrates and chemicals were purchased from Sigma-Aldrich.

Organism

All *P. ostreatus* monokariotyc and dikaryotic strains were maintained through periodic serial transfers and kept at 4℃ on agar plates in the presence of 2.4% potato dextrose and 0.5% yeast extract (PDY) (Difco).

Culture conditions

Shacked submerged cultivation was carried out in 100 ml Erlenmeyer flasks (125 rpm) containing 30 ml of PDY supplemented with copper sulphate (final concentration 150 μM) and ferulic acid 2 mM (dissolved in ethanol and added at the second day of growth). The flasks were inoculated with four agar plugs (8 mm diameter) cut from the actively growing part of the colony on a Petri dish and incubated for at least 15 days at 28° C in the dark .

Fructification and basidiospores isolation

P. ostretus mushrooms were cultivated in 500 mL flasks containing 400 g of wheat-straw (65% water content), which were twice autoclaved for 1 h at 121 ℃ separated by a period of 24 h at room temperature. Each flask was inoculated with four agar plug (13 mm diameter), and left to grow at 28 ℃ fo r 30 days in the dark. Fructification was promoted by opening the jars, and placing them in presence of daylight in a chamber at 15±5 ℃ and 90% relative humidity. Primo rdia appeared after a further 15 days of growth, and basidiocarps were harvested 7 days later and weighed [10].
P. ostreatus basidiospores were collected by sporal print on a glass Petri dish, previously sterilised in autoclave (1 h at 121℃). A fresh healthy fruit body of *P. ostreatus* was attached to the cap by eukit® resin under aseptic conditions in such a way that the gills of the fruit body were facing underneath. After 24 h, the lid was removed from the top of the Petri dish holding the spore print.
A spore suspension was prepared in 1 mL of sterile physiological salt solution (0.9% NaCl). Sporal concentration was estimated by counting them in a Thoma chamber on optical microscopy. The basidiospores suspension was plated on PDY solid medium in Petri dishes after appropriate dilution to obtain distinct monosporic colonies and incubated at 28℃. Colonies formed were transferred onto agar slants and tested for production of ligninolytic enzymes.

Mating test
Small pieces of mycelium of two monokaryons (agar plugs 5 mm diameter) were inoculated close to each other on 2% malt extract (Difco) agar plates; after 5–7 days growth the presence of clamp connections, in the interaction zone was checked microscopically.

Enzyme assays
Phenol-oxidase activity was assayed at 25℃ using 2 ,2' –azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [19]. The assay mixture contained 2 mM ABTS and 0.1 M sodium-citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}) for 1 minute. Enzyme activity was expressed in IU.
Enzyme activity was measured in the filtrates from four parallel flasks after removing the mycelia. All measurements were repeated at least in triplicate.

Native polyacrylamide gel electrophoresis
Polyacrylamide gel electrophoresis (PAGE) was carried out at alkaline pH under non denaturing conditions. The resolving and stacking gels contained 9% and 4% acrylamide, respectively. The buffer solution used for the resolving gel contained 50 mM Tris-HCl (pH 9.5), and the buffer solution used for the stacking gel contained 18 mM Tris-HCl (pH 7.5). The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). Gels were stained to visualize laccase activity by using ABTS as substrate, in 0.1M sodium citrate buffer pH3.0. Samples containing 0.015 laccase unities were loaded on each lane.

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

Protein identification by mass spectrometry
Protein band corresponding to the laccase of interest was excised from the native gel. Slices of interest were destained by washes with 0.1 M NH_4HCO_3 pH 7.5 and acetonitrile, reduced for 45 minutes with 100 µl of 10 mM dithiothreitol in 0.1 M NH_4HCO_3 buffer pH 7.5 and carboxyamidomethylated for 30 minutes in the dark with 100 µl of 55 mM iodoacetamide in the same buffer. Tryptic digestion was performed by adding for each slice 100 ng of enzyme in 10 µl of 10 mM NH_4HCO_3 pH 7.5 for 2 hours at 4 ℃. The buffer solution was then removed and were added 50 µl of 10 mM NH_4HCO_3 pH 7.5 for 18 hours at 37 ℃. Peptides were extrac ted by washing the gel slices with 10 mM NH4HCO3 and 1% formic acid in 50% acetonitrile at room temperature.
The peptide mixtures were filtered by using 0.22 µm PVDF membrane (Millipore) and analysed using a 6520 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed in 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as the eluent. The sample was then fractionated on a C18 reverse –

phase capillary column (Agilent Technologies chip) at flow rate of 400nl/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% in 50 min. Peptide analysis was performed using data - dependent acquisition of one MS scan (mass range from 300 to 180 0 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan. Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (0.5 min) of ions from which definitive mass spectral data had previously acquired. Nitrogen at a flow rate of 3 L/min and heated to 325℃ was used as the dry gas for spray d esolvation. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50000 counts. Double and triple charged ions were preferably isolated and fragmented over single charged ions. The acquired MS/MS spectra were transformed in mz.data format and used for proteins identification with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA). Raw data from nanoLC–MS/MS analyses were used to query the *P. ostreatus* database and the Mascot search parameters were: trypsin as enzyme, allowed number of missed cleavage 3, carbamidomethyl as fixed modification, oxidation of methionine, pyro-Glu N-term Q, as variable modifications, 10 ppm MS tolerance and 0.6 Da MS/MS tolerance, peptide charge, from +2 to +3. Individual ions scores >32 indicate identify or extensive homology ($p<0.005$). Protein scores are derived automatically by MASCOT Software from ions scores as non-probabilistic for ranking protein hits.

Analysis of protein sequence

The predicted protein sequence of newly identified laccase POX1 was analyzed by comparison with those of the previously defined *Pleurotus ostreatus* laccases. Alignments of amino-acidic sequences were generated with ClustalW2 (http://www.ebi ac.uk/Tools/clustalaw2/index.html). Signal peptide was predicted with SignalP V2.0 (http://www.cbs.dtu.dk/services/). The amino-acid conservation scoring among the laccase signature sequences was performed by PRALINE (http://www.ibi.vu.nl/programs/pralinewww/).

Results

Production of *P. ostreatus* monokaryotic strains

Two varieties of the fungus *P. ostreatus, var. florida* (ATCC MYA-2306) and *var. ostreatus* (D1208), have been used as parental dikaryotic strains. The two varieties show different morphological and physiological features like size, temperature tolerance, colour, etc., and have been previously extensively characterized for their ability to produce oxidative enzymes [9, 20, 21], hydrophobins [22, 23], and natural compounds [24]. In order to produce new dikaryotic hybrids with increased production capabilities, basidiospores-derived monokaryons, obtained from each variety, have been isolated and analysed. Collected spores from the two different basidiocarps were successfully germinated in solid medium. Microcolonies progeny was microscopically analyzed and monokaryotic state was confirmed by the absence of mycelial clamp connections, as reported by Eichlerov´a I. and Homolka L. [18].

Isolated monokaryons (marked “A” and “D”, for strains derived from the parental dikaryons ATCC MYA2306 and D1208, respectively) were inoculated in PDY submerged culture in order to detect laccase activity during fungal fermentation. Time courses analysis of extracellular laccase activities showed high variability among the strains and a maximum of production between the 9^{th} and the 10^{th} day of growth. Among twenty-eight randomly chosen germinating spores, three monokaryons (5A,

6A, 18A) from the parental strain *P. florida* and three monokaryons (3D, 9D, 11D) from the parental strain *P. ostreatus* exhibit higher or comparable production levels than the two parental strains. Corresponding data are reported in table.1 (a) and (b), derived from three independent experiments and the mean values were taken. The standard deviation for the experiments was less than ±10%. As also reported by Eichlerov´a and Homolka [18], the isolates differ also in morphology and growth rate when they grew on solid media. However, these colony appearances were transient and, in general, not connected with laccase production levels.
Isoenzymatic pattern analyses were performed on the samples derived from extracellular medium of monokaryotic fungal cultures. Native PAGE of laccase isoenzymes stained for laccase activity were carried out and the identity of the laccase isoenzymes was ascertained on the basis of the different electophoretic mobility. Analysis of samples withdrawn from the media at different growth times showed that the activity is associated mainly to the production of three isoenzymes POXA1b, POXA3, and POXC, [9] as reported in figure 1.
Moreover, the monokaryon 6A secretes a new isoform, previously never identified (figure 1). When the protein band, corresponding to the new laccase was excised and analyzed by tandem mass spectrometry, 5 peptide (sequence coverage, 20%; protein summary score, 286) could unambiguously be identified. *P. ostreatus* database search with the raw MS/MS data identified the protein encoded by the predicted laccase transcript POX1. The cDNA of this 'new' native laccase was previously isolated [25], but the corresponding produced protein in an active form was not identified yet.

Dikaryotization of *P. ostreatus* generated by classical crossing

Because laccase production is regulated by multifactorial and mutiallelic expression systems which are dependent on extra- and intra-cellular regulations [13, 14, 26, 27], all crossings among the six monokaryotic strains were performed in order to obtain new high laccase-producers dikaryotic strains (table 2). Anastomosis induction followed by formation of clamp connections (figure 2) was confirmed microscopically at ×45 magnification, indicating that compatibility among two strains and subsequent formation of the corresponding dykarion.
New dikaryotic hybrids derived from the two parental strains were obtained through crossing of selected strains, excepting 5AX9D, that results incompatible. Time course analysis on laccase activity produced by the new dikaryotic strains grown in submerged cultures were perfomed. Six out of the eight dikaryons produced a higher amount of laccase activity. Moreover, three of them (5AxD3, 5AxD11 and 6AxD11) reached a production level of up to 70,000 U/L (figure 3), and were chosen for further investigations.
Effect of ferulic acid inducer [28] on the selected dikaryotic hybrids 5AxD3, 5AxD11 and 6AxD11 was also analyzed. Addition of the inducer to the culture broth positively affects extracellular laccase activity of the analyzed strains. Nevertheless, results indicate that hybrids differentially responded to the inducer presence (figure 4). As a fact, all the selected hybrids were more sensitive to the presence of ferulic acid than the parental strains. Moreover, the 5AXD3 strain resulted the best laccase producer also in inducing conditions, reaching a laccase expression of up to 110,000 U/L.

Laccase fractionation

Time course of the isoenzymatic pattern of the selected dikaryons was performed analyzing the extracellular medium of fungal culture by native PAGE stained for

laccase activity. Analysis of samples withdrawn from the media at different growth times confirmed the presence of the three isoenzymes POXA1b, POXA3, and POXC, as reported above for the corresponding monokaryotic parents, either in the presence or in the absence of inducer.
Furthermore, the strain 6AxD11, in both conditions, secretes the new isoform POX1, previously detected in the monokaryotic parental strain 6A. Relative quantification of laccase isoenzymes, in basal conditions, were also carried out in correspondence of laccase activity peak (9^{th} day). Fungal cultures were harvested and fractionated by anionic exchange chromatography ResourceQ (table 3). The fractions were pooled, concentrated, assayed and analyzed by electrophoretic separation on native PAGE gels stained for laccase activity. The extracellular enzyme mixtures of the strains 5AxD3 and 5AxD11 were showed to contain POXA3 and POXC isoenzymes, the latter being the most abundant (> 90%). Performing the same analysis on the isoenzymatic pattern of 6AxD11, POXA1b laccase and a significant production of POX1 were also detected (35% relative abundance).

Discussion

Laccase enzymes exhibit an extraordinary range of natural substrates (phenols, anilines, inorganic/organic metal compounds, etc) which is the major reason of their attractiveness for several biotechnological applications [29]. More than one laccase isoenzyme, either constitutive or inducible, has been detected in most white-rot fungi [3, 30]. Nevertheless, constitutive extracellular phenoloxidase from basidiomycetes are produced only in small amounts. In according to Galhaup *et al.* [31], one of the major limitations for the large-scale industrial applications of fungal laccases is the low production yields by both wild type and recombinant fungal strains and, as consequence, the high costs for enzyme production [32, 33]. To successfully utilize laccases in industrial applications, the production of large quantities of enzyme at low cost is required, and therefore, any attempt to increase their production from microbial sources could be of considerable interest.
In our study improvement of *P. ostreatus* laccase production was achieved by classical breeding. In particular, basidiospores-derived monokaryons, obtained from *P. ostreatus var. florida* and *var. ostreatus,* were isolated and analyzed. Crossing were performed in order to produce hybrids with favourable cultural characteristics, applicable in large-scale production.
Comparison of the morphological characteristics and laccase levels production of the randomly isolated monokaryons indicated a significantly high genetic variation. The surprisingly high variability between monokaryotic strains for the laccase production may indicate an heterozygosity in genes responsible for the synthesis and secretion of the analyzed enzymes [18].
Moreover, isoenzymatic variety was deeply investigated and a novel laccase protein was identified in one of the selected monokaryotic strains, undetectable in parental dykarion *P. florida*. The new laccase protein was unambiguously identified as POX1 through MS/MS analysis and querying of the *P. ostreatus* genome data bank. Discovery of POX1 proves the production of a functional protein by the related encoding transcript, previously identified by Giardina *et al*. [25]. The new isoenzyme POX1 was also expressed by one of the selected dikaryotic strains, the 6AxD11, either in basal and inductive analyzed conditions. Not surprisingly, the 6A phenotype related to the laccase expression resulted co-dominant in the corresponding dikayriotic strain 6AxD11. As a fact, during *P. ostreatus* dikaryotization process and

dikaryotic mitotic division, the two parental nuclei remain independent and probably silencing on the homologous loci coding for laccase enzymes does not occur [34].
All compatible crossings were performed and allowed the identification and characterization of three new high laccase producer *P. ostreatus* strains. Laccase production levels of the dikaryotic hybrids increased up to 4 folds respect to the maximum level of dikaryotic parents. The extraordinary enhanced laccase activity in dikaryotic strains (hybrid of high-yielding monokaryons), as well as certain physiological differences, may be due to genetic recombination effects, occurred during basidia formation [15].
As well as reported by Chaudhary *et al.* [16] and Sawashe and Sawant [17], results indicate that classical breeding is an useful method for obtaining isolates with interesting combination of enzyme and growth characteristics without inducing physics or chemical mutagenization. The classical natural method of hybrid preparation represents a considerable advantage in comparison with the other common methods (e.g. mutagenization), because undesirable side-effects (e.g. pleiotropic) can be avoided. In addition, new generated dikaryotic strains, in according with current legislation (European Directive 2001/18/CE) are still considered as GRAS and thus could be good candidates for their use in bioprocesses as natural strains.
This study also extends recent reports [9, 10] that demonstrated the production of multiple laccase isoforms in the basidiomycete white rot fungus *P. ostreatus*, enlarging the assortment of these industrially useful enzymes. Further investigations will reveal the peculiar molecular aspects and the biochemical properties of POX1 laccase.

References

[1] Ten Have R., Teunissen P.J. Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chem Rev*. (2001); 101(11):3397-413.
[2] Cullen D. and Kersten P. Fungal enzymes for lignocellulose degradation. *In: Kinghorn JR and Turner G (eds) Applied molecular genetics of filamentous fungi.* New York: Chapman & Hall (1992) Chapter 4.
[3] Giardina P., Faraco V., Pezzella C., Piscitelli A., Vanhulle S., Sannia G. Laccases: a never-ending story. *Cell Mol Life Sci*. (2010); 67(3):369-85.
[4] Thurston C.F. The structure and function of fungal laccases. *Microbiology*. (1994); 140:19-26.
[5] Gianfreda L., Xu F., Bollag J.M. Laccases: a useful group of oxidoreductive enzymes. *Bioremediat J*. (1999); 3:1-26.
[6] Hatakka A. Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS Microbiology Reviews.* (1994); 13:125-135.
[7] Arora D.S. and Sharma R.K. Ligninolytic fungal laccases and their biotechnological applications. *Appl Biochem Biotechnol* (2010); 160(6):1760-88.
[8] Piscitelli A., Pezzella C., Giardina P., Faraco V. and Sannia G. Heterologous laccase production and its role in industrial applications. *Bioengineered Bugs*. (2010); 1:4, 1-11.
[9] Pezzella C., Autore F., Giardina P., Piscitelli A., Sannia G. and Faraco V. The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. *Current Genetics.* (2009); 55:45-57.
[10] Lettera V., Piscitelli A., Leo G., Birolo L., Pezzella C., Sannia G. Identification of a new member of *Pleurotus ostreatus* laccase family from mature fruiting body. *Fungal Biology.* (2010); doi:10.1016/j.funbio.2010.06.004.

[11] Irie T., Honda Y., Watanabe T. and Kuwahara M. Efficient transformation of filamentous fungus *Pleurotus ostreatus* using single -strand carrier DNA. *Appl Microbiol Biotechnol* (2001); 55:563-565.
[12] Irie T., Honda Y., Hirano T., Sato T., Enei H., Watanabe T. and Kuwahara M. Stable transformation of *Pleurotus ostreatus* to hygromycin B resistance using Lentinus edodes GPD expression signals. *Appl Microbiol Biotechnol* (2001); 56(5-6):707-709.
[13] Kothe E. Mating-type genes for basidiomycete strain improvement in mushroom farming. *Appl Microbiol Biotechnol* (2001); 56(5 -6):602-12.
[14] Kaur J., Sodhi H.S. and Kapoor S. Breeding of *Pleurotus florida* (oyster mushroom) for phenotypic pigmentation and high yield potential. *Journal of the Science of Food and Agriculture* (2008); 88:2676-2681.
[15] Xu J., Horgen P.A., Anderson J.B. Somatic recombination in the cultivated mushroom *Agaricus bisporus. Mycol Res* (1996); 100:188–192.
[16] Chaudhary A., Dwivedi R.R. and Singh R.P. Evolution and evaluation of hybrids of oyster mushroom, in Proceedings of an International Conference on Mushroom Biology and Biotechnology, NRCM, Solan, India. Mushroom Society of India Publication, Solan, H.P., India, (2007):51.
[17] Sawashe S.G. and Sawant D.M. Studies on evaluation of new strains of Pleurotus spp. obtained through hybridization. In Proceedings of an International Conference on Mushroom Biology and Biotechnology, NRCM, Solan, India,. Mushroom Society of India Publication, Solan, H.P., India, (2007):52.
[18] Eichlerov´a I., Homolka L. Preparation and crossing of basidiospore-derived monokaryons useful tool for obtaining laccase and other ligninolytic enzyme higher-producing dikaryotic strains of *Pleurotus ostreatus*. *Antonie van Leeuwenhoek.* (1999); 75:321-327.
[19] Palmieri G., Giardina P., Bianco C., Scaloni A., Capasso A., Sannia G. A novel white laccase from *Pleurotus ostreatus*. *The Journal of Biological Chemistry.* (1997); 272:31301-31307.
[20] Das N., Sengupta S., Mukherjee M. Importance of laccase in vegetative growth of *Pleurotus florida*, *Appl Environ Microbiol.* (1997); 63:4120-4122.
[21] Giardina P., Aurilia V., Cannio R., Marzullo L., Amoresano A., Siciliano R., Pucci P., Sannia G. The gene, protein and glycan structures of laccase from *Pleurotus ostreatus*. *Eur J Biochem.* (1996); 235:508-515.
[22] Armenante A., Longobardi S., Rea I., De Stefano L., Giocondo M., Silipo A., Molinaro A., Giardina P. The *Pleurotus ostreatus* hydrophobin Vmh2 and its interaction with glucans, *Glycobiology.* (2010); 20:594-602.
[23] Peñas M.M., Asgeirsdóttir S.A., Lasa I., Culiañez-Macià F.A., Pisabarro A.G., Wessels J.G., Ramírez L. Identification, characterization, and in situ detection of a fruit-body-specific hydrophobin of *Pleurotus ostreatus*. *Appl Environ Microbiol.* (1998); 64:4028-4034.
[24] Venkateshwarlu G., Chandravadana M.V., Meera P., Tewari R.P., Selvaraj Y. Volatile flavour compounds from oyster mushroom (*Pleurotus florida*) in submerged culture. *Flavour and fragrance journa.l* (2001); 15:320-322.
[25] Giardina P., Cannio R., Martirani L., Marzullo L., Palmieri G. and Sannia G. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus. Applied and Environmental Microbiol.* (1995); 61: 2408–2413.
[26] Soden D.M. and Dobson A.D. Differential regulation of laccase gene expression in *Pleurotus sajor-caju. Microbiology* (2001); 147:1755-1763.

[27] Elisashvili V., Kachlishvili E. Physiological regulation of laccase and manganese peroxidase production by white-rot basidiomycetes. *J Biotechnol.* (2009); 144:37-42.
[28] Palmieri G., Giardina P., Sannia G. Laccase-mediated Remazol Brillant Blue R decolourization in a fixed-bed bioreactor. *Biotechnol Prog* (2005); 21(5):1436-41.
[29] Kunamneni A., Camarero S., Garcı´a-Burgos C.' Plou F.J., Ballesteros A., Alcalde M. Engineering and applications of fungal laccases for organic synthesis. *Microb Cell Fact* . (2008); 7:32.
[30] Mayer A.M., Staples R.C. Laccase: new functions for an old enzyme. *Phytochemistry*. (2002); 60:551–565.
[31] Galhaup C. and Haltrich D. Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Applied Microbiology and Biotechnology*. (2001); 56, 225–232.
[32] Hou H., Zhou J., Wang J., Du C., Yan B. Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Process Biochem*. (2004); 39:1415–1419.
[33] Minussi R., Pastore G., Dura´n N. Laccase induction in fungi and laccase/N-OH mediator systems applied in paper mill effluent. *Bioresour Technol*. (2007); 98:158–164.
[34] Eric U. Selker, Epigenetic phenomena in filamentous fungi: useful paradigms or repeat-induced confusion? *Trends in Genetics*. (1997); 13(8): 296-301.

Table 1: Maximum laccase production of basidiospore-derived monokaryons: (a) monokaryons A from parental strain *P.ostreatus* MYA-2306; (b) monokaryons D from parental strain *P.ostreatus* D1208

(a)

Strain	Activity (U/mL)
	9th day
Parental strain MYA-2306	**17.3**
1A	1
2A	3.36
3A	2.6
4A	2.44
5A	**36.6**
6A	**17.10**
7A	17.11
8A	7.33
9A	3.18
11A	9.17
13A	2.66
15A	12.5
17A	2.08
18A	**26.3**

(b)

Strain	Activity (U/mL)
	9th day
Parental strain D1208	**15.8**
D1	0.65
D2	4.2
D3	**5.1**
D4	0.015
D5	0.03
D6	3.3
D7	1.3
D8	1.02
D9	**16.6**
D10	1.21
D11	**7.8**
D12	0.83
D13	1.49
D14	0.38
D15	2.62

Table 2: Compatibility tables of selected higher laccase producers monokaryons: A X D crossing

Strain	3D	9D	11D
5A	+	+	+
6A	+	+	+
18A	+	-	+

Table 3: L**accase isoenzymes produced by dikaryotic hybrids cultures. Fractionation was performed by anionic exchange chromatography ResourceQ. Relative quantification of laccase isoenzymes were carried out in correspondence of laccase activity peak (9th day), in basal condition.**

Strains	Activity U/L (day)	Isoenzymatic	
5AxD3	**47,000 ±18,000 (9)**	POXA3	<1%
		POXC	99%
6AxD11	**53,000 ± 13,000 (9)**	POXA1b	2%
		POXA3	15%
		POXC	48%
		POX1	35%

5AxD11	**45,000 ± 8,000 (9)**	POXA3	2,2%
		POXC	97,8%
Parentale	**17,000 ± 2,000 (9)**	POXA3	20%
		POXC	80%

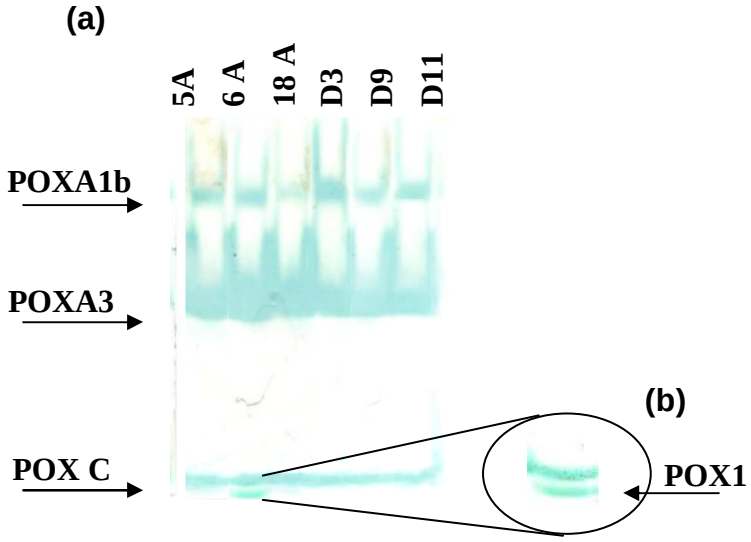

Figure 1: **Zymograms of laccase isoenzymes produced by the selected monokaryotic strains in PDY culture broth. Samples containing 0.015 U of laccase activity collected in the day of maximum production (9th days) were used. Known laccase isoforms POXA1b, POXA3 and POXC were loaded as standards. (b) Zoom of POX1 laccase identified in the monokaryotic strain 6A.**

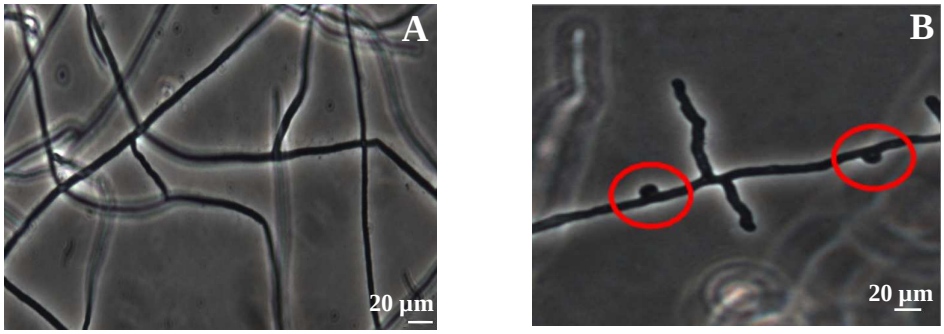

Figure 2: (A) *P. ostreatus* monokaryotic hyphae observed on optical microscope (×45 magnification); (B) *P. ostreatus* dikaryotic hyphae having clamp connections. Clamp structure is highlighted in red.

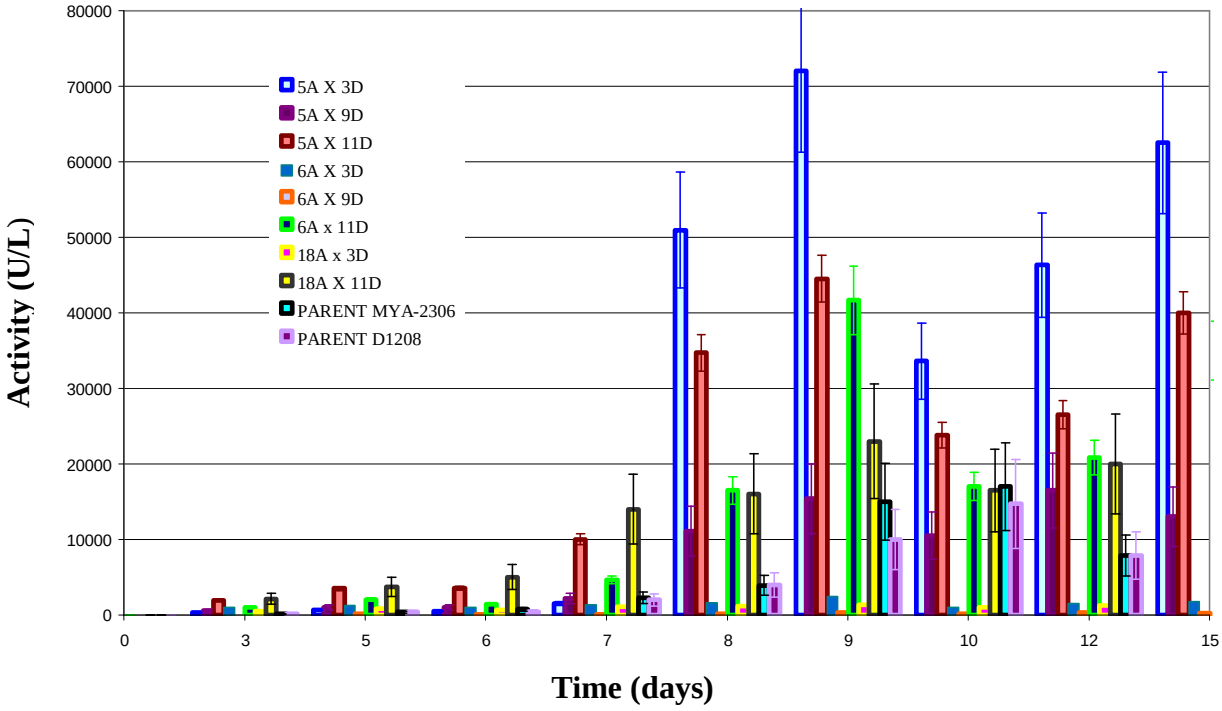

Figure 3: Time course analysis on extracellular laccase activity produced by dikaryotic strains, in basal condition (PDY+150 µMCuSO$_4$).

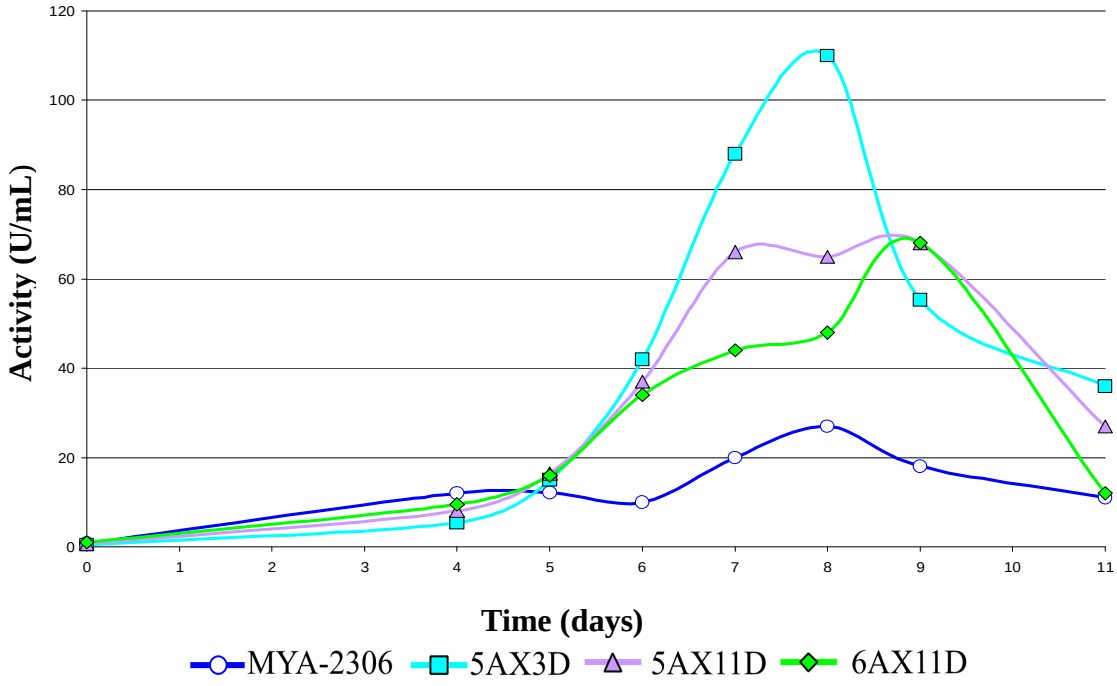

Figure 4: Time course analysis on extracellular laccase activity produced by dikaryotic strains, in addiction of ferulic acid (2mM final concentration).

PUBLICATIONS

- Molecular determinants of peculiar properties of a *Pleurotus ostreatus* laccase: analysis by site directed mutagenesis. F. Autore; **C. Del Vecchio**; F. Fraternali; P. Giardina; G. Sannia; V. Faraco. *Enzyme and Microbial Technology*. (2009); 45:507–513.
- Enhancement of laccase production by *Pleurotus ostreatus* breeding for Biotechnological purposes. V. Lettera, **C. Del Vecchio**, S. Vanhuelle and G.Sannia; Italic 5– Science & Technology of Biomass: Advances and Challenges, September 2009.
- Fungal laccases: versatile tools for lignocellulose transformation. A. Piscitelli, **C. Del Vecchio**, V. Faraco, P. Giardina, G. Macellaro, A. Miele, C. Pezzella, and G. Sannia. Submitted to *Comptes Rendus Biologies; Elsevier ediction* Ottobre 2010.
- Low impact strategies to improve ligninolytic enzyme production in filamentous fungi: the case of laccase in *Pleurotus ostreatus*. V. Lettera, **C. Del Vecchio**, A. Piscitelli, G.Sannia. Submitted to *Comptes Rendus Biologies; Elsevier ediction*. Ottobre 2010.
- *Pleurotus ostreatus* classical breeding: an useful tool to improve and increase laccase variability on dicaryotic strains. **C. Del Vecchio**, V. Lettera, G. Leo, C. Pezzella, A. Piscitelli, Birolo L. and G. Sannia. *Paper in preparation*.

COMUNICATIONS

- Bio-remediation strategies for the treatment of industrial colored wastewaters. Pezzella C, **Del Vecchio C**, Faraco V, Miele A, Giardina P, Sannia G. X National Biotechnology Congress., 17-19 September 2008, Perugia, Italy.
- The white rot fungi PO322 and PC374 and their enzymes for the bioremediation of industrial coloured wastewaters. Pezzella C., **Del Vecchio C.**, Faraco V., Giardina P., Miele A., Piscitelli A., Sannia G. Last Annual Meeting SOPHIED. (Novel Sustainable Bioprocesses for European Colour Industries), 20-30 May 2008, Malaga, Spain.
- Wild type, mutant and new laccases from *Pleurotus ostreatus*: a wide selection of decolorizing tools. Piscitelli A., Autore F., **Del Vecchio C.**, Faraco V., Festa G., Giardina P., Miele A., Pezzella C., Sannia G. Last Annual Meeting SOPHIED (Novel Sustainable Bioprocesses for European Colour Industries), 20-30 May 2008, Malaga, Spain.
- Selection of “better performing” laccases through directed evolution. Miele A., Faraco V., Piscitelli A., **Del Vecchio C.**, Giardina P. and Sannia G. Young Scientific Forum, 2-4 June 2009, Prague, Czech Republic.
- Selection of “better performing” laccases through directed evolution. Miele A., Faraco V., Piscitelli A., **Del Vecchio C.**, Giardina P. and Sannia G. 34th FEBS Congress. 5-9, June 2009, Prague, Czech Republic.
- *Pleurotus ostreatus* classical breeding: an useful tool to improve fungal lignolytic system on dycayotic strains. C**. Del Vecchio**, V. Lettera, A. Bazes and G. Sannia. 1st Symposium on biotechnology. 28 April-1 May 2010, Reims, France.
- Fungal breeding: the power of love! **C. Del Vecchio**, V. Lettera, A.Bazes, P. Giardina and G. Sannia. OxiZymes 9th International Symposium on Peroxidases. 14-16 June 2010, Leipzig, Germany.

• Low impact strategies to improve laccase production in *Pleurotus ostreatus*. V. Lettera, **C. Del Vecchio**, Lucia Guarino, Christian-Marie Bols, Giovanni Sannia. The Fourth Annual Workshop of *COST FP0602*. 21- 24 September 2010, Cesme, Izmir, Turkey.

EXPERIENCES IN FOREIGN LABORATORIES

13 July 2008- 31 July 2008: King's College of London, under the supervision of Prof. F. Fraternali.

Appendix

ENHANCEMENT OF LACCASE PRODUCTION BY PLEUROTUSOSTREATUS BREEDING FOR BIOTECHNOLOGICAL PURPOSES

V. Lettera[1,2], **C. Del Vecchio[1,2]**, S. Vanhulle[3] and G. Sannia[1]
[1]*Department of Organic Chemistry and Biochemistry, University of Naples Federico II, Italy*
[2] *School of Biotechnological Sciences, University of Naples Federico II, Italy*
[3]*Université Catholique de Louvain Microbiology Unit, Belgium*

Abstract

Pleurotus ostreatus var. florida is a industrially useful homobasidiomycete involved in biodelignification, oxidative enzyme production and environmental bioremediation (1,2). Because genetic transformation and mutagenic treatments produce strains not suitable for natural or safe processes, to achieve improvements of this species, it is necessary to optimise specific traits of natural strains by classical cross-hybridization. Mating of this fungus is controlled by a bifactorial tetrapolar genetic system (3): basidiospores, a product of meiosis, can be collected and germinated individually to give rise to single spore monokaryotic cultures. Monokaryons present stable morphological and physiological characteristics. These single-spore isolates can be then inter-crossed to identify their mating type compatibility to form hybrid dikaryons.
The major aim of breeding is to generate variability and combine desirable features from different monokaryotic strains. The present study show a correlation in laccases production, secretion and growth rate between monokaryons and theirs dykaryotic derivates. Moreover it suggest a procedure to select specific monokaryotic strains before the breeding. New dikaryotic strains with improved efficiencies are still considered by the legislation as GRAS and can be good candidates for their use in bioproduction as natural strains.

Introduction

Basidiomycetes have been widely recognized as very efficient lignin degraders. This capacity is assumed to result from the activities of numerous enzymes; among these phenol oxidases (laccase) (4) and peroxidases (lignin peroxidase, manganese-dependent peroxidase) (5) are two the most intensively studied groups of lignin-modifying extracellular enzymes.
Ligninolytic enzymes and the corresponding producing organisms are widely considered to have potential for industrial applications such as biodegradation of environmental pollutants (e.g. polycyclic aromatic hydrocarbons, textile dye, etc.); stain bleaching, bioconversion of lignin, biobleaching and biopulping of wood chips or delignification of agricultural plant residues (6). Sufficient production and secretion of these enzymes is, among other things, necessary for the efficiency and effectiveness of the fungus.
Separation of individual nuclei from cytoplasm after protoplasting resulted in remarkable changes in qualitative and quantitative characteristics of fungal strains (7). The process of meiosis in the basidium and subsequent formation of four uninucleate basidiospores during the fructification of *Pleurotus ostreatus* has a similar effect.

In our present work, we have studied the behaviour of monokaryotic isolates and dikaryons obtained by crossing of characterized basidiospore-derived monokaryons in the hope of obtaining new fast growing species.

Experimental

1. Organism and culture conditions

Dikaryotic strain of *P. ostreatus* (Jacq.: Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) and of two commercial *P. ostreatus* (MBA-0001 and Mba-0002) were maintained through periodic transfer at 4℃ on potato dextrose agar pl ates in the presence of 0.5% yeast extract (Difco). Mycelium was grown in 100 ml shacked flasks (125 rpm) containing 30 ml of PDY with 150 µM CuSO4. The flasks were inoculated with four agar plugs (8 mm diameter) cut from the actively growing part of the colony on a *Petri* dish and incubated for 13 days at 28℃enzyme C in the dark.

2. Enzyme activity determinations

Enzyme activity was measured in the filtrates from three parallel flasks after removing the mycelia.a water reservoir.

Spectrophotometric assays of laccase activity were carried out using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate (12). The assay mixture contained 2 mM ABTS and 0.1 M sodium-citrate buffer, pH 3,0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M-1 cm-1) for 1 minute. Each assay was done in triplicate**.**

3. Native PAGE

Native Polyacrylamide gel electrophoresis (PAGE) was carried out at alkaline pH under nondenaturing conditions. The separating and stacking gels contained 9% and 4% acrylamide, respectively. The buffer solution used for the separating gel contained 50 mM Tris-HCl (pH 9,5), and the buffer solution used for the stacking gel contained 18 mM Tris-HCl (pH 7,5). The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8,4). Gels were stained to visualize laccase activity by using ABTS as the substrate in sodium citrate buffer 0,1M pH3.

4. *P. ostreatus* fructification at laboratory scale

Spawn was prepared on boiled wheat grains and straw mixed with $CaSO_4$ and $CaCO_3$, autoclaved at 120℃ for 20 min, cooled and inoculat ed with 7 plugs of mycelial agar aseptically in Erlenmeyer flasks at 27±1℃ for 30–4 0 days until complete mycelial covering of the substrate.

Flasks were opened and incubated at 15℃ and humidi fied twice daily using mist spray. Fruit bodies appeared 2–3 weeks after the opening of the flasks, and were then harvested when their margin started rolling upwards.

5. Basidiospore isolation

P. ostratus basidiospores were collected in a spore collecting glass *Petri* dish (Fig. 2) that was pre-sterilised in autoclave. A fresh healthy fruit body of *P. ostreatus* was attached to the cap by eukit® resin under aseptic conditions in such a way that the gills of the fruit body were facing underneath. After 24 h, the lid was removed from the top of the *Petri* dish holding the spore print. A spore suspension was prepared in 1ml sterile water. Spore concentration was estimated by counting them in a *Thoma* chamber on optical microscopy. the basidiospores suspension was plated on solid medium in *Petri* dishes after appropriate dilution to obtain distinct monosporic colonies and incubated at 28℃. Colonies formed were transferred onto agar slants and tested for production of ligninolytic enzymes.

6. Mating tests

Small pieces of mycelium of two monokaryons (agar plugs 5 mm diameter) were inoculated close to each other on the solid malt extract 2% in a Petri dish and after 5–7 days of growth the presence of clamp connections in the interaction zone was checked microscopically.

***7*. Estimation of radial growth rate**

As a criterion to select better growing isolates mycelium extension rate on solid media was used. This was estimated by measuring the diameters of four individual colonies grown separately on solid agar PDY medium in *Petri* dishes inoculated with agar plugs (5 mm diameter) cut with an injection needle from the actively growing part of colony on another *Petri* dish. All measurements were repeated three times and done in triplicate.

Results and discussion

1. Characterizaion of monokaryotic strains derived from sporogenesis of *P. ostreatus* and *P. ostreatus* var. Florida

It is well known that many *Pleurotus* spp., except *P. tuberregium* and few other, are interfertile (8): in order to produce oyster mushroom hybrids with favourable cultural characteristics, applicable in large-scale production, basidiospores-derived monokaryons, obtained from the laccase high-producer *P. ostreatus* va. Florida and from two hybrids currently available on the market (MBA-0001 and MBA-0002), were isolated an analysed. The surprisingly high variability of laccase production indicates heterozygocity in genes responsible for the synthesis and secretion.

In the set of 45 randomly chosen germinating spore we found 8 isolated with an higher or comparable laccase production whereas 16 strains showed a variable lower production. Time courses of secreted laccase production showed a maximum on the 9^{th} day, two days before the peak of production of the *P. ostreatus* var. Florida dikariotic strain. Moreover, laccase zymogram revealed a different secretion pattern of isoenzymes for some monokaryotic strains during the growth; this should responsible of the different amount of total laccase production.

Estimation of radial growth rate on solid media showed that, after a short period of adaptation, monokaryons generally grow slowly than their dikaryotic parents. Growth rate was not directly correlated with laccase production, but more in general higher-producing isolated displayed lower growth rates.

Isolates differed also in morphological characteristics showing three main types of colony appearance:

- dense cotton like mycelium with regular colony edge, fast growing, similar to the dykariotic strains
- flat, sparse, submerged mycelium with irregular edge or spiral growth, slow growing
- dense cotton like mycelium with irregular colony edge, slow growing

Data showed no correlation between laccase secretion and macroscopic morphological features.

2. Isolation of new hybrid dikaryotic strains

The above mentioned results stimulated us to study the behaviour of dikaryons prepared by crossing of characterized basidiospore-derived monokaryons in order to obtain fast growing higher-producing stable dikaryotic isolates.

Spore isolates from *P. ostreatus* var. florida and *P. ostreatus* were crossed within and between species for a total of 45 combinations. All compatible crossings were performed and four dikaryons were selected as higher-laccase producer. One of the selected dikaryons exhibits a radial growth rate significantly higher than that of all dikaryotic strains.

Conclusions

Results indicate that crossing is a useful alternative method for mushrooms breeding without inducing physics or chemical mutegenization. Laccase production seems to be the most important parameter to take into account in order to select specific monokaryotic strains. It is quite probable that this finding could hold for other enzyme and other basidiomycetes.
The surprisingly high variation of enzymatic activity production arising from a single dykariotic strain calls upon further study on the regulative mechanism of laccase synthesis/secretion and on the ability of these strains to respond to the presence of endogenous extra cellular signal.

References

1 Kirk, T.K.; Farrell R.L. Annu Rev Microbiol. 1987, 41, 465-505.
2 Hammel, K.E. Environ Health Perspect 1995,103 Suppl 5, 41-43.
3 Casselton, L.A.; Olesnicky N. S. Microbiol Mol Biol Rev 1998, 62, 55–70.
4 Pezzella, C.; Autore, F.; Giardina, P.; Piscitelli. A.; Sannia. G.; Faraco, V. Curr Genet. 2009 Feb 55, 1, 45-57.
5 Giardina P.;Palmieri G.;Fontanella B.;Rivieccio V.;Sannia G. Archives of Biochemistry and Biophysics 2000, 376, 1, 171-179.
6 Singh Arora, D.; Kumar Sharma, R. 2009 Applied biochemistry and biotechnology.
7 Keller, U. Appl. Environ. Microbiol. 1983, 46, 580–584.
8 Isikhuemhen O. S.; Moncalvo, J.; Nerud, F.; Vilgalys, R. 2000 Mycological Research 104, 6, 732-737.

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Low impact strategies to improve ligninolytic enzyme production in filamentous fungi: the case of laccase in *Pleurotus ostreatus*

Vincenzo Lettera, **Claudia Del Vecchio**, Alessandra Piscitelli, Giovanni Sannia

Department of Organic Chemistry and Biochemistry, University of Naples Federico II
Complesso Universitario Monte S. Angelo - Via Cinthia 4, IT-80126 Napoli

Phone: +39081674327; fax: +39081674313
*Corresponding author e-mail: vincenzo.lettera@unina.it

ABSTRACT
Pleurotus ostreatus belongs to a subclass of white-rot fungi producing many ligninolitic enzymes such as laccases that represent promising tools for biodelignification, industrial oxidative processes and environmental bioremediation. the ever-increasing demand of laccases for industrial applications requires the production of large quantities of enzymes at low cost. The present work was carried out to reduce laccase production costs in *Pleurotus ostreatus* liquid fermentations trough two different approaches. In the former, screening of fungal spent media as natural laccase inducer was performed eliminating the presence of potentially toxic/recalcitrant and expensive exogenous inducers in culture broth. In the latter, improvement of different strains of *P. ostreatus* crossed by classical breeding and screened for their laccase productivity was performed, avoiding genetic transformation and mutagenic treatments that could produce organisms not suitable for "natural or safe processes" according to the European legislation . A laccase production level close to 80,000 U/L by combining the two approaches was achieved. Autoinduction and classical breeding represent promising tools for the improvement of fungal fermentation without affecting the disposable costs that also depend on the eco-compatibility of the whole process.

KEYWORD: white-rot fungi , laccase, autoinduction, spent medium, classical breeding.

INTRODUCTION

The white-rot basidiomycete fungus *P. ostreatus* is one of the most active micro-organisms degrading lignin, a complex aromatic biopolymer that is extremely recalcitrant to degradation [1]. This fungus produces different oxidative enzymes, with broad substrate specificity, which can also be used to degrade a vast range of toxic aromatic pollutants [2, 3]. Among these enzymes the production of several laccase (E.C. 1.10.3.2) isoenzymes is prominent [4]. The variety of laccase isoenzymes is related to the diversity of their roles: lignin synthesis/degradation [1, 5], fruit bodies development [6], pigment production [7], cell detoxification [8], etc [9]. Moreover, laccases result in biotechnologically relevant products because of their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants. These features are suitable for several different applications in industrial effluents disposal, medical diagnostics, bioremediation to degrading pesticides and explosives in soils, delignification processes in paper industries and in cosmetics formulation as additive [10]. Owing to the successful use of laccases in the above-mentioned biotechnological applications, the ever-increasing demand requires the production of large quantities of enzyme at low cost. As a fact, several production strategies have been adopted along with process optimization to achieve better process economics.

Concurrently, studies on laccase producing organisms have been intensified in the recent years. The overexpression in suitable hosts and optimization of laccase production from different microorganisms would provide means to achieve high titers. On the other hand, several methods have been used for strain improvement in *Pleurotus spp.* including selection, hybridization and gene transformation [11, 12, 13, 14]. Based on current legislation (European Directive 2001/18/CE), genetic transformation and mutagenic treatments produce strains not suitable for "natural or safe processes". Therefore the construction of genetically modified organisms can not be chosen to improve the addressed quality of the fungus, and breeding should be based on classical genetic approaches. This last technique is based on the mating of two monokaryotic compatible strains of interest, whose hyphae are able to fuse and give rise to a dikaryotic mycelium in which the two parental nuclei remain independent [13, 14]. Production of the monokaryotic strain, germinating from uninucleate basidiospores, is achieved when the fungus enters into a reproductive phase triggering basidiocarp formation: during basidia formation, karyogamy takes place immediately before the onset of the meiosis giving rise to four uninucleate basidiospores. *Chaudhary et al.* [15] developed single spore isolates from the white-rot fungi *Pleurotus djamor, P. ostreatus var. florida, Pleurotus citrinopileatus* and *Hypsizygus ulmarius*. The hybrids showed improved mycelial growth rate compared to parental strains. In another work *Sawashe* and *Sawant* [16] developed hybrid cultures which required a significantly shorter period for spawn run as compared to the parent species.

Selecting new hybrid strains for enzyme production could be viewed as a solution to make the entire process cost effective, and further enhancement using inducers may be added to the benefit.

Use of inducers to enhance laccase production has been widely practiced in fungi especially in white-rots, where induction of laccase production by aromatic compounds is well established [17]. Increasing in laccase production has been also achieved in presence of other compounds like aminoacids [18], plant extracts [19] and copper [20, 21] in the growth medium. However, use of above mentioned inducers enhance production cost, because of their price and their likely toxicity which could negatively affect cost of wastewater disposable. In order to avoid these problems, studies on fungal autoinduction mechanisms [22] are being carried out by several groups. Autoregulations, and the signal molecules involved in, have been clearly elucidated in dimorphic fungi like *Candida albicans* and *Saccharomyces cerevisiae* [23]. However, several compounds have been correlated to the regulation of different aspects of fungal physiology in diverse classes of the kingdom mycota [22]. *Schimmel et al.* [24] reported a specific autoinduction effect on lovastatin synthesis in *Aspergillus terreus,* when a spent medium solution extracted from the fungal submerged culture is used to condition a new fresh growth of the same strain. Conversely, in the literature no information about laccase autoinducers is available.

Aim of the present work has been to exploit both approaches: conditioning *P. ostreatus* growth by spent medium solution extracted from liquid culture and breeding the fungal strains by classical crossing. Finally, the potential exploitable effect of spent medium solutions on laccase production was combined with improved capabilities of new hybrids derived from the breeding of two *P. ostreatus* varieties.

MATERIALS AND METHODS

Organism

All *P. ostreatus* monokariotyc and dikaryotic strains were maintained through periodic serial transfers and kept at 4°C on agar plates in the presence of 2,4% potato dextrose and 0.5% yeast extract (PDY) (Difco).

Monokaryotic progeny were identified by a progressive number followed by the (lower case) letter of the parent strains (strains no."f" and no."o", respectivelly). New dikaryotic varieties were classified by progressive numbers followed by the two lower case letters and separated by the "X" (no.f X no.o).

Fructification and basidiospores isolation

Mushrooms of two commercial dikaryotic strain of *P. ostratus*, *P. ostreatus* variant *Florida* (strain F) and *P. ostreatus* variant *ostratus* (strain O), were cultivated in 500 mL jars containing 400 g of wheat-straw (65% water content), which were sterilized by steam heated (121°C) in autoclave for 1 h at 121°C. Sterilization procedure was repeated a second time after an incubation time of 24 h at room temperature. Each jar was inoculated with four agar plug (13 mm diameter), and left to grow at 28°C for 30 days in the dark. Fructification were promoted by opening the jars, and placing them in presence of daylight in a chamber at 15±5°C and 90% relative humidity. Primordia appeared after a further 15 days of growth, and basidiocarps were harvested 7 days later and weighed [25].

P. ostreatus basidiospores were collected by sporal print on a glass *Petri* dish, previously sterilised in autoclave (1 h at 121°C).

A spore suspension was prepared in 1ml sterile physiological salt solution (0,9% NaCl). Sporal concentration was estimated by counting in a *Thoma* chamber on optical microscopy.

Mating test

The basidiospores suspension was plated on PDY agar medium after appropriate dilution. Vegetative mycelium colonies were examined by phase-contrast microscopy for clamp connections, the appearance of colony characteristics specific for dikaryon. Colonies lacking clamps were subcultured in PDY agar slants at 28°C and inoculated in pairs on 2% malt extract agar plates, so that their mycelia would fuse. Compatible monokaryons were identified by the production of clamp connections.

Culture conditions in liquid culture

CONDITION 1 (C1), FOR SPENT MEDIA PREPARATION

Mycelium of variety P. *florida* was grown in 1 l shaken flasks (125 rpm) containing 300 ml of GYM (Glucose, Yeast extract, Mineral solution) formulated as follw: 10 g/l glucose; 3,8 g/l yeast extract (Difco) 2 g/l H_2KPO_4; 0,5g/l MgSO4 $7H_2O$; 0,1 g/l $CaCl_2$ $2H_2O$; biotin 10 mg/l; thiamine 10 mg/l and 10 ml of mineral stock solution (0,5 g/l $MnSO_4$ $5H_2O$; 1 g/l NaCl; 0,1 g/l $FeSO_4$ 7 H_2O; 0,1 g/l $CoCl_2$ 6 H_2O; 0,1 g/l $ZnSO_4$ 7 H_2O; 0,01 g/l $CuSO_4$ 5 H_2O; 0,01 g/l $AlK(SO_4)_2$; 0,01 g/l H_3BO_3; 0,01 g/l $NaMoO_4$ 2 H_2O); final pH5. Except where indicated, all chemicals were obtained from Sigma Chemical Co. 5-day-old culture were homogenized by Ultra-Turrax® T25 Basic interconnected with S18N-19G dispersing tool (3 flashes of 30 seconds at 24.000 rpm separated by 30 seconds of stand-by) and 1 milliliter of homogenate was transferred in 1-l flasks containing 300 ml of GYM broth. The cultures were grown in shaken flasks at 125 rpm and incubated at 28°C in the dark for 17 days.

CONDITION 2 (C2), FOR HYBRID STRAINS GROWTHS

Submerged cultivation was carried out in 100 ml Erlenmeyer flasks containing 30 ml of PDY with copper sulphate (final concentration 150 μM) on rotary shaker (125 rpm). The flasks were inoculated with four agar plugs (8 mm diameter) cut from the actively growing part of the colony on a *Petri* dish and incubated for at least 17 days at 28°C in the dark.

Liquid-liquid extraction of spent media

Extractions were performed on *P. florida* samples by adding ultra pure chloroform (Carlo Erba reagents) to 250 ml of *P. ostreatus* harvested growth medium (condition 1) using a 1:1 v/v ratio. The mixture was subjected to horizontal and rotary shaking for 2 minutes (min). The procedure was repeated twice for each sample. After 10 min decantation, organic phase was removed and concentrated up to 1000 times using a Heidolph Laborota 4000 rotary

evaporator. The liquid-liquid extraction was used to prepare conditioning solution SM7, SM10, SM13 and SM16 (spent medium 7, 10, 13 and 16 days old, respectively).

Conditioning by spent medium solutions

The liquid-liquid extraction of 300 ml spent GYM medium were concentrated up to 1000 times, sterilized by filter membrane (cut-off 0,22 μm, Millipore®) and used to condition 300ml of basal medium of a fresh growth (C1). Conditioning of GYM basal medium was performed using extracted spent medium solutions supplemented at the time of inoculation.

Protein, biomass and glucose concentration determinations.

Protein concentration was determined using the BioRad protein assay kit (BioRad, Hercules, California), following the manufacturer's instructions, with bovine serum albumin as standard. Biomass was dried by drying oven at 65°C overnight and estimated gravimetrically. Glucose concentrations were determined by the glucose oxidize method [26]. Each assay was performed in triplicate.

Enzyme assays

Phenol-oxidase activity was assayed at 25°C using 2,2'–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [27]. The assay mixture contained 2 mM ABTS and 0.1 M sodium-citrate buffer, pH 3,0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}) for 1 minute. Enzyme activity was expressed in UI. All measurements were repeated at least in triplicate.

Native polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out at alkaline pH under non denaturing conditions. The resolving and stacking gels contained 9% and 4% acrylamide, respectively. The buffer solution used for the resolving gel contained 50 mM Tris-HCl (pH 9,5), and the buffer solution used for the stacking gel contained 18 mM Tris-HCl (pH 7,5). The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8,4). Gels were stained to visualize laccase activity by using ABTS as substrate, in sodium citrate buffer 0,1M pH3. Samples containing 0.0015 laccase unities were loaded on each lane.

RESULTS AND DISCUSSION

Analysis of P. ostreatus growth model in liquid culture.

Avoiding false positive induction derived from biotransformation of unknown compounds (generally present in complex media), *P. ostreatus* var. *florida* was grown in a semisynthetic medium containing glucose as main carbon source, and yeast extract, as nitrogen source. As shown in figure 1, three phases of the basal growth, forming the typical behaviour of filamentous fungi in liquid cultures, have been detected. Starting from the inoculation time (t=0 day), the lag phase was displayed for 2 days. During this phase no relevant glucose consumption in the medium was detected. Increasing of glucose consumption rate occurred in the trophophase up to the complete depletion of the main carbon source (7^{th} day). During the same time the fungal culture reached highest cell density, measured as mycelial dry weight (7.2 ±0.5 g/L). However, between the 5^{th} and the 7^{th} fermentation day the rapid increase of glucose uptake and the decrease of growth rate indicate the beginning of the idiophase, when the anabolic pathways of the cultured fungus are altered to produce different biomolecular compounds (secondary metabolites). As a fact, the beginning of exponential growth is related to the start-point of glucose consumption, while cellular lyses (figure 2) in the last phase of growth should be a consequence of carbon starvation.

Time course analysis of extracellular laccase activity production was carried out and the production profile was evaluated in parallel with the fungal growth. As previously reported [28], laccase synthesis does not appear to be related only to the hyphal growth, because the enzyme activity does not parallel biomass production (figure 1). Laccase activity peaked on the 5^{th} and the 13^{th} day, reaching 4,000 and 3,000 U/L respectively, and decreased

dramatically thereafter. The first laccase production increment, rising up to the 5th day, is probably related to the development of fungal biomass in liquid culture, whereas the latter is probably connected to the idiophase phenomena, where activation of secondary metabolism and cellular autolysis occurs (figure 2).
Laccases were analyzed by native PAGE and stained with ABTS. Analysis of samples withdrawn from the media at different growth times indicates that the activity is associated mainly to the production of three isoenzymes POXA3, POXA1B and POXC [4], as reported in figure 3. The same isoenzymatic pattern was observed in correspondence of the two maximum production levels, although the band intensities changed during the time course: detectable levels of POXA1b and POXA3 activity production were only found in correspondence with the first maximum of laccase production (4th-5th day), while no significant difference in the relative amount of POXC isoenzyme was detected at different times of growth (4th, 5th and 13th day).
Taking into account the whole data acquired during the time course analysis, secondary metabolism activated in the idiophase could be associated to the second maximum of laccase production, although a cause-effect correlation can not be formulated at this stage. It has been previously reported that *P. ostreatus* produce several natural compounds during fermentation in submerged culture during the idiophase [29, 30]. The reasons why fungi produce secondary metabolites are still unknown and most of these molecules have not been credited with a biological role [30]. Although no comparative analyses about dynamic variation of exo-metabolites during fermentation in submerged culture was performed, it is evident that under conditions of nutrient limitation, morphological alterations and mycelium changes variations in secondary metabolism dynamically occur. Moreover, many evidences strongly indicate that *Pleurotus spp.* displays the ability to synthesize lignin-related compounds [31, 32]. These extracellular metabolites could regulate laccase expression similarly to other chemical-related inducers [32], as ferulic acid [33].

Increasing of laccase expression in P. ostreatus by spent medium solution
In order to investigate the above mentioned hypothesis, metabolites excreted by *P. ostreatus* were extracted from the fungal fermented broth, starting from the beginning of the idiophase (7th day). Spent media solution derived from the culture broth 7, 10 13, and 16 days old (SM7, SM10, SM13 and SM16, respectively) were used to condition liquid fermentation of *P. ostreatus* grown in basal condition (C1).
Analyses were performed monitoring biomass growth, total secreted protein and laccase production profiles after adding spent medium solutions at the inoculation time (t = 0 days). Conditioning growth media by SM10, SM13 and SM16 caused an increase of laccase activity up to 5 folds in correspondence of the first peak at the 4th and the 5th day (figure 4), whereas no induction of laccase expression was induced at the 13th day. No significant variation of the other parameters was observed ($p<0,005$).
No relevant difference in the enzymatic pattern of samples collected at 4th, 5th and 13th day, from basal and conditioned growths was observed. Such data indicate that the presence of spent medium solutions in liquid culture affect general mechanisms of laccase expression and/or secretion and that the increase of laccase activity at the 4th and the 5th day does not depend on the over-expression of a specific isoenzyme. This behaviour does not correspond to the laccase expression trend observed in the basal growth during the second phase of enzyme production (late part of the idiophase). As a fact, activity dramatically decreases after the13th day. After the 13th day, when the last phase of carbon starvation stress take place, different mechanisms could deactivate/repress any biological synthesis, including laccase expression. As a fact, quiescence or lyses of hyphae could cause an insensitivity of the fungal cells to the presence of any inducer.

Moreover conditioned fungal cultures result not responsive to the induction during the idiophase, while a laccase production increase during the trophophase occurs. Probably, chemicals contained in the spent medium solutions, that were supplemented at the time of inoculation, were metabolized or degraded by the fungal enzymatic activities.

Increasing of laccase expression in P. ostreatus by classical breeding

The breeding strategies of new varieties of industrially useful fungi like *P. ostreatus* are defined by the breeding objectives and the legal constraints imposed to the breeding technology used. This last aspect is of the greatest importance in the case of *wildtype* microorganisms which are considered edible, GRAS and eco-compatibles. In the framework of local and international legislations, in fact, the use of genetically modified microorganisms (GMMs) in industrial bioprocesses could increase the waste disposable cost and preclude the potential conversion of biomass in bioproducts as animal fodder (European Communities Guidance notes for risk assessment outlined in annex 3 of council directive 90/219/EEC on the contained use of genetically modified micro-organisms). This prevents the use of genetic-engineering based technologies for breeding. Consequently, our work was focused on classic breeding, in order to evaluate the capability of this technique to improve safe strains for laccase production. As reported in literature [34], heterozygocity in genes responsible for laccase expression triggers high variability of enzyme production and increases average production in basidiospores derived monokaryons obtained from a single dikaryotic strain. As a fact, certain monokaryotic isolates produce much higher titres of enzymes than the parental strain.

P. ostreatus var. *florida* (strain F) and *P. ostreatus* var. *ostreatus* (strain O) are the dikaryotic fungi used in the present work. These varieties differ in several morphological and physiological features like size, colour, temperature tolerance, etc. Both strains have been extensively characterized in previous studies for their ability to produce oxidative enzymes [4, 35, 36], hydrophobins [37, 38], and natural compounds [29]. In order to produce new dikaryotic hybrids with increased production capabilities, basidiospores-derived monokaryons, obtained from both strains, were isolated and analysed. Collected spores from the two different basidiocarps were successfully germinate in solid medium. Microcolonies progeny was microscopically analyzed and monokaryotic state was confirmed by the absence of mycelial clamp connections.

Monokaryotic strains “f” and “o”, isolated among twenty-eight randomly chosen germinating spores, were inoculated in the PDY production medium (C2) in order to enhance laccase production during fungal fermentation. Time courses analysis of extracellular laccase activities showed high variability among the strains and a maximum of production between the 9^{th} and the 10^{th} day of growth (figure 5). Three monokaryons from the parental strain F (5f, 6f, 18f) and three monokaryons from the parental strain O (3o, 9o, 11o) exhibit higher or comparable production levels than the two parental strains. As also reported by Eichlerov´a and Homolka [34] , the isolates differ also in morphology and growth rate when subcultured on agar media in *Petri* dishes (data not showed). However, colony appearance, whose morphological patterns are transient, could not be connected with laccase production levels.

Anastomosis induction, followed by formation of clamp connections, indicated compatibility among two strains and the formation of the corresponding dykarion. All compatible crossings, among the six monokaryotic strains were performed. Crossing selected strains, new dikaryotic hybrids were obtained, excepting for the pairs coming from the same parent (f X f and o X o) and the crossing 5f X 9o, that resulted uncompatible (Table 1). These dikaryons were studied for their laccase production yields when grown in production medium (C2). Four hybrids reached laccase expression levels higher than those of compared to the related monokarions (figure 6) up to 70000 U/L.

Laccase induction by new dikaryotic hybrids through spent medium solution.

As above-mentioned, because laccase production is regulated by multifactorial and mutiallelic expression systems which are dependent on extra- and intra-cellular regulations [4, 39], effect of SM16 on the partially characterized dikaryotic hybrids was tested. Results indicate that hybrids grown submerged cultures could differentially respond to the autoinduction mechanisms, increasing enzymatic production. As a fact, addition of the inducer solution to the culture broth (C2) at the time of the inoculation increases laccase production levels, for all but one of the selected hybrids, up to 3 times (figure 7). Surprisingly, the best laccase producer among the selected hybrids resultes insensitive to the presence of the inducer. The insensitive strain, seems therefore to be deregulated.
This study has allowed to get new insights in improving fungal laccases production: classical breeding and autoinduction mechanisms. These represent promising tools for the improvement of fungal fermentation without affecting waste disposable cost that also depend on the safe and eco-compatibility of the whole process.

ACKNOWLEDGEMENTS

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REFERENCES

[1] T.K. Kirk, R.L. Farrell, Enzymatic "combustion": the microbial degradation of lignin, Annu Rev Microbiol 41 (1987) 465-505.
[2] K.E. Hammel, Mechanisms for polycyclic aromatic hydrocarbon degradation by ligninolytic fungi, Environ Health Perspect 103 (1995) 41-43.
[3] E. Rodriguez, M.A. Pickard, R. Vazquez-Duhalt, Industrial dye decolorization by laccases from ligninolytic fungi, Curr Microbiol 38 (1999) 27–32.
[4] C. Pezzella, F. Autore, P. Giardina, A. Piscitelli, G. Sannia, V. Faraco, The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members, Curr Genet 55 (2009) 45-57.
[5] D.M. O'Malley, R. Whetten, W. Bao, C. Chen, R.R Sederoff, The role of of laccase in lignifications, Plant J 4 (1993) 751–757.
[6] U. Kües, Y. Liu, Fruiting body production in Basidiomycetes, Appl Microbiol Biotechnol 54 (2000) 141-152.
[7] M. Nagai, M. Kawata, H. Watanabe, M. Ogawa, K. Saito, T. Takesawa, K. Kanda, T. Sato, Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies, Microbiology 149 (2003) 2455-2462.
[8] C. Eggert, U. Temp, J.F. Dean, K.E. Eriksson, Laccase-mediated formation of the phenoxazinone derivative, cinnabarinic acid, FEBS Lett 376 (1995) 202-206.
[9] A.V. Bolobova, A.A. Askadskii, V.I. Kondrashchenko, M.L. Rabinovich, *Teoreticheskie osnovy biotekh nologii drevesnykh kompozitov: Fermenty, modeli protsessy* (Theoretical Bases of Biotechnology of WoodComposites: Enzymes, Models, and Processes), Bezborodov AM, Moscow: Nauka 2002.
[10] A. Kunamneni, F.J. Plou, A. Ballesteros, M. Alcalde, Laccases and their applications: a patent review, Recent Pat Biotechnol 2 (2008) 10-24.
[11] T. Irie, Y. Honda, T. Watanabe, M. Kuwahara, Efficient transformation of filamentous fungus *Pleurotus ostreatus* using single -strand carrier DNA, Appl Microbiol Biotechnol 55 (2001) 563-565.
[12] T. Irie, Y. Honda, T. Hirano, T. Sato, H. Enei, T. Watanabe, M. Kuwahara, Stable transformation of *Pleurotus ostreatus* to hygromycin B resistance using *Lentinus edodes* GPD expression signals, Appl Microbiol Biotechnol 56 (2001) 707-709.
[13] E. Kothe, Mating-type genes for basidiomycete strain improvement in mushroom farming, Appl Microbiol Biotechnol 56 (2001) 602-612.

[14] J. Kaur, H.S. Sodhi S. Kapoor, Breeding of *Pleurotus florida* (oyster mushroom) for phenotypic pigmentation and high yield potential, Journal of the Science of Food and Agriculture 88 (2008) 2676-2681.
[15] A. Chaudhary, R.R. Dwivedi, R.P. Singh, Evolution and evaluation of hybrids of oyster mushroom, in Proceedings of an International Conference on Mushroom Biology and Biotechnology, NRCM, Solan, India, Mushroom Society of India Publication, Solan, H.P., India, (2007) 51.
[16] S.G. Sawashe, D.M. Sawant, Studies on evaluation of new strains of *Pleurotus* spp. obtained through hybridization. In Proceedings of an International Conference on Mushroom Biology and Biotechnology, NRCM, Solan, India, Mushroom Society of India Publication, Solan, H.P., India, (2007) 52.
[17] M.C. Terrón, T. González, J.M. Carbajao, S. Yagüe, A. Arena-Cuenca, A. Téllez, A. Dobson, A.E. González, Structural close-related aromatic compounds have different effects on laccase activity and on lcc expression in the lignolytic fungus Trametes sp. I-62, Fungal Genet Biol 41 (2004) 954-962.
[18] S. Dhawan, R.C. Kuhad, Effect of amino acids and vitamins on laccase production by the bird's nest fungus *Cyathus bulleri*, *Bioresour Technol* 84 (2002) 35-38.
[19] O. Ardon, Z. Kerem, Y. Hadar, Enhancement of laccase activity in liquid cultures of the ligninolytic fungus *Pleurotus ostreatus* by cotton stalk extract, J Biotechnol 51 (1996) 201-207.
[20] J.K. Dittmer, N.J. Patel, S.W. Dhawale, S.S. Dhawale, Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency, FEMS Microbiol Lett 149 (1997) 65-70.
[21] M.C.N. Saparrat, F. Guillén, A.M. Arambarri, A.T. Martínez, M.J. Martínez, Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*, Appl Environ Microbiol 68 (2002)1534-1540.
[22] U. Ugalde, Autoregulatory Signals in Mycelial Fungi The Mycota. Growth, differentiation and sexuality. Fischer R (2006) 203-213.
[23] D.A. Hogan, Talking to Themselves: Autoregulation and Quorum Sensing in Fungi, Eukaryotic Cell 5 (2006) 613-619.
[24] T. G. Schimmel, A. D. Coffman, S. J. Parsons, Effect of Butyrolactone I on the Producing Fungus *Aspergillus terreus*, Appl Environ Microbiol 64 (1998) 3707–3712.
[25] V. Lettera, A. Piscitelli, G. Leo, L. Birolo, C. Pezzella, G. Sannia, Identification of a new member of *Pleurotus ostreatus* laccase family from mature fruiting body, Fungal Biology (2010) doi 10.1016/j.funbio.2010.06.004.
[26] J.B. Lloyd, W.J. Whelan, An improved method for enzymic determination of glucose in the presence of maltose, Anal Biochem 30 (1969) 467-470.
[27] G. Palmieri, P. Giardina, C. Bianco, A. Scaloni, A. Capasso, G. Sannia, A novel white laccase from *Pleurotus ostreatus*, The Journal of Biological Chemistry 272 (1997) 31301-31307.
[28] S. Tlecuitl-Beristain, C. Sánchez, O. Loera, G.D. Robson, G. Díaz-Godínez, Laccases of *Pleurotus ostreatus* observed at different phases of its growth in submerged fermentation: production of a novel laccase isoforms, Mycol Res 112 (2008)1080-1084.
[29] G. Venkateshwarlu, M.V. Chandravadana, P. Meera, R.P. Tewari, Y. Selvaraj, Volatile flavour compounds from oyster mushroom (*Pleurotus florida*) in submerged culture, Flavour and fragrance journal 15 (2001) 320-322.
[30] G.N. Dalimova, Z.R. Akhmedova, Biodestruction of Lignins by the Basidiomycete *Pleurotus ostreatus* 37 (2001) 83-85.
[31] A.M. Calvo, R.A. Wilson, J.W. Bok , N.P. Keller, Relationship between secondary metabolism and fungal development, Microbiol Mol Biol Rev 66 (2002) 447-459.
[32] T. Scheel, M. Hofer, S. Ludwig, U. Holker, Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds, Appl Microbiol Biotechnol 54 (2000) 686-691.
[33] A. Leonowicz, J. Trojanowski, Induction of a new laccase from the fungus *Pleurotus ostreatus* by ferulic acid, Microbios 13 (1975) 167-174.
[34] I. Eichlerov´a, L. Homolka, Preparation and crossing of basidiospore-derived monokaryons useful tool for obtaining laccase and other ligninolytic enzyme higher-producing dikaryotic strains of *Pleurotus ostreatus*, Antonie van Leeuwenhoek 75 (1999) 321-327.
[35] N. Das, S. Sengupta, M. Mukherjee, Importance of laccase in vegetative growth of *Pleurotus florida*, Appl Environ Microbiol 63 (1997) 4120-4122.
[36] P. Giardina, V. Aurilia, R. Cannio, L. Marzullo, A. Amoresano , R. Siciliano, P. Pucci, G. Sannia, The gene, protein and glycan structures of laccase from *Pleurotus ostreatus*, Eur J Biochem 235 (1996) 508-515.
[37] A. Armenante, S. Longobardi, I. Rea, L. De Stefano, M. Giocondo, A. Silipo, A. Molinaro, P. Giardina, The *Pleurotus ostreatus* hydrophobin Vmh2 and its interaction with glucans, Glycobiology 20 (2010) 594-602.
[38] M.M. Peñas, S.A. Asgeirsdóttir, I. Lasa, F.A. Culiañez-Macià, A.G. Pisabarro, J.G. Wessels, L. Ramírez, Identification, characterization, and In situ detection of a fruit-body-specific hydrophobin of *Pleurotus ostreatus*, Appl Environ Microbiol 64 (1998) 4028-4034.

[39] V. Elisashvili, E. Kachlishvili, Physiological regulation of laccase and manganese peroxidase production by white-rot Basidiomycetes, J Biotechnol 144 (2009) 37-42.

TABLE AND FIGURE CAPTIONS

Table 1 – mating taste between selected monokaryons derived from the parent strain F (5f,6f and 18f) and O (3o, 9o, 11o): + indicates a compatible interaction, clamp connection formed;- indicates an incompatible interaction, no clamp connection formed.

Figure 1 – *P. ostreatus* 17 days fermentation profile in basal condition: extracellular laccase activity, secreted protein concentration, glucose consumption and biomass increasing are reported as U/ml ( ), 10* mg/l (.); g/l ( ), g/l(….) respectively.

Figure 2 – light microscopy observation of fungal pellet at the 5th (A) and the 12th (B) day of growth in liquid basal medium. In picture 3B it's possible to appreciate vacuolization and cell lyses. Bar, 12 µm.

Figure 3 – Zymograms of laccase isoenzymes in the basal condition (C1). Samples containing 0.015 U of laccase activity collected at different times (4th, 5th, and 13th day) were used.

Figure 4 – Laccase activity per litre of culture in *P. ostreatus* growth medium (C1) in absence (line A) and in the presence of SM7 (line B), SM10 (line C), SM13 (line D) and SM16 (line E).

Figure 5 – Time course of laccase activity secreted by monokaryotic strains "f" and "o" in PDY medium (C2). Enzymatic activity of parental varieties F and O are also reported. standard deviation <20%

Figure 6 – Laccase activity of *P. ostreatus* selected hybrids in PDY culture (C2) after 12 days of growth.

Figure 7 – Maximum laccase activity secreted by *P. ostreatus* selected hybrids in PDY medium (C2) in absence (light) and in presence (dark) of SM16 laccase inducer. standard deviation <20%.

	5f	6f	18f	3o	9o	11o
5f	-	-	-	+	+	+
6f	-	-	-	+	-	+
18f	-	-	-	+	+	+
3o	+	+	+	-	-	-
9o	+	-	+	-	-	-
11o	+	+	+	-	-	-

Table 1.

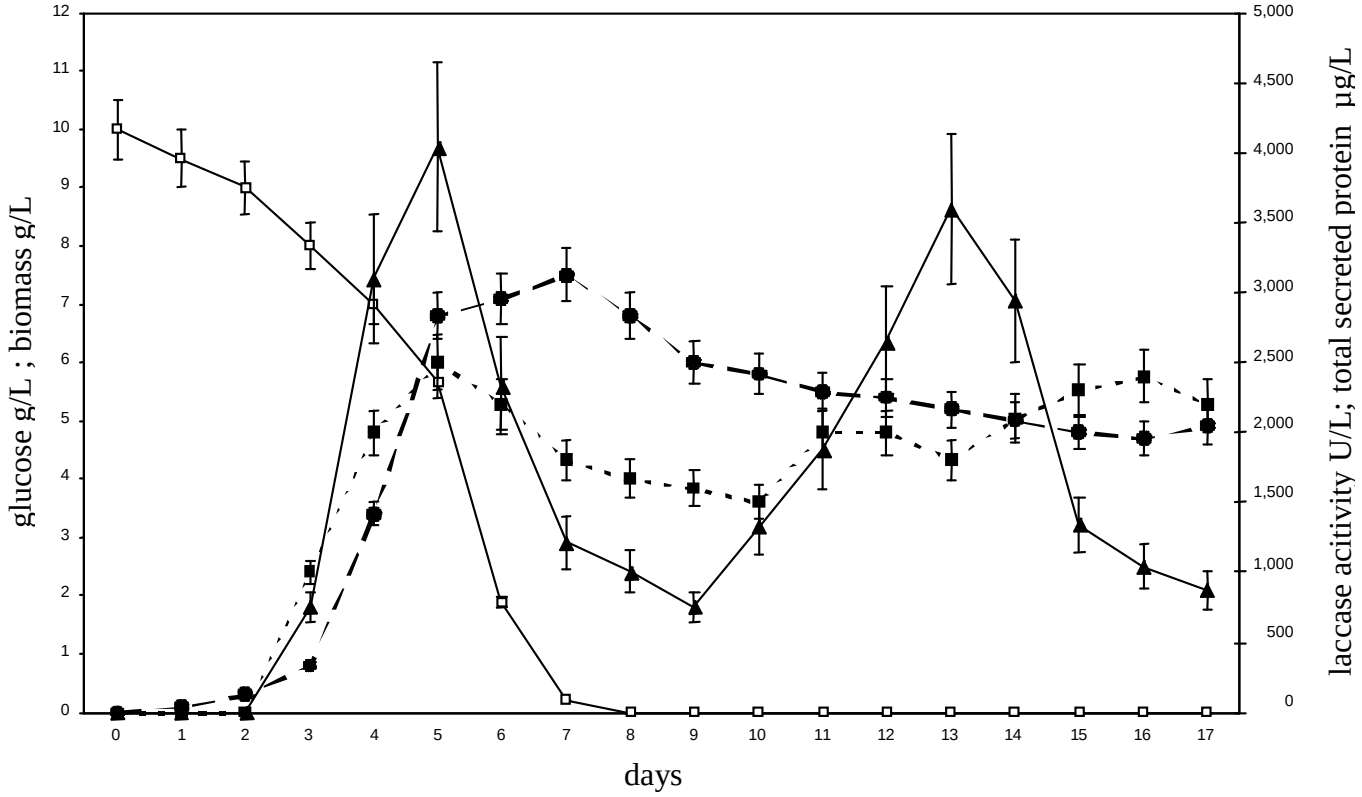

Figure 1.

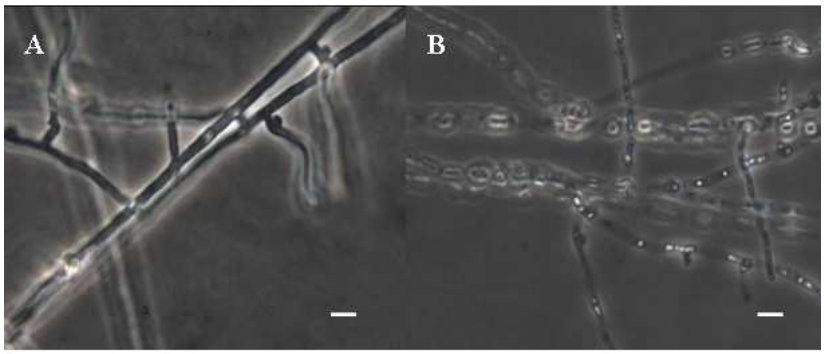

Figure 2.

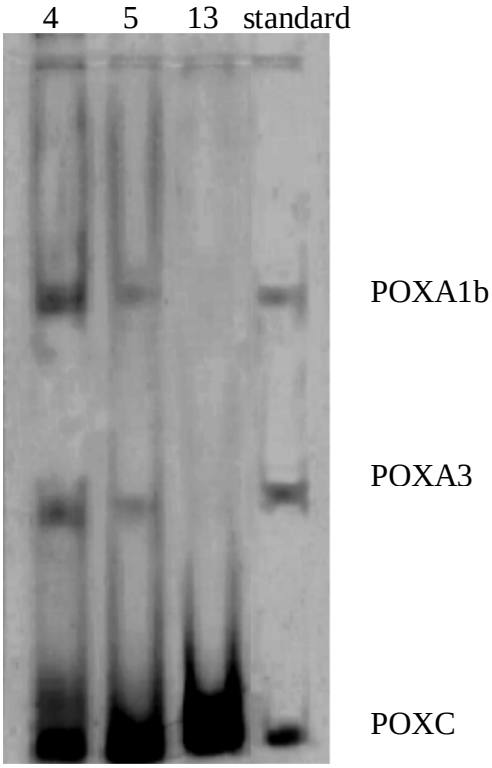
4
5
13
standard
POXA1b
POXA3
POXC

Figure3.

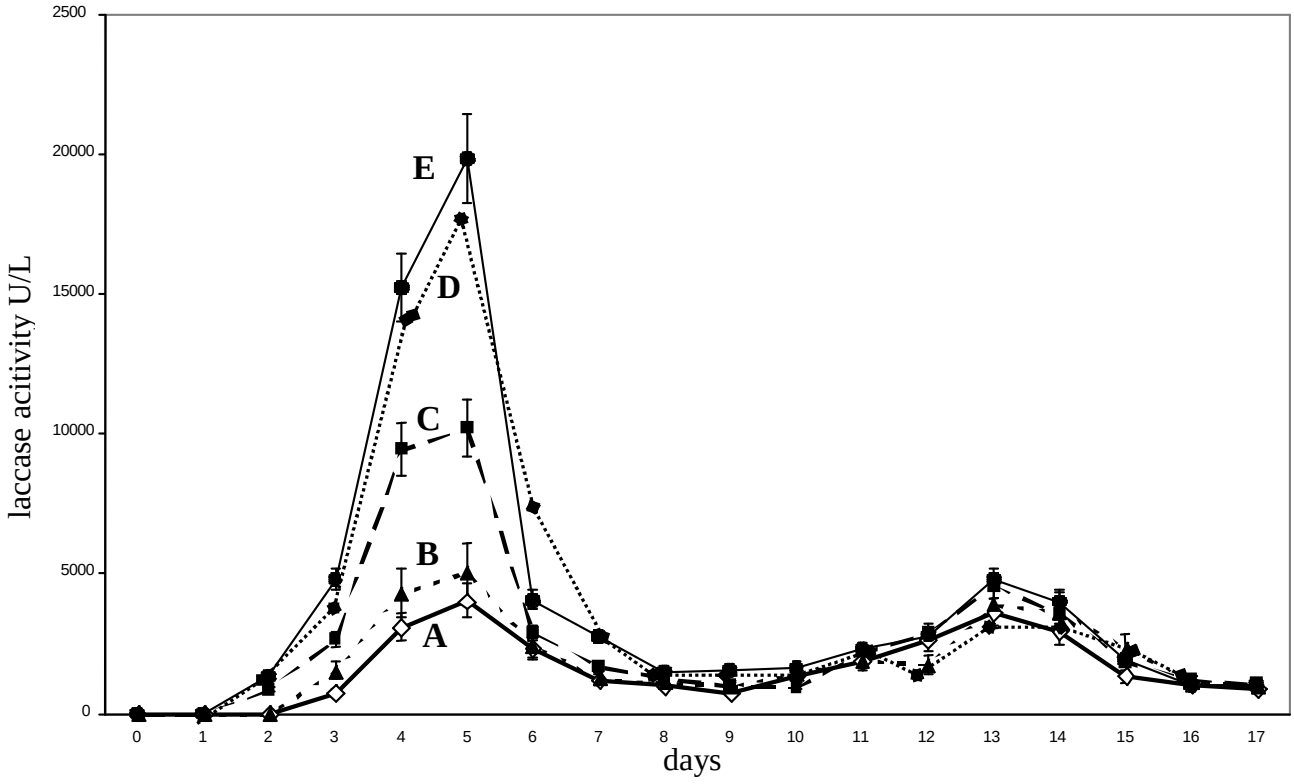
laccase acitivity U/L
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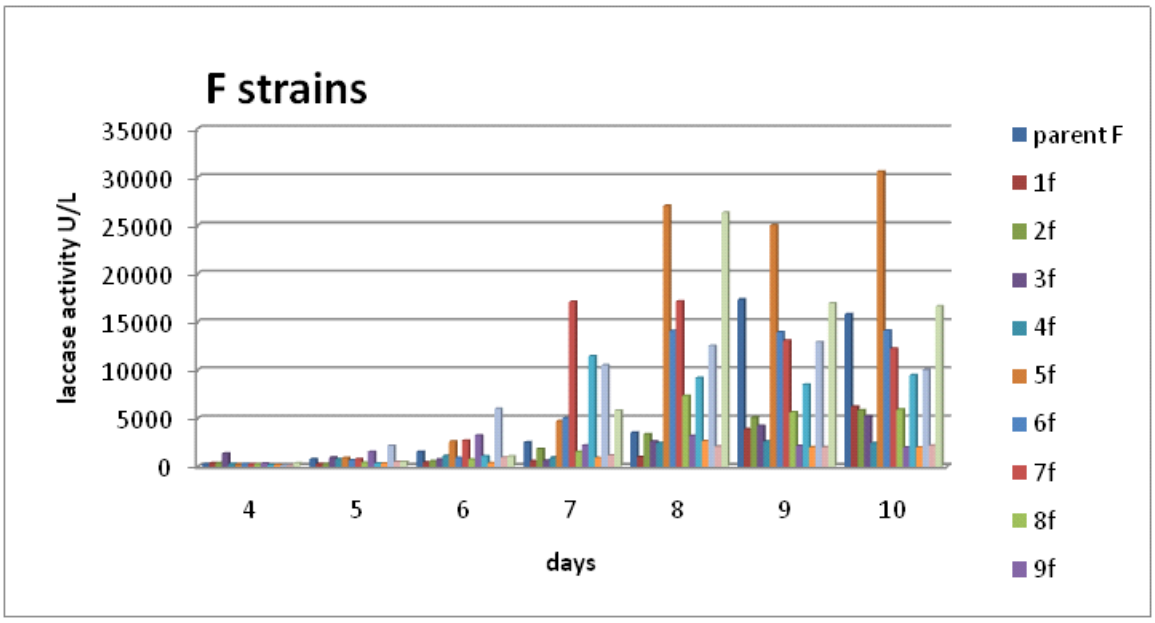
Figure4.



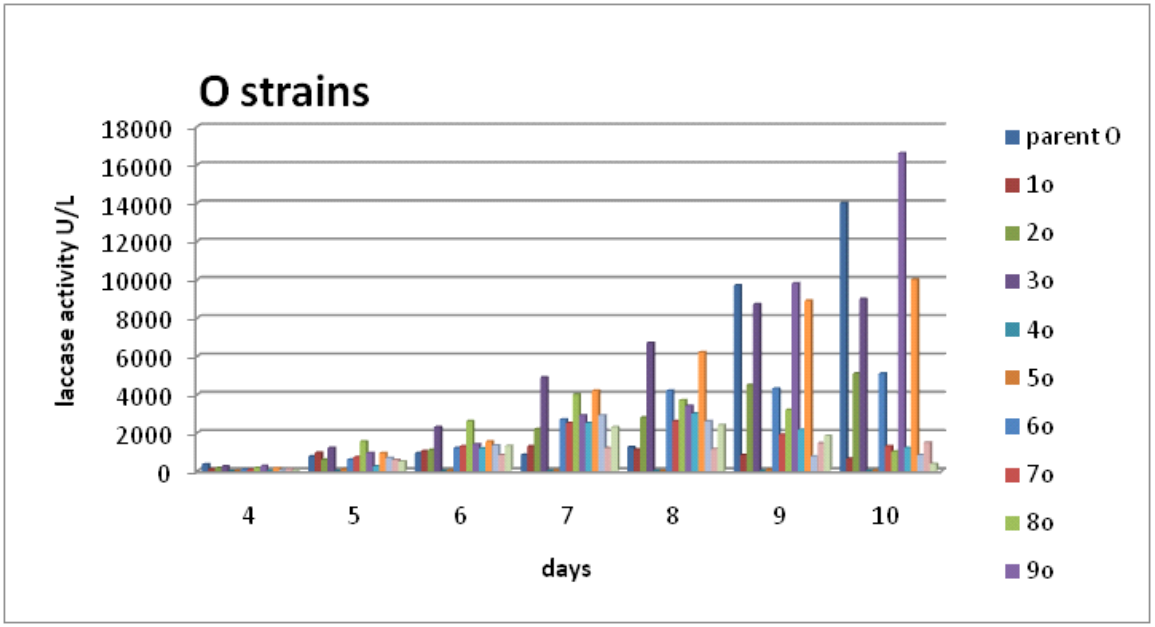


Figure5.

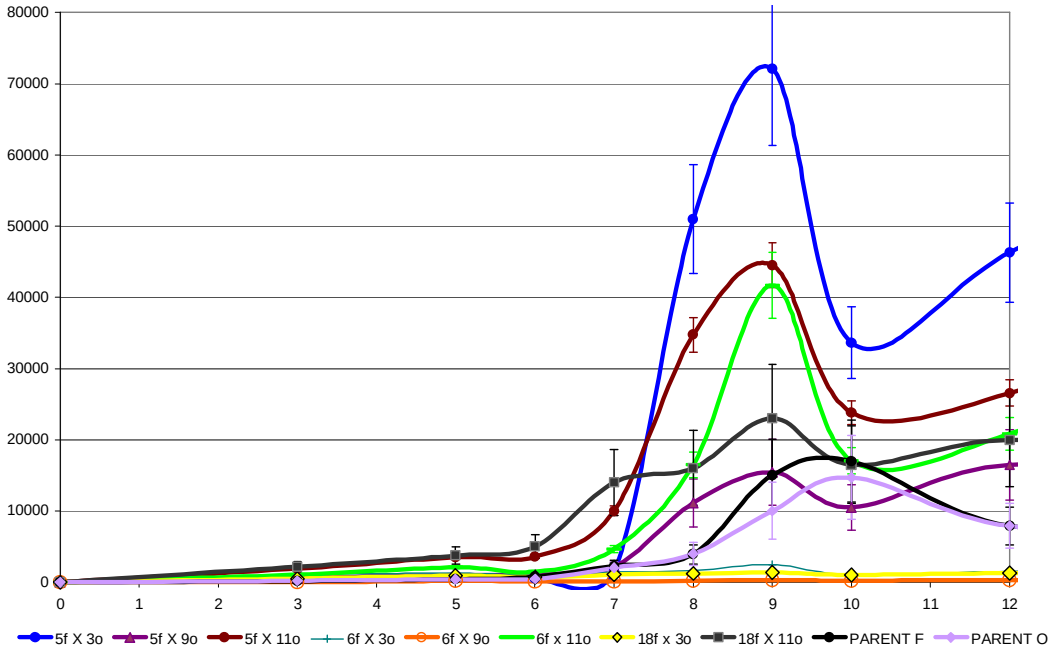


Figure6.

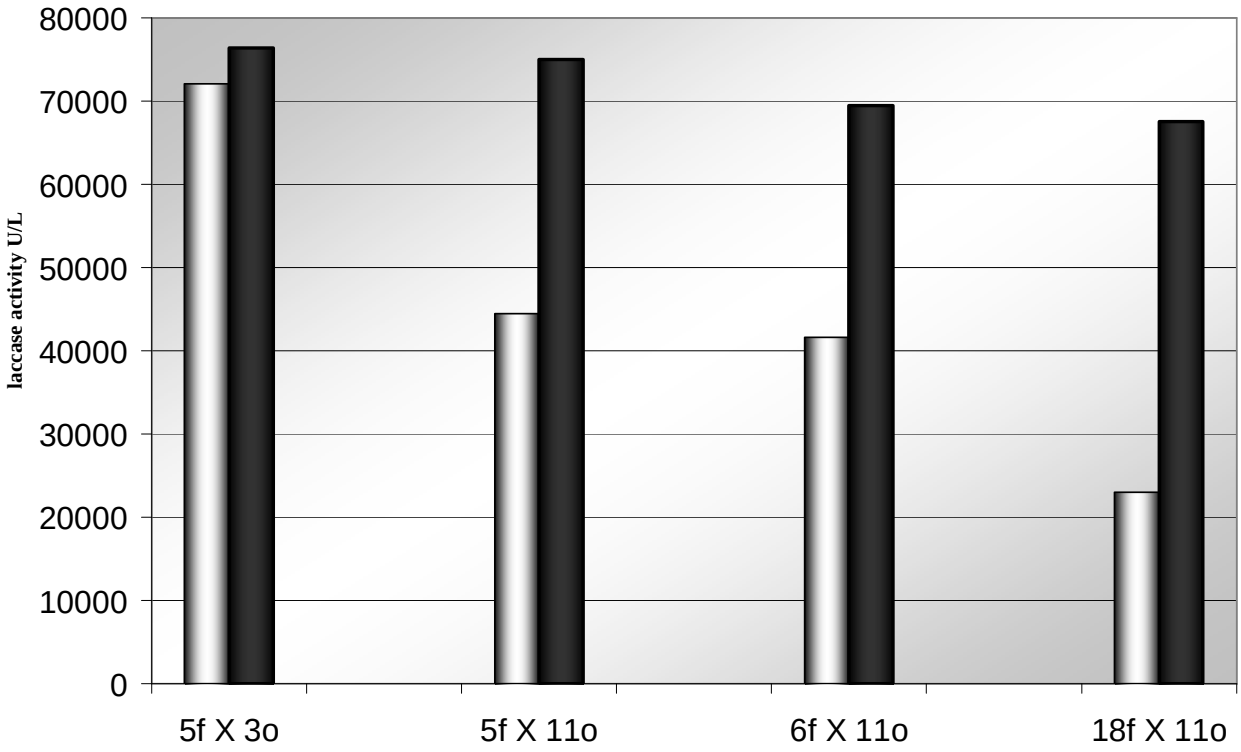


Figure7.

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Fungal laccases: versatile tools for lignocellulose transformation

Alessandra Piscitelli, **Claudia Del Vecchio**, Vincenza Faraco, Paola Giardina, Gemma Macellaro, Annalisa Miele, Cinzia Pezzella, and Giovanni Sannia

Dept. Organic Chemistry and Biochemistry, University of Naples Federico II, via Cinthia 4, 80126-Napoli, ITALY
Corresponding author : sannia@unina.it

ABSTRACT
Conversion of lignocellulosic materials to useful, high value products normally requires a pre-treatment step to degrade or loosen the recalcitrant and heterogeneous lignin fraction. The development of "green tools" for the transformation of lignocellulosic feedstocks is highly demanded for a sustainable exploitation of such resources. This multi-faceted challenge is being addressed by an ever-increasing suite of ligninolytic enzymes isolated from various sources. Among these, fungal laccases are known to play an important role in lignin degradation/modification processes.
The white-rot fungus *Pleurotus ostreatus* expresses multiple laccase genes encoding isoenzymes with different properties. The availability of established recombinant expression systems for *P. ostreatus* laccase isoenzymes has allowed to further enrich the panel of *P. ostreatus* laccases by the construction of mutated, ''better performing'' enzymes through molecular evolution techniques. New oxidative catalysts with improved activity and stability either at high temperature and at acidic and alkaline pH have been isolated and characterized.
Key words: High Redox Potential Laccases; Lignin; Directed Evolution; Industrial Application.

INTRODUCTION

Lignocellulose is a complex of carbohydrate polymers (cellulose and hemicellolose) tightly bound to lignin, and is a major constituent of a wide variety of materials including waste materials from agriculture, forestry, wood-based industries, and municipal solid waste (1). These materials are produced in abundance, and represent a good option for conversion to useful, high value products. Lignocellulose conversion requires a pre-treatment step to degrade or loosen the recalcitrant and heterogeneous lignin fraction. This multi-faceted challenge is being addressed by an ever-increasing suite of ligninolytic enzymes isolated from various sources. Among these, fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are known to play an important role in lignin degradation/modification processes. These enzymes can be successfully applied to paper manufacturing, enhancement of fibre properties, production of improved forages and pre-treatment of lignocellulosic biomasses for fuel production.
Biotechnology can contribute to the development of "green tools" for the transformation of lignocellulosic feedstocks by providing tailor-made biocatalysts based on the oxidative enzymes responsible for lignin attack in nature (1). With this purpose, laccases are currently being improved using (rational and random-based) protein engineering (2).
Laccase is one of the oldest enzymes reported and it is arousing great interest in the scientific community because of its very basic requirements (it just needs air to work and its only

released by-product is water) and huge catalytic capabilities, making it one of the ‘‘greenest’’ enzymes of the 21st century (3). This enzyme is produced by various fungi, plants, and certain bacteria or insects (4). Laccase is able to catalyze direct oxidation of *ortho* and *para*-diphenols, aminophenols, polyphenols, polyamines, and aryl diamines as well as some inorganic ions. It couples the four single-electron oxidations of the reducing substrate to the four electron reductive cleavage of the dioxygen bond, using four Cu atoms distributed against three sites, defined according to their spectroscopic properties. Typical metal content of laccase includes one type-1 (T1) copper, and one type-2 (T2) and two type-3 (T3) copper ions, with T2 and T3 arranged in a trinuclear cluster (TNC).

Fungal laccases exhibit a similar molecular architecture organized in three sequentially arranged cupredoxin-like domains. Each of them has a greek key β-barrel topology (4). T1 is located in domain 3, whilst the TNC cluster is embedded between domains 1 and 3 with both domains providing residues for copper coordination. The structure is stabilized by two disulfide bridges between domains 1 and 3 and between domains 1 and 2.

Laccases are commonly classified as low-medium and high redox potential laccases (HRPLs) according to their redox potential at the T1 site ranging from +430 mV in bacterial and plant laccases to +790 mV in some fungal laccases. The latter are by far the most important from a biotechnological point of view (5). HRPLs are typically secreted by ligninolytic basidiomycetes, the so-called white-rot fungi (3).

The white-rot fungus *Pleurotus ostreatus* expresses multiple laccase genes encoding isoenzymes with different properties, being the physiological significance of this multiplicity still unknown (6). Among these, POXA1b, in addition to its high redox potential (+650 mV) (7), is highly stable at high temperature and in the pH interval of 7 to 10 (8). Thus this enzyme is a suitable scaffold for directed evolution experiments, since the likelihood of achieving required improvements without affecting its stability is high.

This article reports the optimization of the functional properties of POXA1b laccase expressed in the yeast *Saccharomyces cerevisiae* (9). We integrate these results with a structural analysis of some of the generated mutants that allowed us to suggest some of the reasons, at a molecular level, for their enhanced activity.

EXPERIMENTAL

2.1 Strains, media, and plasmids

The *S. 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 plasmid used for *S. cerevisiae* expression was pSAL4 (copper-inducible *CUP1* promoter). *S. cerevisiae* was grown in selective medium (6.7 g l^{-1} yeast nitrogen base w/o amino acids and ammonium sulfate; 5 g l^{-1} casaminoacids; 30 mg l^{-1} adenine; 40 mg l^{-1} tryptophane; 50 mM succinate buffer pH 5.3; 20 g l^{-1} glucose).

2.2 Random mutagenesis

Random mutations were introduced with low, medium, and high frequency of mutation, into the

POXA1b encoding cDNAs using GeneMorphTM PCR Mutagenesis Kit (Stratagene, La Jolla, CA). EP-PCR was performed with primers POXA1bfw (ATAAAAGCTTGAATTCATGGCGGTTGCATTCG) and POXA1brev (TAAGGATCCAAGCTT TTATAATCATGCTTC).

2.3 Construction of mutant library

The cDNA resulting from EP-PCR on *poxa1b* cDNA were cloned in pSAL4 expression vector, digested with SmaI and BglII restriction enzymes, by using homologous recombination expression system of *S. cerevisiae*. Yeast transformation and selection was performed as already reported (10).

2.4 Library screening

Single clones grown on plate were picked and transferred into 96-well plates containing 30 µL of selective medium per well. Plates were incubated at 28°C, 250 rpm for 24 h. After 24 h, 130 µL of selective medium 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 1500 g, 4°C, and a suitable volume of supernatant was transferred to a new 96-well plate to perform laccase assay. Phenol oxidase activity was assayed at 25°C using 2 mM 2,2' -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε 36,000 M^{-1} cm^{-1}), using Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA). Enzyme activity was expressed in international units (U). Cultures in shaken flasks were also performed. Pre-cultures (10 mL) were grown on selective medium at 28°C on a rotary shaker (150 rpm). A volume of suspension sufficient to reach a final 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. Optical density and laccase activity determination were daily assayed.

2.5 Screening of library for stability

The collection of 3300 mutants obtained by random mutagenesis of POXA1b laccase was analyzed in three different screenings. First and second screening were effectuated in 96-well plate, while the third screening was effectuated in shaken flask. In the first screening the library was analyzed after one day growth in 96-well plate. The supernatant was incubated for 48 hours at pH 3 in Robinson and Britton buffer, and then activity towards ABTS assayed. The positive clones were further analyzed during a three days growth in 96-well plate. Cellular density and laccase activity production were followed for three days. Every day the supernatant was incubated for 96 hours at 37°C at pH 3 in Robinson and Britton buffer. Laccase activity was analyzed every 24 hours, in this phase only clones that showed greater stability if compared to the wild-type enzyme at pH 3 were selected.

2.6 Molecular modelling

The structure of POXA1b was obtained by homology modelling from the crystal structure of *Trametes versicolor* (1GYC pdb entry), with which it shares 60% sequence identity. The last 16 residues of POXA1b were modelled using the coordinates of the corresponding residues at the C-terminus of the crystal structure of the *Melanocarpus albomyces* laccase (1GWO pdb entry). 3D model and *in silico* mutants were generated using the SWISS-MODEL web server by means of the project mode option that allow to select the template and control the gap placement in the alignment. Refinement of the models has been performed by molecular dynamics simulations. Simulations on the wild-type POXA1b and on the *in silico* generated mutants 1M9B and 3M7C were performed with the GROMACS package as already described (10).

2.7 Stability assays

Stability at pH values was measured using citrate-phosphate buffer adjusted at pH 3, 5 and 7.0, and Tris–HCl buffer adjusted at pH 10.

2.8 r4 cDNA construction

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

2.9 DNA sequencing

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

2.10 Decolourization experiments

Batch decolourization experiments have been performed incubating (1 mL final volume of reaction) crude preparation of laccase containing different enzyme amounts (0.1 U, 1 U and 3 U) in the four wastewater models, prepared as already described (11). Performances of selected laccases in model wastewater decolourization were evaluated by recording light absorption spectra between 280 and 800 nm at different times (10 min, 20 min, 1 h, 2 h, 3 h e 24 h), and comparing them with the corresponding spectra of controls (the waste incubated with the supernatant of yeast cultures transformed with the empty expression vector). Decolourization was calculated as the extent of decrease of spectrum area recorded between 380 and 740 nm with respect to a control sample. All spectra were recorded after 1:100 dilution of the sample in milliQ water. All experiments were carried out in duplicates, and the mean values were taken. The standard deviation for the experiments was less than ±5%.

RESULTS AND DISCUSSION

The white-rot fungus *P. ostreatus* expresses multiple laccase genes encoding isoenzymes with different properties, being the physiological significance of this multiplicity still unknown (6). Investigation of the recently released *P. ostreatus* genome (http://www.jgi.doe.gov/sequencing/why/50009.html) has disclosed a complex multicopper oxidase family of up to twelve members. The availability of established recombinant expression systems for *P. ostreatus* POXA1b (9) has allowed the improvement of enzymes features through a combination of rational and random mutagenesis (10, 12, 13). Our starting point was the high redox potential laccase POXA1b (14), which exhibits remarkable stability at alkaline pHs (8). The idea behind the evolution strategy is to create an ideal biocatalyst, able to oxidise a wide assortment of substrates, and stable in a broad range of pHs. Thus, different screening criteria were applied to search for such catalysts.

3.1 First generation

A library of almost 1100 mutants with low, medium and high range of mutations was generated by error-prone PCR (EP-PCR) using *poxa1b* cDNA as template (10). Screening this library for variants with improved activity at pH 3 using the non-phenolic substrate ABTS has allowed the selection of one mutant, named 1M9B. It shows a single mutation (L112F) leading to an improvement of activity but a decrease of stability with respect to the wild-type enzyme (POXA1b) in all the analyzed conditions. In position 112 there is a generally conserved leucine in all laccases from basidiomycetes, although a phenylalanine seems to be conserved in laccase sequences from ascomycetes. POXA1b 3D model shows that the residue 112 is located in the channel through which the solvent has access to the oxygen-reducing T2/T3 site. To elucidate the role played by this mutation, Molecular Dynamic (MD) simulations were performed on the model of the mutant and compared with those of POXA1b. The analyses show a movement of the subdomain around position 112 as a consequence of a conformational rearrangement due to the presence of the bulkier residue of phenylalanine. A significant effect generated by the mutation is observed in the permeability to water of the T2/T3 channel. Residue F112 is located at the entrance of the channel and its steric hindrance affects the passage of water molecules toward the TNC. As a fact, a larger number of water molecules in the T2/T3 channel has been observed for 1M9B. These data could suggest an increased affinity of this mutant toward oxygen molecules, thus justifying its improved specific activity.

3.2 Second generation

1m9b cDNA was used as template for a second round of EP-PCR at low and medium frequency of mutation (10). A second generation library of 1200 clones was obtained and screened using the same *criterium* described before. Three mutants, 1L2B, 1M10B (L112F,

K37Q, K51N), and 3M7C (L112F, P494T), were selected showing an activity increase of up to three fold with respect to POXA1b.
Concerning 1M10B mutations, it finds out how directed evolution can get the same result of natural evolution, preserving the properties of mutated aminoacids. As a fact, positions 37 and 51 are generally occupied by amidic residues. The mutant 3M7C displays an high activity and an up to two fold increased stability at acidic and neutral pH, as well as at high temperature. 1M10B variant is more stable at alkaline pH (about two fold), whereas its stability is almost equivalent to that of POXA1b in the other tested conditions. The mutation P494T is located in the C-terminal loop that has already been ascertained to affect the function of fungal laccases (4). MD simulations of this mutant and comparison with the wild-type POXA1b revealed a lower flexibility of the subdomain around position 112 probably responsible of its higher stability. On the other hand, an increased mobility of loops forming the reducing substrate binding site, has been observed in 3M7C leading to higher accessibility of water molecules to the T1 copper site, and to an increased activity of the enzyme.
First and second generation libraries (2300 clones) were then screened for variants with improved activity at pH 5 using either the non-phenolic substrate ABTS, and the phenolic one 2,6-dimethoxyphenol (DMP).
When DMP was used as substrate, two new variants (2L4A and 3L7H) endowed with higher enzyme activity (about three fold increase) than the wild-type laccase were selected (12). Both mutants doubled the stability of the wild-type enzyme at pH 5. Q272H mutation found in 2L4A may stabilize the protein structure at pH 5 allowing additional interactions– electrostatic and hydrogen bonds– between the positively charged imidazolic ring of His272 and the side chain of Asp287.
After screening with ABTS, one clone, 1L9A, was selected, showing an increase of about three fold with respect to wild-type. Besides the parental mutation (L112F), 1L9A also presents the mutation R284H, located in the loop Gly282-Thr289. This loop may play an important role in protein stability (12). As a fact, concerning its properties, the mutant increases stability at pH 5 (1.5 fold), while loosing the high characteristic stability of POXA1b at alkaline pH.

3.3 Rational design

During the engineering of POXA1b, some of the beneficial mutations discovered in the early stages of evolution were merged in the rational designed R4 mutant. Synthesis of a laccase joining mutations of 3M7C and 1M10B variants (10) was performed to combine the increased stability of 1M10B at alkaline pH and the improved catalytic efficiency of 3M7C (13). Joining these mutations a two fold increase in laccase activity with respect to wild-type enzyme was obtained. The main improvement due to the chimer construction is a slight increase in stability at high temperature, and even more at neutral (about four fold) and alkaline pH values (about two fold).

3.4 Third generation

The increased stability of R4 makes it an appropriate scaffold to carry out directed evolution (15). In fact, more stable enzymes should also be more susceptible to evolution since they have higher ability to tolerate functionally beneficial but destabilizing mutations. Therefore, directed evolution of R4 was chosen as strategy to improve its performances (13). A library of 1000 clones with low, medium and high average of mutation frequency was obtained through EP-PCR. When this new collection was screened by assaying activity towards ABTS at pH 3, two mutants, 4M10G and 1H6C, with higher activity (about four fold increase) than that of POXA1b wild-type were selected. Both mutants display higher stability than POXA1b at pH 5 (almost four fold). 1H6C also retains R4 stability features at pH 10 and at pH 7. Sequence analyses of the selected mutants led to the identification of the mutations V126I for the 4M10G variant and V148L for the 1H6C mutant, besides those of the parental R4 enzyme.

Position 148 is located in a closely packed region of the domain 2 (16) adjacent to the reducing substrate binding site. The substitution of V with the larger L causes a close contact of the leucine side-chain with the aromatic ring of Y208, thus changing the conformation of the loop 204–208 forming the bottom of the reducing substrate binding site (16). This change could, in turn, influence the oxidation rate of the reducing substrate but also the interaction between the domains 2 and 3 (13).

Libraries generated after three rounds of molecular evolution (3300 clones) were then screened for variants with improved activity at pH 8 using the phenolic substrate 2,6 DMP. One variant, 1L10A, was selected for its higher activity (more than four fold increase) at pH 8 respect to wild-type POXA1b . This mutant loses wild-type stability at pH 7, while raises stability at pH 5 of three fold.

To complete the panel of laccase-based biocatalysts, new selection rounds have been performed on POXA1b mutants libraries. This step has been aimed to develop new laccases more stable than the wild-type at pH 3. One mutant, 3L2A, with higher stability (up to three fold increase) was selected. This selected variant shows an increased stability at neutral and alkaline pH respect to wild-type, besides the higher stability at pH 3.

3.5 Application of improved laccases

Laccase improvement, along with elucidation of their structure-activity-stability relationships allows to design and develop more suitable systems for specific industrial applications either in the bioremediation field or for lignocellulose conversion, increasing their real exploitation. An ideal catalyst should be stable and active in extreme conditions of pH, normally found in the industrial wastewaters, as well as able to operate on a wide range of substrates. Here we describe, as an example, the application of mutated laccases to the decolourization of industrial wastewaters. In these experiments, models of acid, direct and reactive dye wastewaters from textile industry -defined on the basis of discharged amounts, economic relevance and representativeness of chemical structures of the contained dyes (11)- were used. POXA1b variants represent good candidates thanks to their properties best suited for wastewater conditions. Seven out twelve of selected POXA1b variants show an increased decolourization ability with respect to wild-type toward the acid model. As a fact, a two fold increase in decolourization percentage respect to that of POXA1b has been obtained. In particular the mutant 3M7C shows an higher decolourization capacity (up to 40% decolourization after 3 hours of incubation). No significant decolourization has been obtained towards the others analyzed wastewaters, such as reactive and direct models.

3.6 Conclusion

Although some laccases are being employed successfully in industry, no natural laccase combines the desired attributes of being stable and active over a wide range of temperatures and pH values with a high reduction potential (17). High-potential laccases represent a starting point to create an ideal catalyst, endowed with all these desirable characteristics, through directed evolution experiments.

In this research, three generation of libraries have been screened using different criteria, and twelve variants have been selected for their improved features (Table 1). Although no *a priori* selection criterium for “more stable laccase” has been applied, some mutations with beneficial impacts on total enzyme activity also significantly contributed to protein stability in different environmental conditions. This work represents an example of how random and rational approaches can be combined for the engineering of protein function, and how such a strategy could provide an inventory (Fig. 1) of enzymes better-suited for different industrial applications.

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REFERENCES

[1] A.T Martìnez, F.J. Ruiz-Dueñas, M.J. Martìnez, J.C del Rìo, A. Gutiérrez, Enzymatic delignification of plant cell wall: from nature to mill, Curr Opin Biotechnol 20 (2009) 348–357.

[2] A. Piscitelli, C. Pezzella, P. Giardina, V. Faraco, G. Sannia, Heterologous laccase production and its role in industrial applications Bioengineered Bugs 1 (2010) 254 –264.

[3] D. Maté, C. Garcìa-Burgos, Eva Garcìa-Ruiz, A.O. Ballesteros, S. Camarero, M. Alcalde, Laboratory evolution of high-redox potential laccases, Chem Biol 17 (2010) 1030–1041.

[4] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, G. Sannia, Laccases: a never-ending story, Cell Mol Life Sci 67 (2010) 369–385.

[5] M. Alcalde, Laccase: biological functions, molecular structure and industrial applications, in: J. Polaina, A.P. MacCabe (Eds.), Industrial enzymes: structure, functions and applications. Dordrecht, The Netherlands: Springer, 2007, pp. 459–474.

[6] C. Pezzella, F. Autore, P. Giardina, A. Piscitelli, G. Sannia, V. Faraco, The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members, Curr Genet 55 (2009) 45–57.

[7] A.M. Garzillo, M.C. Colao, V. Buonocore, R. Oliva, L. Falcigno, M. Saviano, A.M. Santoro, R. Zappala, R.F. Bonomo, C. Bianco, P. Giardina, G. Palmieri, G. Sannia, Structural and Kinetic Characterization of native laccases from *Pleurotus ostreatus*, *Rigidoporus lignosus*, and *Trametes trogii*, J Protein Chem 20 (2001) 191–201.

[8] P. Giardina, G. Cennamo, C. Greco, G. Palmieri, G. Sannia, Protease mediated processing of a Cu-induced laccase in *Pleurotus ostreatus*: a natural approach to improve protein stability, Prog Biotechnol 21 (2002) 113–120.

[9] A. Piscitelli, P. Giardina, C. Mazzoni, G. Sannia, Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, Appl Microbiol Biotechnol 69 (2005) 428–439.

[10] G. Festa, F. Autore, F. Fraternali, P. Giardina, G. Sannia, Development of new laccases by directed evolution: functional and computational analyses, Proteins 72 (2008) 25–34.

[11] V. Faraco, C. Pezzella, A. Miele, P. Giardina, G. Sannia, Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes, Biodegradation 20 (2009) 209–220.

[12] A. Miele, P. Giardina, G. Sannia, V. Faraco, Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation, J Appl Microbiol 108 (2010) 998–1006.

[13] A. Miele, P. Giardina, E. Notomista, A. Piscitelli, G. Sannia, V. Faraco, A semi-rational Aapproach to engineering laccase enzymes, Mol Biotechnol 46 (2010) 149–156.

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

[15] J.D. Bloom, S.T. Labthavikul, C.R. Otey, F.H. Arnold, Protein stability promotes evolvability, Proceedings of the National Academy of Sciences of the United States of America 103 (2006) 5869–5874.

[16] T. Bertrand, C. Jolivalt, P. Briozzo, E. Caminade, N. Joly, C. Madzak, C. Mougin, Crystal structure of a four-copper laccase complexed with an arylamine: Insights into substrate recognition and correlation with kinetics, Biochemistry 41 (2002) 7325–7333.

[17] C.J. Rodgers, C.F. Blanford, S.R. Giddens, P. Skamnioti, F.A. Armstrong, S.J. Gurr, Designer laccases: a vogue for high-potential fungal enzymes? Trends Biotechnol 28 (2010) 63–72.

Figure legends

Fig. 1 Ribbon representation of POXA1b model. The mutated residues are in licorice rendering. Trinuclear coppers and T1 copper are highlighted in van der Waals representation (yellow and blue, respectively).

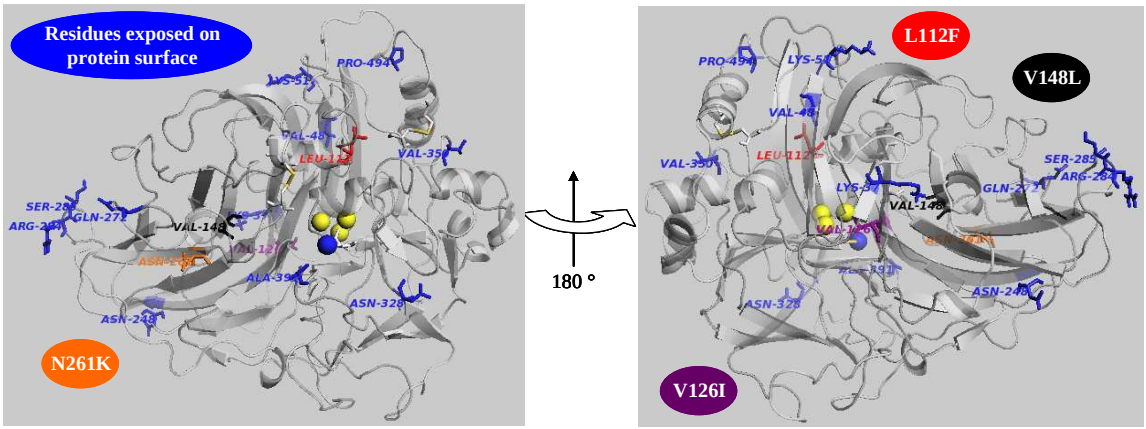

Fig. 1

Table 1. Properties of selected POXA1b variants.

Mutants	Substitution	Activity	Stability				
			pH3	pH5	pH7	pH10	60°C
1M9B	L112F	1.5 x	-	-	-	-	-
1L2B	L112F, N248Y, N261K, V350I	2.5 x	=	+	-	=	=
1M10B	K37Q, K51N, L112F	2.5 x	=	+	=	++	=
3M7C	L112F, P494T	3 x	+	++	++	=	+
2L4A	L112F, Q272H	2.7 x	-	++	=	=	=
3L7H	L112F, S285N, N328S	2.7 x	-	++	-	-	=
1L9A	L112F, R284H	3 x	-	+	=	-	=
R4	K37Q, K51N, L112F, P494T	2.5 x	-	=	+++	++	+
1H6C	K37Q, K51N, L112F, P494T, V148L	4.5 x	-	+++	+++	++	+
4M10G	K37Q, K51N, L112F, P494T, V126I	4.5 x	-	++	++	+	=
1L10A	K37Q, V48I, K51N,A391T	4.5 x	-	+++	-	+	=
3L2A	K37Q, K51N, L112F, S285N	3 x [a]	++	=	+++	++	=

[a] this value represent an increase of stability at pH3 with respect to the wild-type enzyme