# DEVELOPMENT OF OXIDATIVE BIOSYSTEMS FOR THE TREATMENT OF INDUSTRIAL COLOURED WASTEWATERS

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*How to climb mountains:*
*Choose the mountain you want to climb*
*Find out how to reach the mountain*
*Learn from someone who has been there before*
*Dangers, seen from close to, are controllable*
*The landscape changes, so make the most of it*
*Respect your body*
*Respect your soul*
*Be prepared to go the extra mile*
*Be joyful when you reach the top*
*Promise yourself to discover another mountain*
*Tell your story*
*P.Coelho*

*Alla mia famiglia e ad Enzo*

## INDEX

## SUMMARY

The increasingly stringent environmental regulations on hazardous wastes has 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.
The present research program explores new strategies for the bio-treatment of dye-contaminated wastewaters produced by textile industries. Two kind of processes have been specifically analysed: biodegradation and biosorption based processes. Their effectiveness was tested on three textile wastewater models (Acid, Direct, Reactive), by assessing and comparing their performances in terms of decolourisation ability, COD and toxicity reduction of the treated effluent.
The inactivated biomasses of three Mucorales fungi have been tested as potential candidates for dye biosorption. They displayed good sorption capabilities towards all the tested synthetic effluents, giving rise to decolourisation percentages up to 94% and decrease in COD up to 58%, coupled to a significant toxicity reduction.
Biodegradation based processes, have been explored from a multiplicity of perspectives, by testing the degrading performances of whole-cell as well as enzyme-based biosystems, in both free and immobilized forms. The white-rot fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium* and their oxidative enzymes were the object of this investigation. Both fungi showed good decolourisation and detoxification capabilities towards the model wastewaters, exhibiting different specificities. As far enzymatic biosystems, the study has been specifically focused on laccases from *P. ostreatus*. Optimal conditions for laccase production by *P. ostreatus* have been defined and an extra-cellular enzyme mixtures, with known laccase isoenzyme composition, was produced and tested for its decolourisation potential, showing the best performances toward Acid wastewater model.
The same laccase preparation was successfully immobilized on perlite, a siliceous inert carrier, and preliminary tested for the conversion of the reactive dye Remazol Brilliant Blue R. The immobilization process was optimized with reference to the immobilization yield and to the dye adsorption capacity of the solid biocatalyst. Stability and catalytic parameters of immobilized laccases, in comparison with those observed for free enzyme, were also assessed.

A parallel session of the project was aimed at identifying and at characterizing new members of *P. ostreatus* laccase gene family. This research pathway was ultimately targeted at enriching the knowledge of this complex enzymatic family as well as at developing new bio-catalysts for wastewater treatment. The existence of a laccase gene clustering was demonstrated in *P. ostreatus* and three new laccase genes were cloned, thus enlarging the panel of these biocatalysts up to seven members. A “laccase subfamily” consisting of three laccase genes was identified. cDNAs coding for two of the new laccases were isolated and expressed in yeasts in order to characterize the recombinant proteins. A peculiar intron-exon structure was revealed for one of the new laccases, along with a high instability of the recombinant enzyme due to lability of its copper ligand.

## *RIASSUNTO*

I coloranti sintetici sono ampiamente utilizzati in diversi settori dell'industria, in quello tessile in particolare. La produzione mondiale annua è infatti stimata intorno alle 70.000 tonnellate, di cui una percentuale variabile tra il 10 ed il 40% è rilasciata nelle acque di scarico dell'industria. L'impatto sull'ambiente di tali scarichi è notevole: molti dei convenzionali metodi di trattamento chimico-fisici si rivelano, infatti, inadeguati o troppo costosi se rapportati alla complessa struttura chimica delle molecole coloranti e agli ingenti volumi da trattare. In questo contesto, il ricorso al biorisanamento costituisce una tecnologia valida, economicamente sostenibile, e a ridotto impatto ambientale da applicare in alternativa ai tradizionali processi di smaltimento.
Due differenti strategie di biorisanamento possono essere applicate al trattamento dei reflui industriali: il bioassorbimento e la biodegradazione.
Il bioassorbimento consiste nella rimozione delle sostanze inquinanti presenti nei reflui da parte di biomasse microbiche, vive o inattivate. Alcuni funghi, grazie alla peculiare composizione chimica e strutturale della loro parete cellulare si sono rivelati particolarmente efficaci nell' assorbire sostanze tossiche 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.
La biodegradazione sfrutta, invece, il naturale potenziale degradativo di diverse specie microbiche (batteri, lieviti, funghi), per la rottura e la successiva trasformazione (ed eventuale completa mineralizzazione) delle sostanze coloranti. 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 -tra cui lignina perossidasi, manganese perossidasi e laccasi- i quali, per la loro bassa specificità di substrato si rivelano in grado di degradare diverse classi di inquinanti, inclusi molti coloranti sintetici.

Entrambe le strategie di biorisanamento sono state esaminate in questo progetto di tesi. Particolare attenzione è stata rivolta ai processi di biodegradazione dei reflui, esplorandone diversi aspetti: dall'impiego di biosistemi microbici (funghi white-rot *P. ostreatus* e *P. chrysosporium*) a quelli enzimatici -in forma libera o immobilizzata-, estendendo lo studio alla ricerca di nuovi geni codificanti attività ossidasiche nel fungo *P. ostreatus*.
Il presente progetto di tesi si articola pertanto in due linee di ricerca : 1) Lo sviluppo e l'applicazione di nuovi biosistemi da applicare al trattamento dei reflui colorati e 2) L'isolamento di nuovi membri della famiglia della laccasi da *P. ostreatus* e la loro caratterizzazione mediante espressione eterologa.

**1) Sviluppo e applicazione di nuovi biosistemi per il trattamento dei reflui colorati**

Questa sezione del progetto mira allo sviluppo di biosistemi per il risanamento dei reflui dell'industria del colore, basati su catalizzatori microbici ed enzimatici. Comune denominatore di questa sezione è l'analisi delle *performance* degradative dei biosistemi in esame in condizioni prossime a quelle dei reflui reali. In una fase preliminare del progetto (nell'ambito del progetto europeo SOPHIED-Sustainable Oxidative Processes for Healthy Industrial European Dyes-) sono stati infatti definiti tre reflui modello (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, nonchè per la struttura e la reattività dei coloranti contenuti. Questi in sintesi i risultati ottenuti in relazione ai vari biosistemi analizzati:

- *Biosistemi microbici per la biodegradazione dei reflui modello*

Le potenzialità applicative dei funghi *white-rot P. ostreatus* and *P. chrysosporium* sono state analizzate impiegandoli quali catalizzatori microbici del processo di decolorazione. Colture in liquido dei due funghi sono state allestite in ciascuno dei reflui modello, in assenza ed in presenza di nutrienti, monitorando i parametri chiave del processo: percentuale di decolorazione, tossicità e COD (Chemical Oxygen demand) residua. *P. ostreatus* si è rivelato efficace nel decolorare il refluo Acid producendo un 54% di decolorazione dopo 7 giorni di trattamento in assenza di nutrienti, e una riduzione del 30% della COD. In presenza di nutrienti, il livello di decolorazione raggiunge il 66%, sebbene in questo caso non sia accompagnata da una riduzione della COD. La tossicità dei reflui al termine del trattamento è stata valutata con il sistema LUMISTOX. Tale sistema utilizza il batterio *V. fisheri* quale organismo test e si basa sulla misura dell'inibizione della naturale luminescenza del batterio indotta da sostanze tossiche. In entrambi i casi, il trattamento con il fungo comporta una significativa detossificazione del refluo. Inoltre, l'analisi delle attività ossidasiche secrete nel mezzo di coltura nel corso del trattamento, rivela la produzione di livelli costanti di attività laccasica, suggerendo il coinvolgimento delle laccasi nel processo di decolorazione.

Il trattamento con il fungo *P. chrysosporium* si è rivelato invece efficace sul refluo Direct, producendo un 46% di decolorazione dopo un solo giorno di trattamento e fino all'87% di decolorazione dopo 7 giorni. Il trattamento risulta efficace solo in presenza di nutrienti e non è accompagnato dalla riduzione della COD. Non è stato possibile, in questo caso, correlare la decolorazione osservata alla produzione di specifiche attività ossidasiche potenzialmente coinvolte nel processo, a causa dell'interferenza nelle misure di attività enzimatica prodotta dalla colorazione residua del refluo. Per lo stesso motivo, non è stato possibile valutare, con il sistema LUMISTOX, la tossicità residua del refluo Direct trattato con *P. chysosporium* data la scarsa riproducibilità dei risultati ottenuti.

Il trattamento con entrambi i funghi si è rivelato invece inefficace sul refluo Reactive, probabilmente a causa di fattori quali, le condizioni di pH estreme, l'elevata concentrazione salina o l'eccessivo contenuto in coloranti, che inibiscono la crescita del fungo.

I risultati ottenuti, oltre a dimostrare le potenzialità di applicazione dei biosistemi microbici esaminati, evidenziano le diverse specificità di degradazione dei due funghi, suggerendo, quale prospettiva futura, l'idea di impiegarli in co-colture, al fine di combinarne proprietà e potenzialità.

*Biosistemi enzimatici in fase libera: laccasi da P. ostreatus*

Nell'ambito dello sviluppo di biosistemi enzimatici, lo studio è stato incentrato su una classe di enzimi ossidativi particolarmente interessante dal punto di vista applicativo, le laccasi fungine. Le laccasi sono 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. Esse trovano svariate applicazioni biotecnologiche: dall'industria alimentare o della carta, al settore tessile, fino ai processi di biorisanamento dei reflui.

In molti funghi le laccasi sono codificate da complesse famiglie di geni, in numero variabile da specie a specie, che danno origine a proteine strettamente relazionate ma dotate spesso di proprietà catalitiche differenti. Il fungo basidiomicete *Pleurotus ostreatus* produce una vasta gamma di isoforme enzimatiche ad attività laccasica, di cui sono stati isolati i corrispondenti geni e cDNA. Cinque isoenzimi sono stai finora purificati e caratterizzati: POXC, il più abbondantemente prodotto in tutte le condizioni di crescita analizzate; POXA1b, dall'insolita stabilità a pH alcalino; POXA1w, particolare per il suo contenuto in ioni metallici; ed infine gli isoenzimi POXA3a e POXA3b, atipici per la loro struttura eterodimerica e particolarmente efficaci nella decolorazione del colorante modello, RBBR.

Diverse colture del fungo *P. ostreatus* sono state allestite al fine di individuare le condizioni ottimali per la produzione di laccasi. La miscela laccasica prodotta nelle migliori condizioni selezionate (130,000 IU/L) è stata caratterizzata per il suo contenuto isoenzimatico ed applicata alla decolorazione dei reflui modello. La stessa, si è rivelata efficace nel degradare il refluo Acid, producendo fino al 35% di decolorazione in 24h. Il tempo necessario ad ottenere lo stesso grado di decolorazione si riduce ad 1h, incrementando di 10 volte la concentrazione di enzima, fino a 1IU/ml. La decolorazione è accoppiata ad una riduzione del 30% della COD e ad una significativa detossificazione del refluo. Il trattamento con la miscela laccasica si è rivelato, invece, poco efficace sui reflui Direct e Reactive. Una parziale decolorazione del refluo Direct si ottiene soltanto riducendo il pH del refluo verso valori più acidi. Nessun effetto si osserva invece sul refluo Reactive anche in seguito a variazioni di pH o a diluizione del refluo. Pertanto, come già osservato nel caso del trattamento con biosistemi microbici, il refluo Reactive si è rivelato estremamente recalcitrante alla biodegradazione.

*Biosistemi enzimatici in fase immobilizzata*

La possibilità di produrre biosistemi enzimatici immobilizzati costituisce un'opportunità interessante da applicare al trattamento dei reflui modello. L'immobilizzazione enzimatica, infatti, oltre a consentire il recupero ed il riutilizzo dei catalizzatori impiegati nel processo, comporta, nella maggioranza dei casi, un incremento della stabilità dell'enzima rispetto alla controparte in fase libera. Questa fase del progetto è stata pertanto finalizzata alla definizione di una tecnica di immobilizzazione di miscele di laccasi prodotte da *P. ostreatus* e all'analisi delle capacità decoloranti del sistema immobilizzato.

La miscela laccasica è stata immobilizzata covalentemente su un supporto siliceo, la perlite. La resa del processo è stata ottimizzata (fino al 70% di attività immobilizzata sul supporto), agendo sia sul grado di derivatizzazione del supporto -variabile nei due passaggi successivi di silanizzazione ed attivazione con glutaraldeide- sia su parametri quali il rapporto attività iniziale/g di solido, il pH, la forza ionica, il tempo e la temperatura di incubazione con il supporto. Il sistema immobilizzato è stato quindi caratterizzato in termini di stabilità e parametri cinetici, ricorrendo per questi ultimi, all'immobilizzazione dell'enzima purificato POXC. I risultati indicano che l'immobilizzazione su perlite comporta un incremento di stabilità del sistema immobilizzato sia a temperatura ambiente che a 4°C rispetto alla controparte in fase libera. Si osserva invece un incremento della $K_M$, ed una riduzione dell'efficienza catalitica dell'enzima immobilizzato nei confronti del substrato ABTS. Alterazioni nella conformazione dell'enzima causate dall'immobilizzazione o la ridotta accessibilità del

substrato al sito attivo dell'enzima immobilizzato, possono plausibilmente spiegare i dati osservati.
Il sistema è stato infine impiegato in un reattore a letto fluidizzato per la decolorazione in continuo del colorante modello RBBR. I primi esperimenti di decolorazione hanno evidenziato il solo adsorbimento del colorante sul supporto, seguito dalla completa inattivazione dell'enzima. Il protocollo di immobilizzazione è stato quindi opportunamente modificato al fine di minimizzare le quantità di colorante adsorbite sul supporto, mantenendo inalterata la resa di immobilizzazione. I risultati preliminari relativi alle prove di decolorazione effettuate con il sistema così ottimizzato, indicano una conversione del 60% del colorante, associata però ad una disattivazione del catalizzatore pari al 70% dell'attività immobilizzata.
I risultati fin qui prodotti non sono sufficienti a valutare l'applicabilità di questo sistema per la conversione dei coloranti. Si rendono infatti necessarie ulteriori analisi, finalizzate alla caratterizzazione dei parametri cinetici di entrambi i fenomeni osservati: la conversione del colorante ed il processo di disattivazione a cui il catalizzatore va incontro. Solo un'attenta analisi di tali parametri consentirà una completa valutazione delle *performances* del sistema ed una stima dei vantaggi connessi all'impiego di biosistemi enzimatici immobilizzati (in termini di volumi trattati, costi e tempi di trattamento) rispetto ai biosistemi in fase libera.

- *Decolorazione dei reflui modello mediante bioassorbimento con biomasse fungine*
In collaborazione con l'Università di Torino sono state esplorate le capacità di biomasse fungine inattivate (derivanti dai funghi zigomiceti, *Cunnighamella elegans, Rhizomucor pusillus e Rhizopus stolonifer*) di decolorare e detossificare i reflui modello mediante bioassorbimento. Il trattamento con le biomasse inattivate ha determinato una sostanziale e rapida decolorazione di tutti i reflui studiati. Fino al 94% di decolorazione può essere ottenuto dopo già due ore di trattamento per i reflui Acid e Direct, mentre il refluo Reactive è decolorato fino al 65% in un tempo più lungo (24 ore). Le biomasse studiate rappresentano dunque un sistema efficace e versatile, esibendo elevate rese di bioassorbimento nei confronti di coloranti di diverse classi chimiche e nelle differenti condizioni di salinità e pH che caratterizzano i tre reflui modello analizzati. Le elevate percentuali di decolorazione ottenute sono inoltre associate ad una significativa detossificazione dei reflui e ad un notevole abbattimento dei valori di COD (fino al 58%) dei tre reflui analizzati.

I risultati descritti in questa sezione hanno dunque consentito di analizzare diverse alternative da applicare al trattamento dei reflui colorati, evidenziandone potenzialità e limiti. I processi basati sulla biodegradazione si rivelano economicamente competitivi, ma sono spesso caratterizzati da tempi di trattamento lunghi o risultano limitati dalle condizioni che caratterizzano i reflui industriali (pH estremi, elevate concentrazioni di sali e coloranti, presenza di additivi e surfattanti). I processi di bioassorbimento, d'altro canto, pur risultando più efficienti e versatili, determinano solo un trasferimento del colorante dalla fase liquida alle biomasse fungine, ponendo così il problema dello smaltimento delle biomasse colorate prodotte alla fine del processo.
Concludendo, una combinazione di differenti tecniche (sia biologiche come quelle descritte, che chimico-fisiche), sostenibili a livello sia tecnologico che economico, e finalizzate alla realizzazione di uno schema integrato di trattamento, sembra essere la soluzione più indicata per lo smaltimento dei reflui dell'industria tessile.

I risultati più significativi, ottenuti con i biosistemi analizzati su ciascun refluo modello, sono riassunti in termini di percentuale di decolorazione (DEC), variazione di COD e tossicità (TOX) nella tabella seguente:

| | ***ACID*** | | | ***DIRECT*** | | | ***REACTIVE*** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Biosistema** | **DEC** | **COD** | **TOX** | **DEC** | **COD** | **TOX** | **DEC** | **COD** | **TOX** |
| *Pleurotus ostreatus* (-) | 54% (7 giorni) | -33% | -4 volte* | - | - | - | - | - | - |
| *Pleurotus ostreatus* (+) | 66% (7giorni) | - | -5 volte* | - | - | - | - | - | - |
| *Phanerochaete chrysosporium* (-) | - | - | - | - | - | - | - | - | - |
| *Phanerochaete chrysosporium* (+) | - | - | - | 87% (7 giorni) | - | ND | - | - | - |
| Laccasi da *P. ostreatus* | 30% (1h) | -30% | -3 volte* | - | - | - | - | - | - |
| Biomasse fungine inattivate** | 94% (2h) | -22% | + | 93% (2h) | -58% | + | 65% (2h) | -47% | + |

(-) e (+) si riferiscono alle prove di decolorazione condotte in assenza ed in presenzadi nutrienti

* Riduzione tossicità ottenuta con il test Lumistox

** migliori risultati ottenuti in esperimenti condotti con tre diverse biomasse fungine

+ Riduzione significativa di tossicità ottenuta con il *Lemna minor* test

**2) Isolamento di nuovi geni codificanti laccasi da *P. ostreatus***

La mancata disponibilità della sequenza completa del genoma di *P. ostreatus* non esclude che la famiglia genica delle laccasi di questo fungo comprenda ancora altri membri non ancora caratterizzati. Da qui l'interesse verso la ricerca di nuove laccasi da *P. ostreatus*, finalizzato non solo ad approfondire la caratterizzazione di questa complessa famiglia enzimatica, ma anche ad ampliare la gamma di biocatalizzatori enzimatici da applicare ai processi di biorisanamento.

Il punto di partenza di questa analisi è costituito dall'isolamento, mediante *screening* di una genoteca genomica in cloni BAC, di un clone (I24) contenente tutti i geni codificanti le laccasi note da *P. ostreatus*. La co-localizzazione dei geni *poxc*, *pox1*, *poxa3*, *poxa1b* nella regione di 150kb contenuta nel clone I24, suggerisce che i membri di questa famiglia genica siano organizzati in *cluster*, supportando l'ipotesi che altri geni codificanti laccasi siano potenzialmente presenti nello stesso inserto genomico. Il clone I24 è stato quindi oggetto di ulteriori indagini finalizzate a confermare tale ipotesi. Due approcci sono stati applicati in parallelo: i) l'analisi per PCR condotta utilizzando oligonucleotidi degeneri, costruiti sulla sequenza aminoacidica conservata dei siti di legame al rame delle laccasi, ha condotto all'isolamento di un nuovo putativo gene codificante laccasi, denominato *pox4*; ii) lo screening di una sub-genoteca plasmidica del clone I24, mediante ibridazione con sonde corrispondenti ai cDNA delle laccasi note da *P. ostreatus*, ha portato invece all'isolamento di due nuovi putativi geni, *pox3* e *pox5*.

All'analisi *in silico* dei tre nuovi geni isolati, ha fatto seguito la ricerca *in vivo* dei trascritti corrispondenti. Esperimenti di RT-PCR sono stati condotti su campioni di RNA estratti dal fungo in diverse condizioni di crescita. Trascritti maturi, codificanti enzimi con putativa attività laccasica, sono stati isolati, nelle condizioni analizzate, per i geni *pox3* e *pox4* ma non per *pox5*. L'analisi delle sequenze aminoacidiche dedotte dai trascritti isolati ha rivelato, per entrambe le proteine, un alto grado di similarità con le laccasi note depositate in banca dati, oltre che la conservazione, nella struttura primaria, di tutte le regioni *consensus* delle laccasi.
Per la caratterizzazione delle nuove putative laccasi POX3 e POX4 -mai isolate in forma nativa dal fungo delle condizioni analizzate- è stata scelta la strategia dell'espressione eterologa.
Si è proceduto quindi all'espressione eterologa di POX3 e POX4 nei lieviti *S. cerevisiae* e *K. lactis*, scelta dettata dalla necessità di modificare post-traduzionalmente le laccasi ricombinanti. Per entrambe le proteine, ed in entrambi i lieviti, la localizzazione nel mezzo extracellulare è garantita dall' utilizzo del peptide segnale omologo alle laccasi, la cui efficacia è stata già verificata nel dirigere la secrezione di altre laccasi da *P. ostreatus* espresse negli stessi ospiti.
In entrambi gli ospiti non è stata rivelata attività fenolo-ossidasica per rPOX4. I livelli di espressione di questa proteina, rivelabili mediante analisi per *western blotting,* risultano inoltre molto scarsi in entrambi i sistemi analizzati. E' ipotizzabile che la proteina rPOX4 sia espressa in forma inattiva, per effetto di uno scorretto processamento o *folding* da parte dell'ospite; in alternativa, rPOX4 potrebbe essere caratterizzata da un'attività specifica molto bassa o essere prodotta a livelli troppo scarsi per poter essere rivelata dalla sensibilità del saggio spettrofotometrico.
La proteina rPOX3 risulta invece prodotta in forma attiva in entrambi gli ospiti. La successiva caratterizzazione di rPOX3 è stata condotta sulla proteina ricombinante prodotta in *S. cerevisiae*, dati i maggiori livelli di produzione ottenuti in quest'ospite rispetto a *K. lactis*. La produzione di rPOX3 è stata ottimizzata agendo su parametri quali: la concentrazione di rame (induttore trascrizionale e cofattore delle laccasi), il tempo di aggiunta dell'induttore, e la temperatura di crescita. In tutte le condizioni analizzate, la curva di produzione di rPOX3 mostra un peculiare andamento: la proteina si accumula nel mezzo di coltura nelle prime fasi della crescita cellulare esibendo un picco massimo di attività (75 IU/L) al secondo giorno, per poi diminuire rapidamente a tempi successivi (l'attività è ridotta a zero entro le successive 24 ore). Misure di attività condotte sui sovranatanti delle colture, raccolti in corrispondenza del picco massimo di attività, indicano che essa diminuisce del 60% dopo solo un'ora di incubazione a temperatura ambiente. Pur non potendo escludere completamente l'azione di proteasi extracellulari sulla proteina ricombinante, il comportamento osservato è in larga misura attribuibile all'elevata instabilità della proteina rPOX3, dal momento che la stabilità della stessa può essere incrementata in seguito all'aggiunta di agenti stabilizzanti. In particolare, l'aggiunta di solfato di rame ai sovranatanti di colture di *S. cerevisiae* esprimenti la laccasi rPOX3, causa un incremento dell'attività laccasica, in misura proporzionale alla sua concentrazione. L'aggiunta del cofattore esercita, inoltre, un effetto stabilizzante sull'enzima. L'instabilità di rPOX3 potrebbe essere quindi relazionata alla perdita del rame e alla conseguente formazione di una apo-laccasi riconvertibile in olo-enzima attivo in seguito all'aggiunta di rame. L'enzima rPOX3 è stato infine parzialmente purificato e caratterizzato, determinandone le costanti di affinità verso due diversi substrati, l'ABTS e il DMP. Il confronto dei valori delle $K_M$ determinati per rPOX3 con quelli già noti per l'enzima rPOXA1b (62% di identità con POX3) espresso nello stesso ospite, indica una

minore affinità di rPOX3 nei confronti di entrambi i substrati. Una possibile spiegazione ai dati ottenuti può derivare dal confronto del modello tridimensionale di POX3 con quello di POXA1b. L'accesso del substrato alla tasca di legame dell'enzima POX3 appare infatti ostacolato dalla presenza di un *loop* più lungo rispetto a POXA1b. Ciò si tradurrebbe in un aumento della costante di affinità per il substrato, con un effetto particolarmente evidente verso substrati stericamente più ingombranti come l'ABTS.

# *Introduction*

## 1. Biotechnology and bioremediation: a new challenge for wastewater treatment

Water is a rare and precious commodity, and only an infinitesimal part of the earth's water reserves (approximately 0.03%) constitutes the resource available for human activities [1]. Besides the problem of water shortage, the huge amount of wastewaters discharged in the environment by the ever-growing industrial activities of the last century, has risen serious environmental concerns about water pollution. In light of this, the recycle of properly treated industrial effluents at the "end of pipe" would be extremely desired.

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.

The term "bioremediation" has been used to describe the process of using microbial or enzymatic processes to degrade or transform hazardous organic contaminants.

A large number of microorganisms have been isolated and applied to both *in situ* and *ex-situ* bioremediation processes in recent years, and the identification of new microbes with novel metabolic potential offers an attractive route to solve environmental problems [2].

Newly developed biotechnological tools can be applied to the analysis of biological processes in the environment, providing new approaches for understanding, managing, and restoring polluted sites. On the other hand, advances in genetic and protein engineering techniques have opened up new avenues towards the design of genetically engineered microorganisms (GEMs) and enzymes with the desired biodegradation properties [3].

The relative cheapness of the processes, the wide degradation potential offered by the different class of microorganisms which can be employed in, and the new frontiers opened by genetic engineering, render bioremediation one of the most promising alternatives for wastewater treatment.

## 2. Dye-containing effluents

*Synthetic dyes*

Dyes have been used since ancient times, when they were extracted by natural sources (plants, lichens, insects or mussels). The introduction of synthetic dyes is attributed to William Henry Perkin in 1856. In an attempt to synthesize an antimalarial drug, he accidentally discovered the world's first commercially successful synthetic dye, the Mauveine, marking the origin of the organic chemical industry of dyes.

With the increased knowledge of chemical organic reactions, newly synthetic dyes with improved quality and characteristics have been developed. As a consequence, 50 years after Perkin's discovery, more or less 90% of dyes on the market are of synthetic origins.

Dyes are generally aromatic organic compounds with complex structures. They are composed of a group of atoms responsible for the dye colour, called chromophores, as well as an electron withdrawing or donating substituent that influences the colour of the chromophores, called auxochrome. 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) [4] 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.
The chemical classes of dyes most frequently used on industrial scale are the azo derivatives. Azo dyes are characterized by the presence of one or more azo linkages and aromatic rings with simple application-technique requirements and high water-fastness. Also reactive dyes are extensively used because of their favourable characteristics of bright colour and low energy consumption during application [5].

*Textile effluents*
More than 70.000 tons of synthetic dyes are annually produced and applied in several industrial activities, up to 40% of which is lost during the dyeing processes. The textile industry, in particular, accounts for two-thirds of the total dyestuff market. It consumes large volumes of water (up to 150 L of water are required for the processing of 1 Kg of dyed fabrics) and generates huge amount of coloured wastewaters. Depending upon customer orders, types of manufactured textile materials and production schedules, textile wastewaters display a great chemical complexity and variability in terms of quantities and pollution load, type of dyes, pH, and temperature [6].
Most of dyes used in the textile industry are very recalcitrant to traditional treatment, since they are designed to resist fading upon exposure to sweat, light, water chemicals including oxidizing agents, and microbial attack. Besides dyes, these 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 the wastewaters from textile industries one of the most difficult to treat [7].
The release of coloured wastewaters in the ecosystem is a remarkable source of aesthetic pollution, eutrophication and perturbation of aquatic life. Moreover, synthetic dyes are toxic, carcinogenic and mutagenic to various organisms, being detrimental to human health [8]. In particular, azo e nitro compounds are reduced in sediments of aquatic bodies, yielding carcinogenic amines that spread in the ecosystem [9].
These concerns have led to new and/or stricter regulations concerning coloured wastewater discharges, compelling the dye manufacturers and users to adopt “cleaner technology” approaches, for instance, the development of new lines of ecologically safe dyeing auxiliaries and the improvement of exhaustion of dyes on to fibre [10].
Nevertheless, the treatment of the large quantities of dyed wastes that are continually being produced, represents the major problem currently experienced by the textile industry.

## 3. Textile Wastewater treatment: which are the new frontiers?
Various methods for textile wastewater treatment have been proposed, falling into three categories: physical, chemical and biological. Although successfully applied in small scale decolourisation processes, physic-chemical methods are largely limited

by their high cost, restricted versatility, interference by other wastewater constituents and the handling of the waste generated (**Table 1**).

| Physical/chemical methods | Advantages | Disadvantages |
|---|---|---|
| Fentons reagent | Effective decolourisation of both soluble and insoluble dyes | Sludge generation |
| Ozonation | Applied in gaseous state: no alteration of volume | Short half-life (20 min) |
| Photochemical | No sludge production | Formation of by-products |
| NaOCl | Initiates and accelerates azo-bond cleavage | Release of aromatic amines |
| Cucurbituril | Good sorption capacity for various dyes | High cost |
| Electrochemical destruction | Breakdown compounds are non-hazardous | High cost of electricity |
| Activated carbon | Good removal of wide variety of dyes | Very expensive |
| Peat | Good adsorbent due to cellular structure | Specific surface areas for adsorption are lower than activated carbon |
| Wood chips | Good sorption capacity for acid dyes | Requires long retention times |
| Silica gel | Effective for basic dye removal | Side reactions prevent commercial application |
| Membrane filtration | Removes all dye types | Concentrated sludge production |
| Ion exchange | Regeneration: no adsorbent loss | Not effective for all dyes |
| Irradiation | Effective oxidation at lab scale | Requires a lot of dissolved $O_2$ |
| Electrokinetic coagulation | Economically feasible | High sludge production |

**Tab. 1 Advantages and disadvantages of the current methods of dye removal from industrial effluents (Robinson et al. 2001)**

Conversely, biological options (bioremediation) have emerged as environmental friendly and cost-competitive alternatives. Bioremediation can be achieved using two different principles: biosorption and biodegradation

## Biosorption

Adsorption based methods produce high-quality treated effluents, as they are effective on different types of dyes and provide high and rapid decolourisation. 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. Different waste products from industrial and agricultural operations, have been tested for dye removal [11]. Despite of their cheapness, these wastes have generally low adsorption capacity, thus are required to be used in large amounts, rising further disposal problems.

Alternatively, the use of microbial biomass (in dead or living form) has emerged as a promising and cost-effective technology. Although a wide variety of microorganisms including algae, yeasts, bacteria and fungi have successfully been used in biosorption, most of the studies have prompted out the advantages linked with the use of living or died fungi as dye absorbers [12]. Fungal biomasses can be produced using relatively simple fermentation techniques and inexpensive growth media; alternatively they are available as by-products of industrial fermentation, representing a potential source of cheap adsorbent materials [13]. The use of dead fungal biomasses, is particularly advantageous since they are not affected by toxic wastes, do not pollute the environment releasing toxins and/or propagules and do not require a continuous supply of nutrients. Moreover, their 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 [13].

Mitosporic fungi and Zygomycetes such as *Aspergillus* spp., *Penicillium*spp., *Myrothecium* spp., *Botrytis* spp., and *Rhizopus* spp., are usually employed because

of their peculiar cell wall structure, whilst only few cases of biosorption employing basidiomycetes have been reported [12]. Fungal cell walls consisting mainly of polysaccharides, proteins and lipids, offer many functional groups for dye interaction, therefore colour removal can be the result of different mechanisms, such as physical (i.e. electrostatic or Van der Waal forces) or chemical binding (covalent and ionic). The adsorption process is hence influenced by many factors including dye/sorbent interaction, dye concentration, sorbent surface areas, particle size, temperature, pH and contact time [14]. In some cases, the external binding can be also followed by dye internalization [15]. Several authors also reported the use of immobilized and inactivated fungal biomasses for dye removal, highlighting the importance of proper definition of operational parameters, such as flow rate, dye concentration and reactor configuration (aerated or rotating mode) for the success of the process [16;17]

**Biodegradation**

Biodegradation can be accomplished by either aerobic or anaerobic processes. Bacterial aerobic treatments are often ineffective in degrading dyes [18]. By contrast, bacteria are mostly applied as mixed microbial consortia for azo-dye decolourisation under anaerobic conditions [19; 20]. Bacterial cytoplasmic azo-reductases play an important role in this process, however, their action results in the production of very toxic aromatic amines. Only a combination of bacterial anaerobic-aerobic treatments has proved successful for both decolourisation (provided by anaerobic treatment) and detoxification (by aerobic degradation of toxic compounds obtained by azo-dye cleavage) of textile wastewaters [21].

By far, the single class of microorganisms most efficient in breaking down synthetic dyes are the white-rot fungi (WRF) [4].

These constitute a diverse ecophysiological group comprising mostly basidiomycetes (and, to a lesser extent, litter-decomposing) fungi capable of extensive aerobic lignin depolymerization and mineralization. This property is based on the WRF's capacity to produce one or more extracellular lignin-modifying enzymes (LMEs), 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. In addition, the secretion of LMEs in the extracellular medium allows fungi to tolerate high concentration of toxic compounds and to degrade very insoluble dyes as well as those which can't enter the cell. Accordingly, all these features promote white-rot fungi as good candidates for wastewater treatment.

## 4. White rot fungi and LMEs

The complex pattern of LMEs produced by white-rot fungi to accomplish lignin degradation comprises several oxidative activities, mainly Manganese 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.

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 are strictly influenced by nutrient levels (often induced under C and N limitation), culture conditions (shaking or stationary) and by the presence of different inducers (metals, aromatic compounds and so on). Frequently, more than one

isoforms of LMEs are expressed by different taxa depending on culture conditions [22].

*Mn peroxidase*

Manganese peroxidases (MnP) 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 [23]. MnP catalyzes the peroxide dependent oxidation of $Mn^{2+}$ to $Mn^{3+}$ which is stabilized by chelators such as oxalic acid [24], itself also excreted by the fungi. Chelated $Mn^{3+}$ acts as a highly reactive (up to 1510mV) low molecular weight diffusible redox-mediator. Thus, MnP are able to oxidase and depolymerise their natural substrate, i.e., lignin as well as recalcitrant xenobiotics, such as nitroaminotoluene and textile dyes [25]. *In vitro* depolymerization can be enhanced in the presence of co-oxidants such as thiols or unsaturated fatty acids (Tween 80) [26].

*Lignin peroxidase*

Lignin peroxidases are N-glycosylated enzymes with molecular masses between 38 and 47 KDa, which catalyzes the 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 LiP in ligninolysis could be the further transformation of lignin fragments which are initially released by MnP. LiP are not essential for the attack on lignin: several highly active WRF and litter-decaying fungi do not produce this enzyme.

Lip have been used to mineralize a variety of recalcitrant aromatic compounds, such as PAHs [27], polychlorinated biphenyls [28], and also dyes [29]. Usually, the natural fungal secondary metabolite veratryl alcohol (VA) acts as redox mediator to stimulate the LiP catalyzed oxidation of a wide range of recalcitrant substrates [30].

*Laccase*

Laccases are multicopper-containing enzymes belonging to the group of blue oxidases, catalyzing the one-electron oxidation of a wide range of aromatic substrates such as a variety of phenolic compounds, as well as diamine and aromatic amines with the concomitant reduction of oxygen to water.

A more detailed description of this class of enzyme is given in the following sections.

*Versatile peroxidase*

Versatile peroxidases (VP) are heme containing enzymes, structural hybrid between MnPs and LiPs, since they can oxidize not only $Mn^{2+}$ but also phenolic and non-phenolic aromatic compounds including dyes, in manganese-independent reactions [31]. VPs have been recently described in *Pleurotus* and *Bjerkandera* [32; 33]. Decolourisation of some textile dyes by VPs from *B. adusta* has been reported only in the presence of oxidizing mediators [34].

*Auxiliary activities*

Many other enzymes produced by fungi are involved in lignin degradation. Several extracellular oxidases (glucose, glyoxal, galactose and aryl alcohol oxidases) have been shown to play an important role in the lignin degrading process since they provide $H_2O_2$, an essential cofactor for peroxidases. The involvement of other enzymatic activities, such as cellobiose dehydrogenase (CDH) and haloperoxidases

in lignin breakdown has also been proposed, although their role is not yet full understood [35].

*Small molecular mediators*

Given the random polymer nature of lignin and the bulk of LME, direct and specific interactions between lignin (or recalcitrant structural analogs) and LME are highly improbable. Rather low-molecular weight and diffusible redox mediators are able to migrate into the lignocellulose complex, providing high redox potential (>900mV) to degrade lignin. They can 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 [36].

Both native and synthetic mediators have been found to promote LMEs activities. Some organic acids, excreted by several fungi may act as $Mn^{+3}$ chelators stabilizing the metal. Veratryl alcohol (VA), a secondary metabolite of several WRF [37], after its oxidation to the VA cation radical by LiP, also acts as a mediator for the degradation of lignin. Nevertheless, LiP is stimulated by VA probably by protecting the enzyme against the damaging effect of $H_2O_2$ [38].

Laccase mediators, ranging from natural (3-HAA) [39], to synthetic ones, have also found several industrial applications since they are able to expand the substrate range of laccases to non-phenolic substrates [40].

## 5. Decolourisation of textile dyes and effluents by WRF and their enzymes

White-rot fungi have shown to have an outstanding potential for the bioremediation of industrial coloured wastewaters. Thanks to their non-specific LMEs these fungi are able to transform a wide range of organic compounds [4].

Most previous studies have focused on few “standard” organisms, i.e. *P. chrysosporium* and *T. versicolor*. More recently, there has been a growing interest in exploring the decolourisation potential of a wide array of fungi, ranging from basidiomycetes to ascomycetes and mitosporic fungi. Experimental evidence indicated that the biodegradation capability of fungal species is very heterogeneous, mainly due to the physiological differences among them and to the variable pattern and expression of their LMEs in the presence of chemically different compounds. This heterogeneity has been exploited for the selection of fungi with high biodegrading capabilities [41; 42] and could turn out to be particularly valuable to meet the variability of textile wastewaters.

The decolourisation of dye-bearing wastewaters may be achieved using either microorganisms or enzymes as biocatalysts. Together with the constraints posed by specific bioprocesses, some distinctive advantages and drawbacks characterize the use of each of these two biosystems.

**Whole-cell based processes**

Some reports suggest the superiority of using whole cells against purified enzyme based processes. Romero and co-workers [43] reported that the lower percentage of decolourisation obtained with laccases, with respect to the *in vivo* process catalysed by *T. versicolor* whole cell, could be ascribed to product inhibition of the enzyme. This inhibition is avoided using the whole fungus, since degradation products can be further metabolized. Furthermore, in many cases, the concerted action of different enzymatic systems, extracellular, wall associated or intracellular (cytochrome P-450

monooxygenase) [44], is necessary to achieve both efficient dye removal and toxicity reduction of treated wastewaters. Moreover, the cells may synthesize several cofactors required to maintain the enzyme activity and to promote dye decolourisation, such as veratryl alcohol [45] or hydrogen peroxide [46].
Few studies addressed the question whether fungal dye decolourisation causes effective dye mineralization. Mineralization rates of 23.1-48.3% for a wide range of $^{14}$C-ring labelled azo dyes after a 12-day incubation with *P. chrysosporium* have been recorded [47]. However the role played by the substitution pattern on dye mineralization rates is a matter of controversy [48], though it is clear that dye decolourisation is not equivalent to dye mineralization.
Although some studies have been conducted on testing the toxicity of the treated effluent/dye solution [49], with also reported examples of effective detoxification, further detailed characterization of the intermediates and metabolites produced during biodegradation is still required to ensure the safety of the decolourized wastewaters.

Beside the contribution of all the activities associated with fungal growth and dye decolourisation, whole cell based treatments exhibited two additional advantages: the relative easiness and cheapness of up-stream processes required to make biocatalytic activity available compared with enzymatic processes, and the possibility to exploit microbial consortia for the concurrent removal of different toxic compounds [50].

*Factors affecting whole cell based processes*
The decolourisation ability of WRF can be substantially increased by carefully optimizing the operational parameters, such as initial dye concentration, nutrient content of the media, and culture conditions (stationary or shaking). [51; 52].
Studies on the effect of type (inorganic or organic) and concentration of nitrogen sources on dye-treatment, were carried out by many authors [53;54;55;56]. For most of the fungi the highest decolourisation rate was reached under nitrogen limitation. In addition, in some cases, the choice of the proper N-source was shown to be crucial to achieve colour removal [57].
Several reports investigated various carbon sources as effective substrate for dye-decolourisation process, showing that the efficiency of colour removal by different fungal species, may be strictly dependent on the type of C-source.
Addition of glucose as carbon source caused a dramatic improvement in dye-treatment catalysed by several fungi [58]; glucose concentration was also shown to finely affect the decolourisation rate [57]. Various cheaper carbon sources have been applied in decolourisation processes to replace glucose (e.g. starch, molasses), showing different results depending on the fungal species [59].
The amount and the type of carbon and nitrogen sources affect dye decolourisation by altering the enzymatic pattern produced by the fungi. Beside the level of LME production, the choice of nutrients especially affects the patterns of LME expression in each fungal species. Thus, judicious manipulation of the culture conditions may allow the production and selection of different mixtures of ligninolytic enzymes, exhibiting selective decolourisation abilities toward specific classes of dyes or effluents [53;55].
Enhanced LME production in the presence of industrial effluents turns out to be an additional advantage during bioremediation processes. For example, during the treatment of a dye-containing textile effluents by the white-rot fungus *Clitocybula*

*dusenii*, both MnP and laccase activities were induced [25]. In another study, D'Souza and co-workers reported a seven fold enhancement of laccase production during the treatment of coloured effluents by a marine fungal isolate [60].
The efficiency of fungal strains in dye removal is also affected by the choice of shaking or stationary conditions. Higher colour removal has been observed in shaking cultures as compared to the stationary cultures, because of better oxygen transfer and nutrient distribution [61].

Although numerous WRF strains have been successfully used for decolourisation of several synthetic dyes, their application 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.
As reported by Abadulla [62], Cu and Fe chelators as well as anionic detergents, sometimes found in real textile industrial effluents, inhibited *Polyporus sp*. and *Trametes villosa* up to 20%. In another report, some fungal species, at first selected for their decolourisation ability on Reactive Black 5, were effective on a real waste only if the dye-bath was diluted, in order to allow fungal growth [63].
The fungal growth, enzyme production and subsequent dye decolourisation are also affected by pH value. The optimum pH for dye decolourisation lies in the acidic range for the majority of tested fungi [54;64]. In contrast, neutral and more basic pH values characterize real textile effluents, rendering their treatment even more troublesome.
The effect of heavy metals on dye decolourisation has also to be taken into account, since they form organo-metal complexes in many dye molecules and, consequently, are present in discrete amount in textile effluents. Their effect has been investigated by many authors [65;66]. In an exemplificative case, Hatvani and Mecs [67] studied the effect of different metals on decolourisation of Poly R-478 by *Lentinula edodes*. They observed that, all the metals caused an increase in laccase and MnP production, when added at very low amount. However, over a metal-dependent threshold concentration, their addition produced an inhibitory effect on decolourisation.

In view of the application of whole cell based processes to real wastewater treatment, the evaluation of the performances of the fungal systems in non-sterile conditions is also an important factor to be considered. Libra et al [68] addressed this issue proposing several strategies to improve the use of *T. versicolor* for RB5 decolourisation under non-sterile conditions. They found that an effective process could be realized by producing fungal inocula in conditions which gave them a selective advantage over bacteria (nitrogen limitation in the medium, cultivation on organic solids).
Considering all the above factors, the selection of fungal species able to cope with unfavourable characteristics of process effluents represents a possible and feasible solution. Junghanss et al [69] for example, reported that aquatic fungi selected for their good decolourisation ability towards several classes of dyes, may better fit the high content of inorganic ions found in industrial dye-containing effluents.

Beside the choice of the fungal strain, the decolourisation of real effluents requires the appropriate design of reactor configuration. There are few reports specifically on

dye decolourisation by fungal whole-cells in continuous bioreactors [57;70]. Their application is still lacking because of the problems encountered with this operation: the short fungal lifetime due to the limitation of nutrients and oxygen required for growth and LME production; the excessive growth of the fungus with hyphae extension, which prevent adequate oxygenation of the medium and decrease the efficiency of mass transfer.
Several reactor configurations have been proposed to overcome these limitations. Mielgo and co-workers [71] set up a pulsed bed bioreactor with immobilized *P. chrysosporium*; Hai and coworkers [72] proposed an efficient system based on fungi immobilized in a membrane-bioreactor; several authors [73;74] investigated the effect of different types of discs for fungal immobilization to allow intermittent contact of the mycelium with the effluent, thus avoiding overgrowth.

**Enzyme based processes**

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.
The involvement of LMEs in the dye decolourisation process by WRF has been confirmed in several independent studies using purified enzymes or WRF cell-free culture supernatants. Sometimes it's difficult to find a correlation between dye decolourisation and the activity of a specific class of LME (MnP, LiP or Lac). Non-optimum conditions of enzyme assays [75], poor enzyme diffusion in the medium [76], simultaneous presence of isoforms with different affinity for both dyes and reaction substrates during measurement, or rapid inactivation of the enzymes by proteolytic degradation [77] can affect this analysis.
*In vitro* decolourisation using purified LME was widely described, e.g., using Lac from *Pyricularia oryzae* [78], *P. ostreatus* [79], *T. versicolor* [4] *Pycnoporus cinnabarinus* [80], LiP from *P. chrysosporium* [81], *T.versicolor* [30], and MnP from *B. adusta* [81], *P. chrysosporium* [82], *T. versicolor* [83].
However, the 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 [46]. Moreover, laccase and peroxidases may act as starters of a chain reaction by generating highly active free radicals which in turn, playing a mediating role, promote the decomposition of those dyes which are not the substrate of LME per se [84].

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. Reducing the costs of LME production by optimising the fermentation parameters is the basic research for industrial applications. On the other hand, new tools to achieve these tasks have being developed:

*Enzyme immobilization*

An increase of enzyme-based process throughput, could be achieved by means of enzyme immobilization. Immobilization of enzymes to water-insoluble supports has

shown to increase their operational stability [85]. Furthermore, enzyme immobilization would allow the reuse of the enzyme and thus decrease the cost of industrial applications. For these reasons, its application to wastewater treatment has gained much interest and various examples have recently been reviewed [86]. Many efforts have being focused on finding cheap and reliable confinement methods in order to realize suitable biosystems for wastewater treatment with a reduced effect on the overall cost of the processes.

*Use of small mediators*

The activities of the LMEs can be further increased by the addition of different low molecular mass mediators. Most of the work in this field has been done on laccases, since their lower redox potential (0.5-0.8V) compared to those of ligninolytic peroxidases (>1.0V) only allows the direct degradation of low redox-potential compounds. From the description of the first laccase mediator, ABTS [87], to the more recent use of the -NOH-type, synthetic mediators such as 1-hydroxybenzotriazolr, violuric acid and N-hydroxyacetanilide or TEMPO, a large number of studies have been performed on the mechanisms of their action [88; 89] and on their application to dye decolourisation.

However, a systematic effort has been conducted to evaluate the potential of several naturally occurring compounds to mediate the oxidative reactions catalysed by laccases with the aim of identifying cheaper, more efficient and eco-friendly mediators for the decolourisation of recalcitrant dyes [90].

Moreover, MnP mediated decolourisation of some azo dyes by *P. chrysosporium* has proved to be enhanced by the addition of Tween 80 [91]. Whereas, LiP produced by *T. versicolor* is able to decolourise RBBR in the presence as well as in the absence of VA [30].

*Heterologous expression*

Recombinant expression of oxidative enzymes in suitable hosts represents a promising tool to obtain higher production yields at and affordable price. Efficient expression systems of LME have been proposed [52]. 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 [92].

In addition, molecular biology tools have been applied to improve the performances of oxidative enzymes. Laccases have been subjected to both rational design and directed evolution and enzymes endowed with improved properties (extended substrate range, catalytic properties and stability) have been produced as “new” enzymatic system more suitable to the unnatural conditions provided by industrial effluents [93].

Thus, the isolation of new genes, the characterisation of their products and further engineering of LMEs for their improvement, are the area of potential future research.

## 6. Oxidative enzymes for wastewater treatment: the case of laccases

Laccase was first isolated in the exudates of *Rhus vernicifera*, the Japanese Lacquer tree by Yoshida in 1883. A few years later its presence was also demonstrated in fungi [94]. More recently, findings of laccase activity in prokaryotes [95] and insects [96] have also been reported.

Although known for a long time, laccases attracted considerable attention only after the beginning of studies on enzymatic degradation of wood by white-rot fungi and

now, fungal laccases are being extensively exploited for many industrial purposes. Laccases, EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase, are part of a larger group of enzymes termed the multicopper enzymes, which includes, among others, ascorbic acid oxidase and ceruloplasmin.

They catalyse the one-electron oxidation of four substrate equivalents, coupled to the four-electron reduction of $O_2$ to water. The catalysis carried out by all members of this family, is guaranteed by the presence of different copper centres, classified on the basis of their spectroscopic properties: one type-1 (T1) copper, one type-2 (T2) and two type-3 (T3) copper ions.

Two histidines 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 functions as an additional axial ligand, the fourth ligand for this copper center is not present in fungal laccases, in which un uncoordinating Phe or Leu occupies this position [97].

Type 1 copper confers the typical blue colour to laccases, which results from the intense electronic absorption at 600nm caused by the covalent copper-cysteine bond. Due to its high redox potential of 500-800mV, Type 1 copper is the site where substrate oxidation takes place. Type 2 site is characterized by the lack of strong absorption features in the visible region and reveals usual EPR spectra. It is strategically positioned close to the Type 3 copper, a binuclear center, spettroscopically characterized by an electron adsorption at 330nm (oxidized form). Type 2 and Type 3 copper ions form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place (Fig.1). 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.

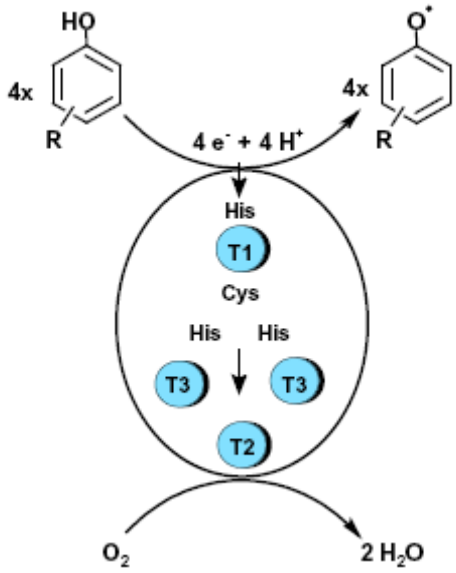


**Fig.1 Catalytic cycle of laccases**

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 [98].

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 [99].

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 [100].

Most fungi produce several isoforms of laccases. The molecular basis for the production of different isoenzymes is the presence of multiple laccase genes. Indeed, several laccase gene families have been described [101;102]. Gene families probably produce closely related proteins that are subtly different in their activities, allowing the transformation of a wider range of substrates or showing differential regulation [103].

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. The localization of laccases seems to be associated with their physiological functions. Extracellular laccases are mainly involved in lignin transformation but they are also reported to be important virulence factors in many fungal diseases by protecting fungal pathogens from toxic phytoalexins and tannins [104].
Intracellular fungal laccases can be involved in transformation of low-molecular-weight phenolic compounds produced in the cell, whilst laccases located in the cell walls and spores can be involved in synthesis of melanin and other substances protecting the cell walls.

**Structural properties**

The molecular properties of laccase and the nature of its copper-containing active sites have been studied extensively by biochemical and spectroscopic methods over the last 50 years. To the present, more than a hundred laccases have been isolated and characterized. Based on the literature, general characteristics of these enzymes can be drawn out.
Typical fungal laccase is a monomeric protein of approximately 60–70 kDa with an acidic isoelectric point around pH 4.0 (**Tab.2**). It seems that there is considerable heterogeneity in the properties of laccases isolated from ascomycetes, especially with respect to molecular weight.

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

**Tab.2 Properties of fungal laccases: n, number of observations; Q25 lower quartile; Q75, upper quartile (Baldrian, 2006)**

Laccases are glycoproteins like many fungal extracellular enzymes; the carbohydrate moiety of the majority of laccases consists of mannose, N-acetylglucosamine, and galactose and constitutes about 45% of the protein mass in laccases of plant origin and about 10-20% of fungal ones. Glycosylation of fungal laccases is one of the main problems for their heterologous production. It was proposed that in addition to the structural role, glycosylation can also participate in the protection of laccase from proteolysis and inactivation by free radicals [105].

Although most of fungal laccases are monomeric proteins, some exceptions of homodimeric, heterodimeric and oligomeric structures have been found [22].
For a long time, attempts to crystallize laccase for X-ray analysis were unsuccessful despite considerable efforts in several laboratories. The major problem was attributed to the presence of carbohydrate chains and their heterogeneity. However, recently, several complete laccase structures from *Coprinus cinereus* (in a copper type-2-depleted form) [106], *T. versicolor* [107;108], *M. albomyces* [109;110], *Bacillus subtilis* [111], *Rigidoporus lignosus* [112], *Pycnoporus cinnabarinus* [113], *Cerrana maxima* [114], have been determined, the last four enzyme with a full complement of copper ions.
The overall structure of *T. versicolor* laccase consists of three consecutively connected cupredoxin-like domains twisted in a tight globule with dimensions of about 65 x 55 x 45 Å. Domain 1 comprises two four-stranded β-sheets and four $3_{10}$-helices. Three of the $3_{10}$- helices are in connecting peptides between the β-strands, and one is in a segment between domain 1 and 2. The second domain has one six-stranded and one five-stranded β-sheet, and like in domain 1, there are three $3_{10}$-helices in peptides connecting individual β-strands and domains 1 and 3, respectively. A $3_{10}$- helix between domains 2 and 3 forms part of a 40-residue-long extended loop region. Finally, domain 3 consists of a β-barrel formed by two five-stranded β-sheets and a two-stranded β-sheets that, together with a α-helix and a β-turn, form the cavity in which the type-1 copper is located. The trinuclear copper cluster (T2/T3) is embedded between domains 1 and 3 with both domains providing residues for the coordination of the coppers. The third domain has the highest helical content with one $3_{10}$-helix and two α-helices located in the connecting regions between the strands of the different β-sheets. Finally, at the C-terminal of domain 3, three sequentially arranged α-helices complete the fold. An α-helix formed by 13 residues at the C-terminal end is stabilized by a disulfide bridge to domain 1 (Cys-85–Cys-488), and a second disulfide bridge (Cys-117–Cys-205) connects domains 1 and 2. Both N-terminal and C-terminal amino acids benefit from hydrogen bonding networks to the rest of the protein.

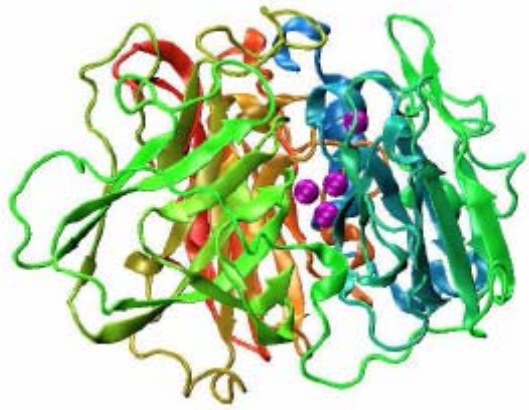

**Fig.2 Ribbon representation of *Trametes versicolor* laccase X-ray structure. Domain 1 is showed in red, domain 2 in green and domain 3 in bleu. Copper ions are show in vdW representation [107].**

Multiple sequence alignments of fungal laccases available in public databases have identified a distinctive sequence signature that distinguishes fungal laccases from plant laccases and other multicopper oxidases. This signature consists of four ungapped sequence segments, L1 to L4, which contain the one cysteine and ten histidines residues serving as copper ligands together with those residues responsible to maintain a local three dimensional fold [115] (Tab.3)
The redox potential of the T1 site determines the efficiency of catalysis, making laccases with high-potential T1 site promising tools for biotechnology applications.
A sum of structural features, ranging form the direct ligands and coordination geometries of T1 site to the nature of the second sphere residues, seem to affect the redox potentials among laccases. A detailed structural comparison between a low

| Laccase | GenBank No. | Ligands of copper sites* | | | |
|---|---|---|---|---|---|
| | | T2 T3 | T3 T3 | T1 T2 T3 | T3T1T3 T1 |
| Ab | AAA17035 | $^{80}$SIHWHGF$^{86}$ | $^{125}$WYHSHLS$^{131}$ | $^{415}$GAHPFHLHGH$^{424}$ | $^{466}$WFLHCHIDWHLEA$^{478}$ |
| Ap | AAB09228 | $^{79}$TIHWHGV$^{85}$ | $^{123}$WWHAHSD$^{129}$ | $^{560}$QNHPMHLHGF$^{569}$ | $^{521}$WFLHCHFERHTTW$^{533}$ |
| Cc | AAD30964 | $^{80}$SIHWHGL$^{86}$ | $^{125}$WYHSHFG$^{131}$ | $^{412}$GPHPFHLHGH$^{421}$ | $^{466}$WFFHCHIEFHLMN$^{478}$ |
| Lt | AAB17191 | $^{80}$TVHWHGI$^{86}$ | $^{125}$FWHAHFS$^{131}$ | $^{469}$ESHLLHLHGY$^{478}$ | $^{530}$WFMHCHFDVHLSW$^{542}$ |
| Ma | CAE00180 | $^{141}$SIHWHGI$^{147}$ | $^{186}$WYHSHFS$^{192}$ | $^{479}$LPHPMHLHGH$^{488}$ | $^{549}$WLFHCHIAWHVSG$^{561}$ |
| Nt | AAC49536 | $^{78}$SIHWHGI$^{84}$ | $^{123}$FWHAHIL$^{129}$ | $^{455}$ENHPIHLHGF$^{464}$ | $^{517}$WFMHCHLEIHTTW$^{529}$ |
| Pc | AAC39469 | $^{83}$SIHWHGF$^{89}$ | $^{128}$WYHSHLS$^{134}$ | $^{414}$TPHPFHLHGH$^{423}$ | $^{468}$WFLHCHIDFHLEA$^{481}$ |
| PM1 | CAA78144 | $^{62}$SIHWHGF$^{68}$ | $^{106}$WYHSHLS$^{112}$ | $^{391}$FPHPFHLHGH$^{400}$ | $^{446}$WFLHCHIDFHLEA$^{458}$ |
| Po | CAA84357 | $^{96}$SIHWHGF$^{102}$ | $^{151}$WYHSHLS$^{157}$ | $^{425}$GPHPFHLHGH$^{434}$ | $^{480}$WFLHCHIDWHLEI$^{492}$ |
| Rv | BAB63411 | $^{57}$TIHWHGV$^{63}$ | $^{102}$WWHAHSD$^{108}$ | $^{431}$TSHPMHLHGF$^{440}$ | $^{491}$WFLHCHFERHTTE$^{503}$ |
| Th | AAA33103 | $^{83}$SIHWHGF$^{89}$ | $^{128}$WYHSHLS$^{134}$ | $^{514}$APHPFHLHGH$^{523}$ | $^{470}$WFLHCHIDFHLEA$^{482}$ |
| Tl | AAC41686 | $^{83}$SIHWHGF$^{89}$ | $^{128}$WYHSHLS$^{134}$ | $^{413}$APHPFHLHGH$^{422}$ | $^{470}$WFLHCHIDFHLEA $^{482}$ |
| Tt | CAC13040 | $^{83}$SIHWHGF$^{89}$ | $^{128}$WYHSHLS$^{134}$ | $^{413}$FPHPFHLHGH$^{422}$ | $^{467}$WFLHCHIDFHLEA$^{479}$ |
| Tv | AAC49828 | $^{82}$SIHWHGF$^{88}$ | $^{127}$WYHSHLS$^{133}$ | $^{413}$APHPFHLHGH$^{422}$ | $^{469}$WFLHCHIDFHLDA$^{481}$ |

**Tab.3 Conservative laccases signatures. Ligands of the active site copper ions are represented in gray colour (Morozova, 2007).** Ab, *A. bisporus* lcc1; Ap, *A. pseudoplatanus*; Cc, *C. cinereus*; Lt, *L. tulipifera* lac2; Ma, *M. albomyces* lac1; Nt, *N. tabacum*; Pc, *P. cinnabarinus* lcc3; PM1, basidiomycetes PM1; Po, *P.ostreatus* pox2; Rv, *R.vernicifera*; Th, *T. hirsuta*; Tl *T. villosa* lacc1; Tt, *T. trogii* lcc1; Tv, *T. versicolor* lcc1

redox potential ($E_0$) *C. cinereus* laccase and a high $E_0$ *T. versicolor* laccase showed that structural differences of the Cu1 coordination possibly account for the different $E_0$ values [108]. This was later confirmed by studies on *R. lignosus* laccase with a high redox potential [112]. However, more effort will be needed to elucidate the relation between the structure of the catalytic site and the substrate preference of different laccase enzymes.

## Industrial applications

Owing to their high and non-specific 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 with some established and lots of emerging biotechnological applications. In food industry, the selective removal of phenol derivatives by laccases is exploited for stabilization and improvement of the quality of different drinks. In pulp and paper industries the treatment of wood pulp with laccases, provides milder and cleaner strategies of delignification that are also respectful of the integrity of cellulose [116].

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 [117]. 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. 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 [118]). Laccases have also found applications in biofuel cells, which could provide power, for example, for small transmitter systems [119]. An emerging field of application is the synthetic

chemistry, where laccases have been proposed be applicable for oxidative deprotection [120] and production of complex polymers and medical agents [121]
Application of laccases in bioremediation are widespread: laccases from different sources have shown to be able to degrade several xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) [122], 2,4,6-trinitrotoluene [123], chlorinated phenolics [124], pesticides [125] and synthetic dyes. They have been successfully applied in Kraft pulp bleaching [126] or in detoxification of agricultural by-products [127] including olive mill wastes and coffee pulp [128].

## 7. Application of the white-rot fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium* to dye decolourisation: state of art

The white-rot fungus *P. ostreatus* belongs to a subclass of white-rot fungi that produces laccases, MnPs, but not LiP. Multiple laccase genes encoding isoenzymes with interesting structural and functional properties are expressed by this fungus. The production of each isoenzyme is differentially regulated by culture conditions. So far, five isoenzymes secreted by the mycelium have been purified and characterized: POXC [129], POXA1w [130], POXA1b [131], and, more recently, the two strictly related isoenzymes POXA3a and POXA3b [132]. POXC is the most abundantly produced in all growth conditions tested so far; POXA1w shows peculiar differences with regard to metal ions content, containing two zinc atoms, one iron atom, and only one copper atom per molecule; POXA1b is the most stable at alkaline pH and shows activity inside the cell or on the cell wall [133]; POXA3a and POXA3b are heterodimeric laccases. Studies on laccase encoding genes have also led to the identification of four different genes and of the corresponding cDNAs, *poxc* (previously named *pox2*) [134], *pox1* (which codes for a laccase isoenzyme not identified yet) [135]), *poxa1b* [136] and *poxa3* [132].
The expression of multiple isoforms of Manganese peroxidases was also reported in *P. ostreatus*. Three isoenzymes (Mnp1, MnP2 and MnP3) have been purified and characterized, whose level of production was shown to be improved in the presence of $Mn^{2+}$ during solid state fermentation [137].
Moreover, this fungus produces a veratryl alcohol oxidase that can participate in the lignin degradation process, either reducing the radical intermediates formed by laccase or MnP, thus preventing their repolymerization, or producing hydrogen peroxide necessary for MnP action [138].
*P. ostreatus* was shown to be able to decolourise a representative antraquinonic dye, RBBR, in solid and liquid culture and the key role played by laccases in this process has been demonstrated. As a fact, when purified enzymes, POXC and POXA3 were used in *in vitro* decolourisation experiments, up to 70-80% colour removal was achieved in the absence of any redox mediators. Interestingly, these laccases differ significantly in their efficiency of decolourisation of the tested dye, showing different catalytic efficiencies ($K_{cat}/K_M$ values) towards RBBR. Furthermore, using a mixture of both POXC and POXA3 an improvement in the reaction rate and in the final level of dye decolourisation were observed, suggesting a concerted action of the two isoenzymes in the decolourisation process [79].
In addition, a crude laccase mixture from *P. ostreatus* cultures was immobilized by entrapment in copper alginate beads and successfully employed in RBBR decolourisation both in batch and in a fixed bed bioreactor. The system provided good decolourisation performances together with a remarkably increase in stability of the immobilized laccases in comparison with the free enzyme preparation [139].

*P. chrysosporium* is a white-rot fungus, able to produce several classes of extracellular peroxidases, such as Manganese and Lignin peroxidases. Its decolourisation ability is well documented in scientific literature with also reported examples of dye mineralization [47]. In one of the earliest studies, Glenn and Gold [140] showed that synthetic dyes were decolourised by this fungus and that inhibitors of ligninolytic activities also inhibited the process of colour removal. In subsequent works, Manganese peroxidases [141] or Lignin peroxidases [142], have been described as the main enzymes involved in decolourisation by this fungus. A novel family of multicopper oxidases has also been described [101] but its involvement in decolourisation processes has never been demonstrated.

## 8. Aim of the thesis

White-rot fungi and their 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 WRF potential by developing new biosystems for the treatment of industrial coloured wastewaters.
Work description has been organised in the following sections:

1) Application of new biosystems for the treatment of industrial coloured wastewaters
   Different kinds of biosystems based on the white-rot fungi *P. ostreatus* and *P. chrysosporium* have been developed and tested for the biodegradation of model wastewaters from textile industry. Both whole-cell and enzyme-based (in free and immobilized form) processes have been explored.
   Biosorption on fungal biomasses (*Mucorales* fungi) was also investigated as a parallel approach in collaboration with University of Turin.
   The performances of the analysed biosystems have been evaluated and compared in terms of decolourisation ability, COD (Chemical oxygen demand) and toxicity reduction of the treated effluent.
2) Isolation of new members of *P. ostreatus* laccase family and their characterisation through heterologous expression:
   Taking into account the role played by laccase in the decolourisation process, the *P. ostreatus* laccase family was investigated in order to enrich the knowledge of this enzyme, thus improving their potential application to wastewater treatment. The assortment of *P. ostreatus* laccases was enlarged up to seven members, by isolation, cloning and heterologous expression of new laccase genes.

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# Section 1

## Application of new biosystems for the treatment of industrial coloured wastewaters

# *Chapter 1*

Whole-cell and free enzyme-based biosystems

Biodegradation
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ORIGINAL PAPER

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

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

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

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## Introduction

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

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

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

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

## Materials and methods

### Microorganisms

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

### Dye containing wastewater models

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

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

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

**Table 1** Composition of colored wastewater models

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

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

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

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

## Enzyme assays

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

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

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

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

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

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

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

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

### Laccase isoenzyme fractionation

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

### Non-denaturing polyacrylamide gel electrophoresis

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

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

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

### Immobilization of laccase mixtures from *P. ostreatus*

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

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

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

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

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

### Analysis of detoxification abilities

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

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

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

### Analysis of COD

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

## Results and discussion

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

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

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

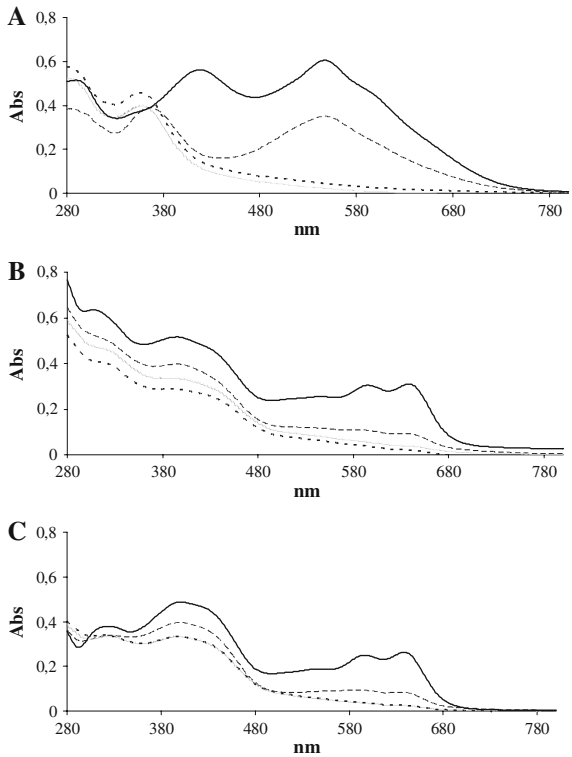


**Fig. 1** Light absorption spectra between 280 and 800 nm of: direct dye wastewater treated by *P. chrysosporium* (**a**), acid dye wastewater treated by *P. ostreatus* in the presence (**b**) and in the absence (**c**) of malt extract, after 0 (——), 1 (– – –), 7 (·····) and 14 days (·····) of treatment

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

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

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

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

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

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

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

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

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

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

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

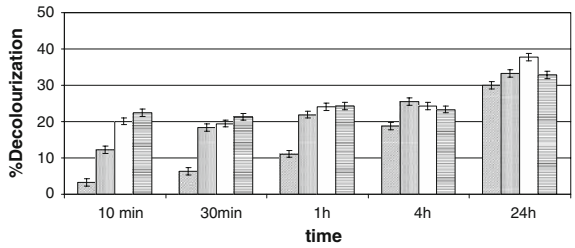


**Fig. 2** Percent of decolourization of acid dye wastewater model after incubation with various amounts (0.1 U/ml, ▨; 1 U/ml, ▩; 2U/ml, □; 10 U/ml, ▤) of laccase mixture, at room temperature, and recording UV-VIS absorption spectra at different times (10 min, 30 min, 1 h, 4 h, 24 h)

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

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

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

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

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

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

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

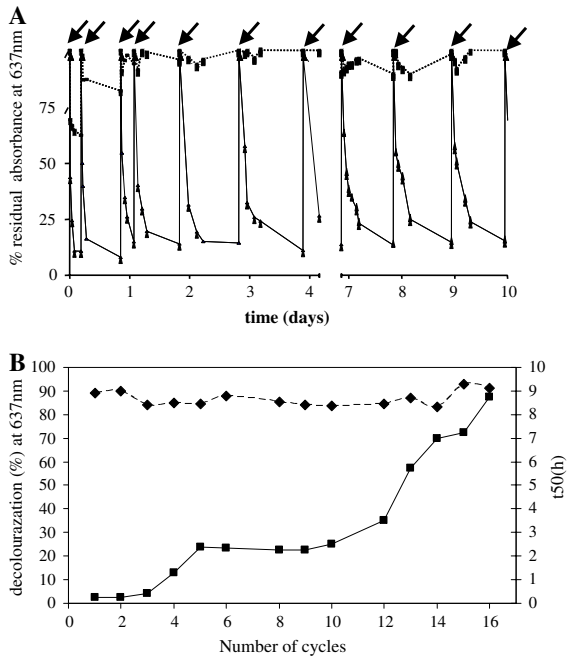


**Fig. 3** Decolourization of acid dye wastewater model obtained by crude laccase mixture entrapped in copper alginate beads. (**a**) Decolourization percentage (residual absorbance at 637 nm) during 10 cycles (▲) compared to that obtained in the presence of alginate beads with no enzyme entrapped (■).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

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

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

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

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

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

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

## Analyses of detoxification and COD reduction abilities

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

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

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

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

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

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

## Conclusions

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

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

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

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

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# *Chapter 2*

Immobilized enzyme-based biosystem

## Immobilization of a laccase mixture on perlite

### 1. Introduction

Extensive research efforts have been dedicated to evaluate the potential applications of laccases in bioremediation processes, particularly for the treatment of industrial coloured wastewaters. However, any biotechnological use of laccases may be hampered by their non-reusability as well as by the high sensitivity to the harsh conditions provided by industrial effluents, i.e. extreme pH values, high salt concentration, presence of organic agents and detergents. Many of these undesirable constraints may be removed by the use of immobilized enzymes. In addition to a more convenient handling of enzyme preparations, the desirable benefits of enzyme immobilization are the easy separation of the enzyme from the reaction volume and reuse of the enzyme. These advantages, coupled to the possibility to employ enzymes in continuously operated bioreactor, are especially attractive to cope with the huge volume of polluted wastewaters demanding remediation. Moreover, it is widely demonstrated that immobilization can improve enzyme stability against thermal and chemical denaturation, by decreasing their flexibility and thus resulting in more suitable catalysts for industrial applications. Several techniques may be applied to immobilize enzymes on solid supports [1], they are mainly based on chemical and physical mechanisms. Both methods offer advantages and drawbacks, thus the choice of a proper technique depends on the application to which immobilized enzymes are addressed. The simplest immobilization techniques are based on the physical adsorption of enzymes on solid carriers. This method provides high enzymatic activity bound to the solid phase since it slightly modifies the enzyme native structure. However, adsorption phenomena depend on ionic strength and pH of the liquid phase so the biocatalyst can suffer from high enzyme leakage, in particular under the harsh and variable conditions provided by the treated wastewaters. The same constraints are valid for entrapment based methods, in which enzyme molecules are entrapped into a proper matrix by a purely physical caging or by covalent binding. In this case, the mechanical properties and the stability of the matrix limit the applicability of this technique to wastewater treatment. Covalent immobilization represents an attractive alternative to obtain enzymatic catalyst for wastewater treatment. This method provides twofold enhancement of biocatalyst performance: i) covalent bonding prevents enzyme leakage even under harsh conditions, ii) enzyme deactivation rate is reduced comparing with that characteristic of enzyme in liquid phase. The stabilization provided by covalent bonding is counterbalanced by partial deactivation occurring when the immobilization is performed. Covalent linkages to the support usually perturb the protein native structure resulting in a reduction of the initial activity available in the liquid enzyme solution. This negative effect can be mitigated by carefully optimizing the immobilization conditions in order to maximize the ratio between immobilized enzyme activity and activity of the primary enzyme solution.

In a previous chapter, *P. ostreatus* laccase immobilization by entrapment in copper alginate beads has been described. Although this system provided high immobilization yields coupled to an effective Acid wastewater decolourisation in batch system, it seems to be not suitable to be employed in continuous decolourisation of dye mixtures, due to the low mechanical stability of alginate beads. Thus, covalent immobilization techniques have been investigated. It is well known that many factors influence the final properties and the performances of covalent

enzyme-carrier systems: the morphology of the carrier (e.g. gel type, monomers, cross-linker, porosity), the polymers chemistry (e.g. type of active groups, their concentration, hydrophobic/hydrophilic balance on the carrier surface) as well as the activation and immobilization procedures (chemical groups involved and accessibility of amino acids for attachment). This means that the selection of the most suitable carrier for a given enzyme may require the screening of many supports and immobilization procedures.
Russo et al. [2] investigated the decolourisation performances of *P. ostreatus* laccases covalently bounded on a commercially available activated carrier (EUPERGIT C 250L©). They assessed dye conversion kinetics of the immobilized laccase mixture. Moreover, they performed a theoretical study that highlighted the relevance of the balance between immobilization efficiency and immobilized laccase deactivation rate on the performance of a continuous decolourisation process carried out in a fixed bed reactor loaded with immobilized laccases.

On the basis of these results, laccase covalent immobilization on a purposely activated inert support has been performed. To this aim, a siliceous inert carrier, perlite, was chosen. Perlite is derived from naturally occurring siliceous rock. It is an amorphous aluminium silicate with more than 70% content of silica. This carrier exhibits several advantages over organic ones, including a greater mechanical stability, resistance toward microbial attack and organic solvents. Several inorganic support materials, such as silica gels, alumina, zeolite have been successfully used for immobilization of enzymes; however, perlite turns out to be a cheaper alternative in comparison to the above mentioned materials.
The choice of such an inert support implies that its surface has to be properly modified in order to offer functional groups for protein binding. Silica based materials modified by inorganic and by organic functional groups have been arouse considerable interest due to the number of possible applications [3] As a matter of fact, surface modification of these materials can be easily achieved and their reactivity may be finely tuned in the derivatization steps [4]. Chemical modification of perlite is mainly related to the presence of silanol groups on its surface. Surface modifications are usually achieved by silanization with appropriate organosilane agents, followed by activation with several crosslinking agents (i.e. glutaraldehyde, carbodimmide).
Silanization is a crucial step in regard to subsequent reproducibility of the chemical functionalization. It provides the reactive amino groups susceptible to the following activation with glutaraldehyde. Although silanization has been extensively studied, the resulting surface coverage, orientation and organization are still a subject of controversy [5]. Despite it resulted in a lower surface concentration of amino groups in comparison with reaction in organic solvents, aqueous silanization with a trifunctional silane APTS (aminopropyltrimethosysilane) has been selected for this study, since it was demonstrated to result in a more stable and uniform immobilized enzyme layer [6].
Glutaraldehyde is a bi-functional reactive agent which has been extensively used as an enzyme immobilizing agent. Although there are many discussions on the composition of the glutaraldehyde solution (monomeric and polymeric forms) and on the structures responsible for its properties, it is generally accepted that it is able of reacting with the surface amine groups of enzyme and carriers, through the formation of Schiff bases and Michael adducts [7].

In the following section the optimization of immobilization technique, including carrier activation, is described. Furthermore, the biocatalyst obtained was preliminary tested for the conversion of the reactive dye Remazol Brilliant Blue R.

## 2. Materials and Methods

*Production of Laccase mixture*

*P. ostreatus* culture conditions and crude laccase extraction were performed according to protocols described in chapter 1.

*Dye*

The anthraquinonic dye Remazol Brilliant Blue R (RBBR) was purchased from Sigma-Aldrich. Powder purity was 50%. Dye concentration was measured by recording optical absorbance at 592nm. The extinction coefficient ($\varepsilon_{592}$=9,000$M^{-1}cm^{-1}$), referred to total powder concentration, was corrected taking into account the purity.

*Perlite pre-treatment and derivatization*

Perlite (SIPERNAT 22©) was provided by Degussa. Solids were sieved in the range 90-150 μm Perlite was pre-treated with 1.2M $HNO_3$ at 60°C for 4 hours following, it was extensive washed with water and dried at 60°C. Carrier derivatization was performed as follows: 0.2g of dry pre-treated perlite was mixed with 4÷0.4% APTS (aminopropyltrimethoxysilane) in 5mL dissolved water and incubated at 80°C for 2h with constant mixing. Then, the suspension was washed thoroughly with 50mM Sodium Phosphate (NaP) buffer pH 6.5 and treated with glutaraldehyde solutions, at different concentration in the same buffer for 2h at room temperature. The activated perlite was extensively washed with the over cited buffer and finally incubated for 1h with a solution of laccase mixture in 50mM Sodium Phosphate buffer pH 6.5 at room temperature. Residual active sites were saturated by 1h incubation with 100mM glycine at room temperature.

*Adsorption experiments*

Dye adsorption on solid particles was determined by incubating 0.2g of solid in a RBBR solution at a pre-set concentration. All the experiments were performed in the conditions usually adopted during dye conversion tests: 20mM Sodium Acetate pH 4.5 at room temperature. Dye concentration in the liquid phase was measured by monitoring optical absorbance at 592nm. The occurrence of adsorption was revealed by the decrease of absorbance in the liquid phase, as well as by an increase of solids colouration. Each experiment was accomplished putting in contact dye solution with the solid particles, once no further change of dye concentration in the liquid was measured (achievement of equilibrium between solids and liquid) the liquid was replaced with fresh dye solution. The cycling operation was repeated until no change in the initial concentration of dye was observed. The overall amount of adsorbed dye was calculated from the difference between the initial and final concentration in the liquid phase for each cycle.

*Assay of immobilized enzyme*

Activity of laccase immobilized on perlite was estimated by measuring the oxidation rate of ABTS in a recirculating fixed bed reactor previously designed for the assessment of activity of enzymes immobilized on granular solids [2]. The device is equipped with a fixed bed reactor, loaded with biocatalyst particles, operated by circulating the liquid phase containing the substrate. The operating conditions were purposely selected in order to prevent mass transfer limitations during the enzymatic conversion so that the assay was carried out under kinetic controlled regime. The operating conditions were suitably adapted to the perlite based biocatalyst. In

particular, the total volume of the reactor and was set at 72mL, the tubular reactor was packed with a fixed amount of solid biocatalyst of 0.18mL, and the liquid was circulated by means of a gear pump at a flow range of 20mL/min.

*Fluidized bed reactor for RBBR conversion*

Conversion of RBBR by immobilized laccases was investigated in a fluidized bed reactor continuously operated with respect to the liquid phase. A sketch of the apparatus is shown in figure 1.

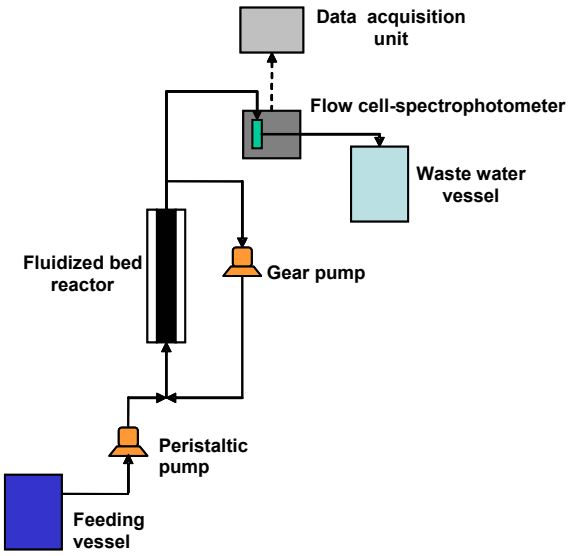


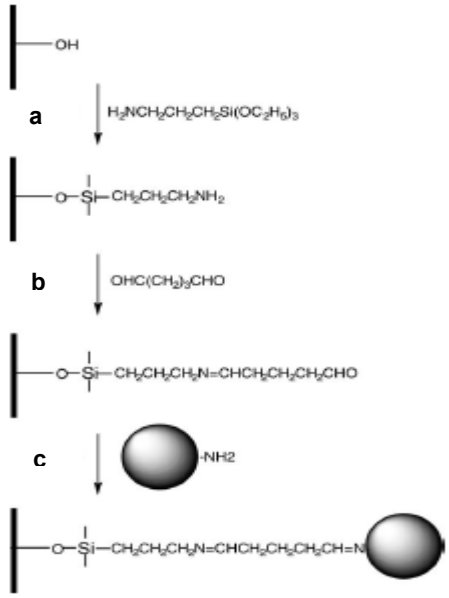

**Fig. 1: Fluidized bed reactor employed for RBBR decolourisation by the means of immobilized laccases**

Biocatalyst particles are placed in a tubular reactor (2.5cm ID, 30cm long). Solid particles were fluidized by the liquid stream delivered by a gear pump (VG 1000 digit, Verder). Dye-bearing liquid solution was fed at different flow rates by the means of a peristaltic pump (Miniplus, Gilson) connected to the feed tank. Dye concentration in the waste stream was measured continuously by means of a spectrophotometer (Cary 50, Varian Inc.) equipped with a flow-cell. Optical absorbance was measured at 592nm. Dye conversion was carried out in conditions assessed as optimal for RBBR decolourisation: 20mM Sodium acetate pH 4.5 buffer at room temperature [8].

## 3. Results and discussion

**Optimization of immobilization process on activated perlite**

In this section the results on the optimization of immobilization of the crude laccase preparation on perlite are reported. The procedure has been described in material and methods section and is schematically drawn in figure 2.




Activity of laccase immobilized on perlite was measured employing a specifically designed device as described in material and methods section [2]. Immobilization yield (Y) is the parameter monitored to optimize the immobilization protocol. It was defined as the ratio between laccase activity expressed by solids biocatalyst and total activity available in the liquid solution at the beginning of the immobilization processes.

**Fig.2: Three step chemical procedure used for laccase immobilization on perlite. a)silanization of pre-treated perlite surface; b)reaction of alkylamine-derivatized perlite surface with glutaraldehyde; c) immobilization of laccase on activated support (Blasi et al. 2005)**

Process optimization has been

carried out assessing the effect of the following operating conditions on the immobilization yield: glutaraldehyde concentration, pH and ionic strength of immobilization solution, time and temperature of incubation, total activity and total protein contents. Table 1 reports the results, in terms of immobilized activity and yield, for each run performed with the adopted conditions.

| Run | Initial activity (IU/g) | Initial protein (mg/g) | Glutaraldehyde % vol | pH (*) | Incubation time and Temperature | Immobilized activity (IU/g) | Immobilization yield (%) |
|---|---|---|---|---|---|---|---|
| R1 | 550 | 4.5 | 1 | 6.5 | 4h RT | 200 | 36 |
| R2 | 550 | 4.5 | 1 | 6.5 | Over night 4°C | 128 | 23 |
| R3 | 550 | 4.5 | 2.5 | 6.5 | 4h RT | 206 | 37.5 |
| R4 | 550 | 4.5 | 2.5 | 6.5 | Over night 4°C | 151 | 27 |
| R5 | 550 | 4.5 | 5 | 6.5 | 4h RT | 206 | 37 |
| R6 | 550 | 4.5 | 5 | 6.5 | Over night 4°C | 236 | 43 |
| R7 | 550 | 7.75 | 1 | 6.5 | 1h RT | 160 | 29 |
| R8 | 550 | 7.75 | 1 | 6.5 | 4h RT | 161 | 29 |
| R9 | 550 | 7.75 | 0.5 | 6.5 | 1h RT | 186.5 | 34 |
| R10 | 550 | 7.75 | 0.5 | 6.5 | 4h RT | 178 | 32 |
| R11 | 80 | 1.33 | 0.5 | 6.5 | 1h RT | 56.5 | 70 |
| R12 | 285 | 4.75 | 0.5 | 6.5 | 1h RT | 136.5 | 48 |
| R13 | 800 | 11.25 | 0.5 | 6.5 | 1h RT | 199 | 25 |
| R14 | 500 | 4.25 | 0.5 | 6.5 | 1h RT | 173.5 | 35 |
| R15 | 500 | 12 | 0.5 | 6.5 | 1h RT | 173.5 | 35 |
| R16 | 500 | 22.5 | 0.5 | 6.5 | 1h RT | 184.5 | 37 |
| R17 | 275 | 2.25 | 0.5 | 5.5 | 1h RT | 52 | 19 |
| R18 | 275 | 2.25 | 0.5 | 6.5 | 1h RT | 137 | 50 |
| R19 | 275 | 2.25 | 0.5 | 7.5 | 1h RT | 120 | 43 |
| R20 | 275 | 2.25 | 0.5 | Mcl 5.5 | 1h RT | 32 | 12 |
| R21 | 275 | 2.25 | 0.5 | Mcl 7.5 | 1h RT | 66 | 24 |
| R22 | 275 | 2.25 | 0.5 | Mcl 6.5 | 1h RT | 42 | 15 |

**Table1: Effects of different experimental parameters on laccase immobilization yield on perlite. Mcl: McIlvaine buffer; (*): All the incubations were performed in 50mM Sodium Phosphate buffer, pH 6.5, unless otherwise indicated**.

*-Effect of glutaraldehyde concentration*

The effect of glutaraldehyde concentration on immobilization yield has been investigated in the range 0.5-5% vol. Results reported in Table 1 (runs 1-3-5-8-10) indicate that immobilization yield is not affected by increasing glutaraldehyde concentration up to 5%. It is worth to note that, in all the tested conditions, no laccase activity or protein were detected in the recovered supernatant, indicating that 100% of the initial protein content of the crude laccase mixture was bounded to the carrier.
These results indicate that the reactive groups obtained with the lowest glutaraldehyde concentration are sufficient to bind the total protein content of the laccase mixtures. Thus, further immobilization experiments were carried out setting glutaraldehyde concentration at 0.5% vol.

*- Effect of pH and ionic strength*

One of the factors that can affect enzyme immobilization is the pH-value of the coupling mixture. The results obtained performing laccase immobilization at pH values 5.5, 6.5 and 7.5 are listed in table 1 (runs R17-R22). The immobilization yield decreased at lower pH values, while it remains constant in the neutral pH range. When the immobilization was carried out at higher ionic strength (runs 21-22) a significant reduction of immobilization yield was observed. In the latter conditions, up to 28% of initial laccase activity was detected in the supernatants. These findings could be a consequence of the heterofunctional nature of the activated matrix: after glutaraldehyde activation, the support may expose unreacted amino-groups which confer some ionic exchanger features to the support [4]. In such type of heterofunctional matrices, a first ionic adsorption of the protein on the amino groups of the support was found to occur before the covalent reaction. Thus, high ionic strength or certain pH values can promote this physical interaction, resulting in lower immobilization yields.

*- Temperature and time of incubation*

The effect of incubation temperature on immobilization yield has been assessed comparing the experiments R1-R10. Lowering the incubation temperature to 4°C did not result in an improvement in the immobilization yield with respect to the reaction carried out at room temperature. In addition, extending the incubation time to over-night incubation (at 4°C) caused a decrease in immobilization yield (compare R1-R3 with R2-R4) or resulted in almost comparable results (compare R5 to R6) with respect to 4 hours room-temperature incubation.

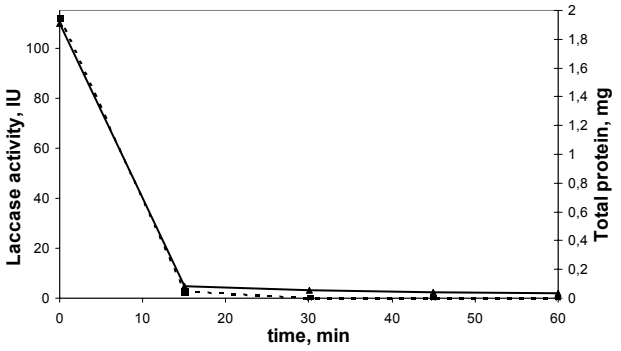



**Fig.3: Laccase activity (IU) and total protein content measured in the liquid phase during the immobilization process (—) Laccase activity; (---) Total protein content**

Fig 3 reports the data of laccase activity and total protein content recorded in the liquid supernatant as a function of the incubation time. After 15 minutes incubation, both measured values become negligible, indicating the immobilization of all the proteins contained in the crude mixture. Hence, further experiments were accomplished fixing incubation time at 1h without any temperature control.

*- Effect of total activity and total protein contents*

A few immobilization tests were carried out by changing the ratio between initial laccase activity and mass of dry support in the range 80-800IU/g (experiments R11-R13). For a given amount of dry support, results showed that the immobilization yield decreased with increasing amount of initial laccase activity while the immobilized activity (IU/g) tend to a constant value (FIG.4).

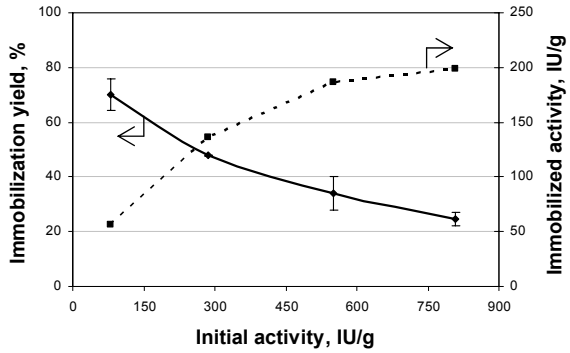



**Fig.4: Immobilization yield versus laccase initial activity. (—) immobilization yield; (---) immobilized activity**

It can be speculated that this effect could be a consequence of protein overcrowding on the carrier surface which may reduce substrate accessibility to the active site.

However, the subsequent series of experiments (runs R14-R16) enabled to rule out this hypothesis. As a fact, when protein content, corresponding to a certain activity (IU/g), was increased by adding BSA no decrease in immobilization yield was observed, as it was expected if the above hypothesis was verified. Moreover, it is worth to note that proteins were completely bounded to the support even at the highest concentration. Both series of data (R11-13 and R14-16) can be accounted for considering that access of substrate to immobilized enzyme was restricted by the irreversible sealing of carrier micro-pores by polymeric components contained in the crude mixture, whose amount increases when a more concentrated laccase mixture was used.

On the basis of the above-mentioned results, the optimal parameters selected are: i) solids activation with 0.5% glutaraldehyde, ii) 1h incubation with laccase mixture (50mM Sodium Phosphate buffer, pH 6.5, 80IU/g support) at room temperature. In these conditions, a maximum immobilization yield of about 70% was achieved. As expected, there is a difference between the actual laccase activity expressed by the biocatalyst and the activity loss in the liquid supernatant. This effect is due to the modifications of the enzyme structures occurring during covalent immobilization: non-productive enzyme orientation, decreased protein flexibility resulting form multi-point attachment, and involvement of crucial amino acids in covalent binding.

## Assessment of stability and catalytic parameters

The performances of the immobilized biosystem have been tested assessing its catalytic parameters and stability. In order to have a suitable amount of immobilized activity on an easily handling amount of solid support, an enzyme support ratio of about 290IU/g was used so, with the aforesaid optimized conditions, an immobilization yield of about 45% was obtained.

Storage stability of the immobilized laccase mixture has been monitored both at room temperature and at 4°C and was compared with that observed for free laccase mixture. Residual activity were determined and expressed as percentages at different time intervals. As showed in Fig.5, the immobilized mixture stored at room temperature displays about 7 fold increased stability with respect to the free laccase mixture ($t_{1/2}$ free enzyme=1.6 days; $t_{1/2}$ immobilized enzyme=11.6 days); while

stability at 4°C shows $t_{1/2}$ increased of about 3 fold ($t_{1/2}$ free enzyme=18.5 days; $t_{1/2}$ immobilized enzyme=61 days).

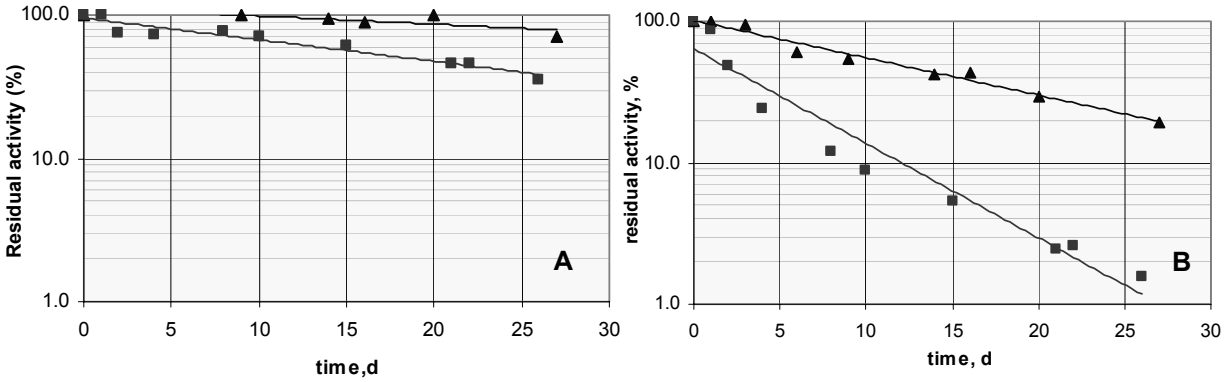



**Fig.5: Stability of immobilized and free laccase mixture monitored at 4°C (A) and at room temperature (B). (Free enzyme ■, Immobilized enzyme ▲)**

The enhanced stability of immobilized laccase may be due to the prevention of structural rearrangement and to the lower flexibility of immobilized form both caused by multipoint attachment to the support [9]. Moreover, immobilization can provide an artificial micro-environment surrounding the enzyme that can alter surface-exposed hydrophilic and/or charged groups and their electrostatic interactions and thus influencing protein structure and function. In particular, the immobilized enzymes were doubly protected by thermal inactivation thanks to the higher number of positive charges on the surface and to the increased hydrophobicity both provided by silanization [10].

To perform a kinetic characterization of the immobilized biocatalyst, purified laccase POXC from *P. ostreatus* has been used rather than the crude mixture. The purified enzyme has been immobilized in the following conditions: 0.2g of activated perlite were incubated for 1h with purified POXC solution (50mM Sodium Phosphate buffer, pH 6.5, 250IU/g support as initial activity) at room temperature, resulting in about 40% immobilization yield (100IU/g support).

Kinetic of immobilized POXC against ABTS has been assessed by means of the recycling fixed bed reactor, commonly employed for activity measurements. Results provided $K_M$=0.44 mM, $K_{cat}$= $1.2 \cdot 10^4$ $min^{-1}$. Comparing these parameters with those characteristics of POXC in liquid phase ($K_M$=$3.0 \cdot 10^{-2}$ $min^{-1}$, $K_{cat}$= $6.2 \cdot 10^5$ $min^{-1}$) both a decreased value for $K_{cat}$ and a higher value for $K_M$ were found. These differences could be a consequence of either the loss of conformational integrity of the immobilized enzyme or lower accessibility of substrate to the active sites of the immobilized enzyme.

## Adsorption

The immobilized system was tested for its decolourisation potential against the model anthraquinonic dye Remazol Brilliant Blue R (RBBR). First of all, the phenomenon of dye adsorption on carrier surface was investigated. Dye adsorption is a fundamental aspect to be addressed in assessing dye conversion by immobilized enzyme, since enzymatic conversion can be masked by rapid adsorption kinetics and large adsorbing capacity of the solid support. Moreover, the adsorption of dyes, as well as of their oxidized products, on the immobilization support can lead to enzyme

inactivation/inhibition [1]. For these reasons, the main goal of this analysis was to quantitatively assess RBBR adsorption on the activated carrier in order to minimize any adverse effect on dye conversion.
No adsorption of RBBR was observed on the inert perlite indicating that the adsorption phenomenon is related to the functional groups resulting from silanization or glutaraldehyde activation. Then, the sample was prepared adopting the optimal conditions defined in the previous section. Results revealed about 71mg RBBR/g perlite. Enzymatic assays performed after dye saturation showed that immobilized laccase activity was completely hindered.
These findings required proper modification of the immobilization protocol in order to limit dye adsorption to a lower value. The effect of several parameters on adsorption capacity and immobilization yield was investigated. In particular, the parameters affecting the surface properties of the support (i.e. APTS concentration and pH, temperature, concentration for glutaraldehyde crosslinking) were tuned.
Results are summarized in Table 2. The adsorption capacity of the silanized perlite rises up to 96.1mg/g (A2 in table 2) with respect to the null adsorption capacity of the untreated perlite. As a fact, reducing the APTS concentration form 4 % to 0.4% vol, keeping the other conditions constant, the adsorption capacity was approximately halved and an undesired drop of immobilization yield was observed. The extent of perlite silanization likely determined the concentration of active amino groups on the carrier surface, a parameter that plays a key role in the adsorption of the dye. Thus, the improvement of the condition for glutaraldehyde activation would provide a reliable solution to saturate the free amino groups available for the interaction with the anionic dye. In run A4 two consecutive activation steps with glutaraldehyde were assessed. However, the adsorption was not significantly affected by this treatment. Rising pH from 6.5 up to 8 during glutaraldehyde reaction provided a further decrease of immobilization yield down to 16.4% together with a negligible effect on adsorption capacity (compare A5 with A3). This effect was assessed on carrier silanized with 4 or 0.4% APTS solutions (Run A5-A6). Finally, an effective reduction of RBBR loading (32.9mg/g), coupled with a satisfying immobilization yield (51.2%), was achieved by incubating the solid silanized with 0.4% APTS with 1% glutaraldehyde at pH 8 and 60°C [6]. The latter operating conditions were selected for further investigation of dye degradation by laccase immobilized on perlite.

| Perlite sample | APTS (%) | Glutaraldehyde activation | Adsorption capacity (mg RBBR/g solid) | Immobilization Yield (%) |
|---|---|---|---|---|
| A1 | 4 | 0.5% (pH 6.5) | 71 | 45 |
| A 2 | 4 | - | 96.1 | - |
| A 3 | 0.4 | 0.5% (pH 6.5) | 28 | 28.6 |
| A 4 | 4 | 0.5% (pH 6.5, 2X) | 64 | - |
| A 5 | 0.4 | 0.5% (pH 8) | 31 | 16.4 |
| A 6 | 4 | 0.5% (pH 8) | 64 | 38.3 |
| A 7 | 0.4 | 1% (pH8 60°C) | 32.9 | 51.2 |

**Tab.2: Adsorption capacity of perlite samples obtained in different experimental conditions**

**Decolourisation experiments**

Decolourisation experiments, aimed at assessing RBBR conversion by immobilized laccases, were carried out in a fluidized bed recycle reactor. The choice of the reactor arises from some observations occurred during preliminary decolourisation attempt performed in a fixed bed reactor. Fixed bed of inert perlite particles were successfully operated on bench scale notwithstanding considerable pressure drops occurred. Unfortunately, when the carrier was coated by the immobilized proteins, the operation of the reactor was completely hindered by *clogging* phenomena (liquid phase can not percolate packed bed).

The fluidized bed was chosen to get round this constraint. Liquid flow rate required for particles fluidization was supplied by the recirculating stream while the stream containing the dye was continuously supplied at a flow rate corresponding to residence time suitable for dye conversion.

Preliminary decolourisation experiments were carried out employing immobilized samples prepared according to two different procedures (C1 and C2 in table 3). Samples C1 and C2 exhibit comparable dye adsorption capacities but, they differed in the amount of immobilized laccase activity. Sample C1 was characterized by an initial activity of 10IU/ml, versus 28IU/ml of sample C2. The reactor was operated according to the following conditions. Total liquid volume was set at 71mL. The first step of the process consisted of saturation of the catalyst with the dye. It was operated by feeding a dye bearing liquid stream at 12.4mL/min without recycling. In this phase, particle fluidization was assured by the supplied liquid stream. The space-time ($\tau \cong 4$min) is set so that enzymatic conversion is negligible and solid saturation is reached within 2 hours. Once saturation was reached, the dye conversion was accomplished lowering inlet flow rate and introducing the recycle stream ($Q_R$ =13 mL/min). A sequence of steady regimes was established in the reactor by decreasing stepwise the liquid feed rate Q keeping $Q_R$ at 13 mL/min. Each step lasted until dye concentration in the effluent approached a steady value. Table 4 reports reactor space-times $\tau$ (i.e. liquid residence time) and dye conversion degrees corresponding to each steady state regime. Dye conversion degrees corresponding to each reactor space-time have been plotted in Figure 6.

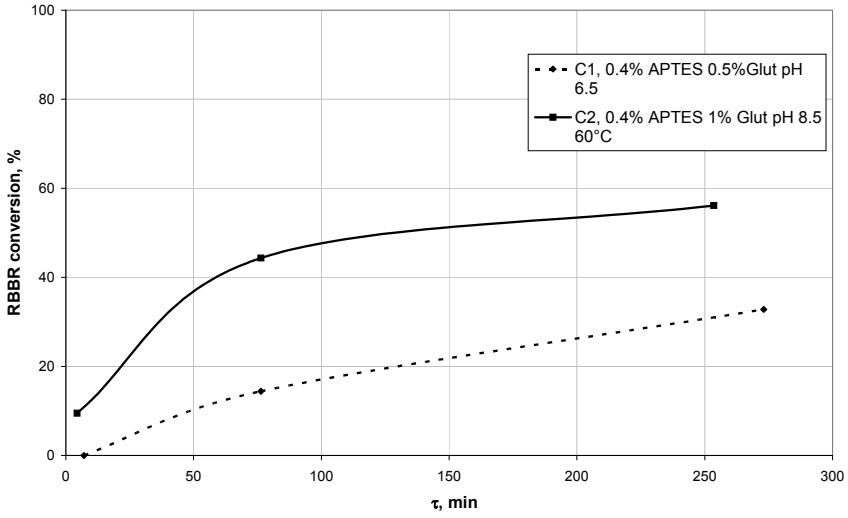



**Fig.6: RBBR conversion degrees plotted versus reactor space-time (τ) . Data refer to decolourisation experiments carried out in fluidized bed reactor operated with immobilized samples C1 and C2.**

| Perlite Sample | Immobilization conditions | Adsorption capacity (mg RBBR/g solid) | Initial activity (IU/mL) | Immobilization Yield (%) | Final activity, (IU/mL) |
|---|---|---|---|---|---|
| C1 | 0.4% APTS 0.5%Glut pH 6.5 | 27.5 | 10 | 23 | 3 |
| C2 | 0.4% APTS 1% Glut pH 8.5 60℃ | 33 | 28 | 51 | 9 |

**Tab.3: Immobilized enzyme samples employed in decolourisation experiments. Initial and Final Activity refers to laccase activity measured on solid carrier at the beginning and at the end of the decolourisation experiment**

| Perlite sample | Inlet RBBR concentration (mg/L) | Recycle Flow rate, Qr (mL/min) | Total Volume (mL) | Dye-feeding rate, Q (mL/min) | Recycle ratio | τ (min) | Outlet RBBR concentration (mg/L) | Dye conversion (%) |
|---|---|---|---|---|---|---|---|---|
| C1 | 41.5 | 13.0 | 71 | 10.0<br>0.9<br>0.3 | -<br>14<br>50 | 7.1<br>76.3<br>273.1 | 41.5<br>35.5<br>27.9 | -<br>14.4<br>32.8 |
| C2 | 39.1 | 13.0 | 71 | 16.0<br>0.9<br>0.28 | -<br>14<br>46 | 4.4<br>76.3<br>253.6 | 35.4<br>21.8<br>17.2 | 9.5<br>44.3<br>56.1 |

**Tab.4: Decolourisation experiments with laccase immobilized on perlite.**

The maximum conversion degree, performed with sample C1, was 33%, measured at a $\tau$=4.6 h. C2 sample provided 56.1% conversion when $\tau$ was equal to 4.2 h. Decolourisation runs with sample C1 and C2 lasted about 80 and 160h with a total volume of treated RBBR solution of 3.5L and 4.5L respectively. Comparing the results obtained with samples C1 and C2, it is evident that the better performance achieved by sample C2 was merely due to the larger amount of immobilized activity per volume unit of carrier provided with sample C2.
It is remarkable to note that, in both decolourisation experiments enzyme activity decays to about 30% of the initial activity, consistently with the similar amount of adsorbed dye (about 30mg/g carrier) showed by both sample C1 and C2. These results allow to confirm that the major biocatalyst deactivation is related with adsorption phenomena (occurring at the same extent for both samples), on the contrary the conversion degree (higher for sample C2) does not influence enzyme activity within the time range investigated (about 160h).
In conclusion, the procedure followed for preparation of sample C2 (table3), allowed to keep satisfactory values of immobilization yield, that mean satisfactory rate of decolourisation, and minimize the adsorption capacity of the solid biocatalyst. This second feature provides the minimum deactivation extent related to the initial fast adsorption phenomenon. The influence of this phenomenon on the overall performance of the bioprocess can be understood observing that the deactivation of the immobilized enzyme occurring during the dye conversion is negligible compared with that deriving form dye adsorption.

On the basis of the reported data, it is evident that further work will be needed to assess the applicability of such immobilized system to dye treatment. Kinetic parameters of dye conversion as well as of deactivation processes (adsorption-related and long-term deactivation) should be inferred. This detailed assessment can provide the tools for the comparison between the dye conversion process by means of immobilized enzyme and by means the free enzyme in homogeneous system.

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# *Chapter 3*

Biosorption-based processes

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# Decolourisation and detoxification of textile effluents by fungal biosorption

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ABSTRACT

Textile effluents, in addition to high COD, display several problems mainly due to toxicity and recalcitrance of dyestuffs. Innovative technologies effective in removing dyes from large volumes of effluents at low cost and in a timely fashion are needed. Fungi are among the most promising organisms for dye biosorption. In this study dye decolourisation, COD and toxicity decrease of three wastewater models after the treatment with inactivated biomasses of three Mucorales fungi cultured on two different media were evaluated. Fungal biomasses displayed good sorption capabilities giving rise to decolourisation percentages up to 94% and decrease in COD up to 58%. The *Lemna minor* toxicity test showed a significant reduction of toxicity after biosorption treatments, indicating that decolourisation corresponds to an actual detoxification of the treated wastewaters.



## 1. Introduction

The textile industry plays an important role in the world economy as well as in our daily life, but at the same time, it consumes a large quantity of water (up to 150 l of water to dye 1 kg of cotton) and generates huge amount of wastewaters (Hai et al., 2007).

Water is a rare and precious commodity, and only an infinitesimal part of the earth's water reserves (approximately 0.03%) constitutes the water resource that is available for human activities. The growth of the world's population and industry has given rise to a constantly growing demand for water compared to the supply available, which remains constant (Allègre et al., 2006). On the global level, the question of the supply of freshwater is becoming more acute every day because water is a valuable asset that must be protected. 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 the 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 (Alinsafi et al., 2006; Hai et al., 2007).

Given 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

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treated in conventional wastewater treatment plants and therefore dyes and their derivates accumulate in the environment (Vandevivere et al., 1998; Fu and Viraraghavan, 2002; Alinsafi et al., 2006). The presence of even trace concentrations of dyes in effluent is highly visible and undesirable. Actually, the release of coloured wastewater in the ecosystem is a remarkable source of aesthetic pollution, eutrophication and perturbations in aquatic life. Dyed effluents usually contain chemicals, including dye itself, that are toxic, carcinogenic, mutagenic or teratogenic to various organisms (Chen, 2006; Novotný et al., 2006; Mathur and Bhatnagar, 2007). Besides, azo and nitro compounds have been reported to be reduced in sediments of aquatic bodies, consequently yielding potentially carcinogenic amines that spread in the ecosystem (Chen, 2006; Hai et al., 2007). The presence of dyes or their degradation products in water even at very low concentrations can also cause human health disorders such as nausea, haemorrhage, and ulceration of skin and mucous membranes, and can cause severe damage to the kidney, reproductive system, liver, brain and central nervous system (Oliveira et al., 2007).

Toxic substances contained in dyed wastewaters should therefore be completely removed before being released into the environment. Physical and chemical purification methods, including the advanced oxidation processes (i.e. application of ozone, hydrogen peroxide, and ultraviolet light, either individually or in combination), are not always applicable. They always involve high costs and therefore their use is restricted in scale of operation and pollution profile of the effluent (Fu and Viraraghavan, 2001; Robinson et al., 2001; Crini, 2006; Hai et al., 2007) and they are practically ineffective. Cost competitive biological degradation is usually rather ineffective and is limited by the long time required and the requirement of microorganisms functioning in strictly controlled environments (Hai et al., 2007).

Recently, several researches have shown that biosorption can be regarded as a valid alternative to chemical–physical methods and to microbial and/or enzymatic biodegradation. Such researches have pointed out the capacity of various microbial biomasses (bacteria, yeasts, fungi and algae) and waste materials from industry and agriculture (i.e. pinewood, corncob, bagasse, rice husk, chitosan, etc.) to absorb or accumulate dyes (Crini, 2006; Ferrero, 2007). Among the various types of biomass, the fungal one has proved to be particularly suitable. Even if the mechanisms regulating biosorption have not yet been fully explained, it seems to take place essentially at the cell wall level (Aksu, 2005; Casieri 2005). The main attractions of biosorption are its high selectivity and efficiency, good removal from large volumes and the potential cost effectiveness. Moreover, both living and dead biomasses can be used to remove hazardous organics; dead cells are obviously preferable for wastewater treatment since they are not affected by toxic wastes and chemicals and do not pollute the environment by releasing toxins and/or propagules (Aksu, 2005). Actually, among the numerous techniques of dye removal, biosorption through activated carbons or organic resins is the procedure of choice and gives the best result as it can be used to remove different types of colouring materials despite the very high costs that point out the necessity to find alternative, less expensive biosorbent materials (Hai et al., 2007; Crini, 2008).

This paper illustrates the decolourisation capacities of the dead biomasses of three Mucorales fungi as displayed in batch biosorption experiments, towards three wastewater models designed to mimic wastes produced during cotton or wool textile dyeing processes. Biomasses were cultured on one medium with glucose as the carbon source and another with cheaper starch, since the production of biomass for industrial-scale bioremediation is expensive and the use of media with alternative carbon sources is a desirable objective. The COD measurements and the *Lemna minor* ecoxicity test were performed before and after treatments to estimate change in toxicity of effluents. Since chitosan is considered one of the most promising dye biosorbents and also the main cell wall component putatively involved in dye biosorption by Mucorales, we were interested in evaluating the actual efficiency of purified chitosan in comparison with the whole fungal cell wall. Hence, batch biosorption experiments were also performed using chitosan from crab shells as biosorbent.

## 2. Materials and methods

### 2.1. Test organisms and chitosan

*Cunninghamella elegans* Lendner (MUT 2861), *Rhizomucor pusillus* (Lindt) Schipper (MUT 2229) and *Rhizopus stolonifer* (Ehrenberg) Vuillemin (MUT 1515) were obtained from the *Mycotheca Universitatis Taurinensis* Collection (MUT, University of Turin, Department of Plant Biology). Starting cultures were lyophilised and cryopreserved until use. They were revitalised on Malt Extract Agar, and mature conidia for the inocula and biomass production were obtained from cultures grown on the same medium in the dark at 24 °C for 1 week.

Chitosan from crab shells was purchased from Fluka (St. Luis, MO).

### 2.2. Dyes and preparation of simulated wastewaters

Three simulated wastewaters, designed to mimic effluents produced during cotton or wool textile dyeing processes, were 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 structural 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 chemical–physical properties and chemical formula of the tested dyes are listed in Table 1. In addition to the dyes, these simulated wastewaters mimic the industrial 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 (300 ppm in total), and had an ionic strength of $4.23 \times 10^{-2}$ and pH of 5; the second wastewater (W2) contained a mix of 4 reactive dyes previously hydrolysed (5000 ppm in total), and had an ionic strength of $1.26 \times 10^{-1}$

**Table 1 – Dyes used in the study, their acronym, CI name, chromophore, chemical class and chemical structure**

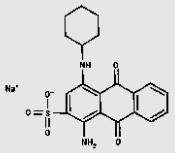
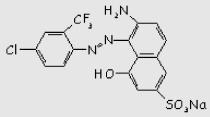
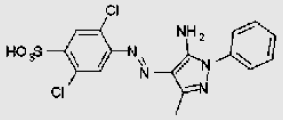
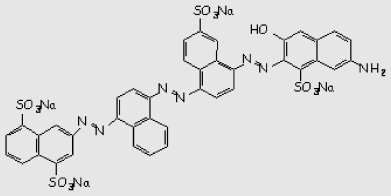
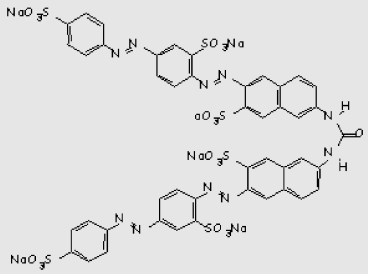
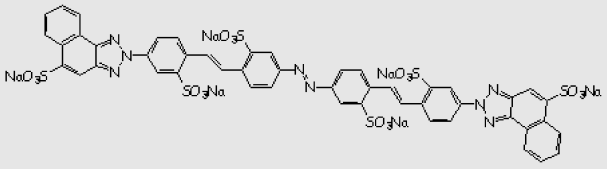
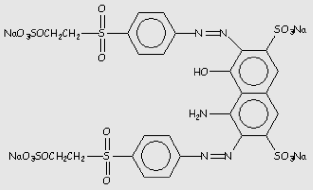
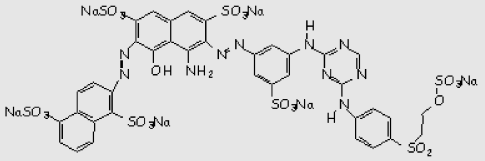
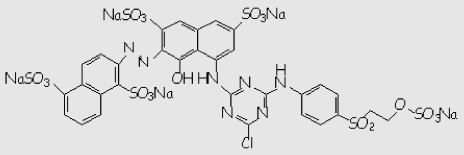
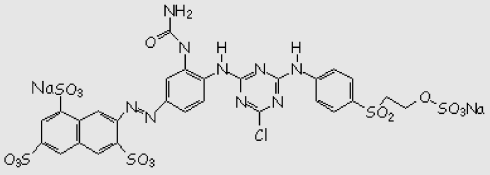
| Acronym | CI name | Chromophore | Chemical class | Chemical structure |
|---|---|---|---|---|
| ABu62 | Acid blue 62 | Anthraquinonic | Acid |  |
| AR266 | Acid red 266 | Azoic | Acid |  |
| AY49 | Acid Yellow 49 | Monoazoic | Acid |  |
| DrBu71 | Direct blue 71 | Trisazoic | Direct |  |
| DrR80 | Direct red 80 | Polyazoic | Direct |  |
| DrY106 | Direct Yellow 106 | Stilbenic | Direct |  |
| RBk5 | Reactive black 5 | Disazoic | Reactive |  |
| Rbu222 | Reactive blue 222 | Disazoic | Reactive |  |
| RR195 | Reactive red 195 | Monoazoic | Reactive |  |
| RY145 | Reactive Yellow 145 | Monoazoic | Reactive |  |

**Table 2 – Effluent composition and pH**

| Effluent | Dyes and salts | Concentration ($g l^{-1}$) | pH |
|---|---|---|---|
| Acid bath for wool (W1) | Abu62 | 0.10 | 5 |
| | AY49 | 0.10 | |
| | AR266 | 0.10 | |
| | $Na_2SO_4$ | 2.00 | |
| Reactive dye bath for cotton (W2) | Rbu222 | 1.25 | 10 |
| | RR195 | 1.25 | |
| | RY145 | 1.25 | |
| | Rbk5 | 1.25 | |
| | $Na_2SO_4$ | 70.00 | |
| Direct dye bath for cotton (W3) | DrBu71 | 1.00 | 9 |
| | DrR80 | 1.00 | |
| | DrY106 | 1.00 | |
| | NaCl | 5.00 | |

and pH of 10; the third wastewater (W3) contained a mix of 3 direct dyes (3000 ppm in total) and had an ionic strength of 1.48 and pH of 9. The exact composition of the 3 wastewaters is listed in Table 2. All the mimmicked wastewaters were sterilised by tindalisation (three 1 h cycles at 60 °C with 24 h interval between cycles at room temperature) before use.

### 2.3. *Fungal biomass preparation and batch biosorption experiments*

The two media used for biomass production were EQ (20 $g l^{-1}$ glucose, 2 $g l^{-1}$ ammonium tartrate, 2 $g l^{-1}$ $KH_2PO_4$, 0.5 $g l^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.1 $g l^{-1}$ $CaCl_2 \cdot 2H_2O$, 10 ml mineral stock solution) and ST, in which 18 $g l^{-1}$ potato starch is used instead of glucose (Fluka, St. Luis, MO).

Since the literature (Ellis et al., 1974) and preliminary tests had shown that *R. stolonifer* cannot use starch as a carbon source, it was cultured on EQ only.

Each isolate was inoculated as a conidial suspension (final concentration of $1 \times 10^5$ conidia $ml^{-1}$) in several 500 ml Erlenmayer flasks containing 300 ml of medium, and incubated at 30 °C for 7 days. To avoid production of aerial mycelium, rich in hydrophobins and poor in adsorbent, biomasses were cultured in agitated condition at 150 rpm with a Minitron Infors orbital shaker (Bottmingen, CH). After incubation biomasses were collected with a sieve (150 μm pore), rinsed several times with distilled sterilised water to remove residual medium and then inactivated in a 9 $g l^{-1}$ NaCl solution by autoclaving at 121 °C for 30 min. They were then collected in sterile conditions and rinsed as already described.

Each biomass was weighed and 3 g of biomass fresh weight or 0.5 g of chitosan dry weight (corresponding to about 3 g of fungal biomass fresh weight) were placed in 50 ml Erlenmayer flasks containing 30 ml of simulated wastewaters. The flasks were incubated at 30 °C in agitated conditions (150 rpm). Each trial was performed in triplicate. Simulated wastewaters without biomass were used as abiotic controls and to assess decolourisation other than that due to biosorption (e.g. photobleaching or complexation).

After 2, 6 and 24 h, 300 μl of simulated wastewaters were taken from each sample, centrifuged at 14,000 rpm for 5 min, to remove disturbing mycelial fragments, and examined with a spectrophotometer (Amersham Bioscences Ultrospec 3300 Pro, Fairfield, CT) to acquire the complete absorbance spectra of the effluents and to calculate the percentage of removed dye (DP, decolourisation percentage) as the extent of decrease of the spectrum area from 360 to 790 nm, with respect to that of the abiotic control. The influence of the incubation time on the biosorption yield was assessed from the increase in decolourisation from the 2nd to the 24th hour, calculated as (DP at T24−DP at T2) × 100/DP at T24.

At the end of the test the biomasses were filtered on filter paper (Whatman type 1), placed in an oven and dried at a temperature of 65 °C for 24 h; then they were weighed and the dry weight of the fungal biomass was calculated subtracting from the dried dyed biomass the amount of the adsorbed dye. The milligrams of adsorbed dye were calculated subtracting the residual amount of dye at the end of the experiment (obtained by the DP) from the initial amount of dye. It has thus been possible to calculate sorption capacity (SC) according to the following formula:

$$SC = \text{mg of removed dye/g of biomass dry weight}$$

The significance of differences ($P \leqslant 0.05$) among the DP values at 2, 6 and 24 h and among SC values was calculated with the Mann–Whitney test (SYSTAT 10 for windows SPSS Inc., 2000).

### 2.4. *COD determination and ecotoxicity test*

Determination of COD with the dichromate method was performed using HACH (COD High range vials) apparatus according to the manufacture's instructions. A calibration curve was obtained using HACH COD standard solution (800 mg $O_2 l^{-1}$). Appropriate dilutions of each sample were assayed.

The *Lemna minor* L. (duckweed) ecotoxicity test was applied to measure the toxicity of the simulated wastewaters before and after the biosorption treatments. The test was performed in triplicates, in 250 ml glass beakers according to the standard ISO/WD 20079 in a working volume of 150 ml and with a sample dilution of 1:10 (ISO 2001). Distilled water was used as the control in the test.

Ten fronds of *L. minor* (2 or 3 fronds per colony) of similar size were used as the inoculum. The test was carried out in a climatic exposure test cabinet, calibrated at 24 ± 2 °C, with fluorescent tubes on top that provided continuous lighting (light intensity, 100 $\mu E s^{-1} m^{-2}$) for 7 days.

At the end of the experiment, fronds number and plant dry weight were used to calculate the growth inhibition ($I_{\mu i}$) by

$$I_{\mu I} = [(\mu_c - \mu_i)/\mu_c] \times 100$$

where $\mu$(average growth rate) $= (\ln N_t - N_0)/T_n$; $N_t$, value of the observed parameter (fronds number or dry weight)

at time $t$; $N_0$, value of the same parameter at time 0; $T_n$, period of time between time 0 and $t$; $\mu_c$, average growth rate of the control; $\mu_i$, average growth rate with the treatment sample.

## 3. Results

### 3.1. Decolourisation

The DP values obtained for the W1, W2 and W3 effluents are listed in Table 3. Substantial decolourisation of W1 was nearly always achieved with DPs from 77.2% to 93.9% after 24 h. In all cases, more than 89% of the total decolourisation was achieved within 2 h; actually the DP values obtained with the biomasses of *R. pusillus* cultivated on ST and that of *R. stolonifer* and with chitosan did not change significantly from 2 to 24 h. The DPs for W2 ranged between 59.3% and 65.1%; as for W1, in all cases most of the total decolourisation was reached within 2 h. The DP values for W3 obtained by fungal biomasses were quite high, ranging from 84.0% and 92.7%, whereas the performance of chitosan was considerably lower (42.9%); in comparison with the other simulated effluents, lower percentage of the total decolourisation was attained within 2 h and in all cases a significant increase of the DP values among 2, 6 and 24 h was observed.

The SC values of the fungal biomasses and chitosan towards the W1, W2 and W3 effluents are shown in Table 4. All the fungal biomasses and chitosan significantly increased their SC passing from W1 to W2 and W3. The highest SCs were displayed by fungal biomasses towards W3, with values always higher than 200 mg of dye $g^{-1}$ of biomass dry weight. Chitosan displayed significantly lower SCs vs W1 and W3 with respect to the fungal biomasses. The medium composition significantly affected the SC of the fungal biomasses in 3 cases: *R. pusillus* vs W1, when the fungus was cultured on EQ proved higher SC than when cultured on ST, and *C. elegans* vs W2 and W3, when the fungus was cultured on ST improved SC in comparison with EQ.

Absorbance spectra during the biosorption experiments show that the fungal biomasses display high affinities for all dyes. The fungal biomasses adsorbed the dyes present in the 3 wastewaters with comparable speed irrespective of their formulas as shown by the absence of modifications in the shape of the absorbance spectra (Fig. 1).

### 3.2. COD

The COD ($mg\,l^{-1}$) values for W1, W2 and W3 before (controls) and after the treatment by the biomasses are listed in Table 5. In most cases a significant decrease in COD was observed. In two cases (*C. elegans* and *R. pusillus* cultivated on EQ vs W1) the COD values increased after the treatment.

### 3.3. Ecotoxicity test

The growth inhibition percentages measured by fronds number and plant dry weight before and after the treatment by the fungal biomasses are reported in Fig. 2. After the treatment the toxicity of the effluents always decreased. In detail, the treatment of W1 by the fungal biomasses caused a decrease, even if not significant, of the growth inhibition measured both as fronds number and dry weight; in all cases *L. minor* resulted even in biostimulation by the treated wastewaters. The toxicity of W2 after the treatment was always significantly lower, considering the fronds number and in one case (*R. pusillus* cultivated on ST) considering the dry weight. The toxicity of W3 was always significantly lower after the treatment, with the only exception of *R. pusillus* cultivated on ST considering the dry weight.

## 4. Discussion

These results show that biosorption treatment by *C. elegans*, *R. pusillus* and *R. stolonifer* biomasses determine a substantial and rapid decolourisation of the 3 tested effluents. Very high DPs that resulted in changes in the appearance of the effluents reducing the strong initial visual impact, were obtained with W1 (up to 94%) and W3 (up to 93%). Good yield of decolourisation (up to 65%) was also achieved with the more concentrated W2 (5000 ppm). Chitosan was generally less effective than the fungal biomasses, and this is particularly true towards W3. Calculation of the increase in decolourisation and monitoring of the decrease in absorbance showed that in nearly all cases the maximum biosorption towards W1 and W2 was reached in the first 2 h, while the decolourisation of W3 proved to be a slower process, since lower percentage of the total decolourisation was attained within 2 h.

Moreover, the absence of modifications in the shape of the absorbance spectra during the biosorption experiments demonstrates that the biomasses removed with comparable efficiency all the dyes present in the same effluent, irrespective of the chromophore (mono-, bi- tri-, poliazoic, anthraquinonic or stilbenic), steric conformation, charge and molecular weight.

The versatility and efficiency of the tested biomasses, previously proved towards single dyes and a mix of ten dyes (Prigione et al., 2008), in this study were confirmed also working with effluents designed to mimic wastewaters produced during cotton or wool dyeing processes. The added value of these results stems from the fact that these wastewaters, prepared using mixed commercially important industrial dyes, contain high concentration of salts and display different pH values, introducing real parameters that often bars the attainment of good biosorption yields (Aksu, 2005). It must be borne in mind that till now, most of the data concerning the exploitation of fungal biomasses in dye biosorption are about single molecules at low concentrations; only few studies with multicomponent dye solutions and high salt concentration, which simulate dye-house or textile-mill effluents have been carried out (Aksu, 2005; Prigione et al., 2008).

The SC of a biomass depends on several biotic and abiotic factors, such as the type of biomass (species, age), the type of sorbates, the presence of other competing ions and the

**Table 3 – Decolourisation percentage of W1, W2 and W3 effluents after 2 h (T2), 6 h (T6) and 24 h (T24) incubation by the biomasses of the species cultured on EQ (*Cunninghamella elegans*, *Rhizomucor pusillus* and *Rhizopus stolonifer*) and ST medium (*Cunninghamella elegans* and *Rhizomucor pusillus*) and by the chitosan, and increase of decolourisation from T2 to T24**

| Effluent | Biosorbent | Medium | Decolourisation percentage (Mean ± st. dev.) | | | Increase of decolourisation from T2 to T24 (%) |
|---|---|---|---|---|---|---|
| | | | T2 | T6 | T24 | |
| W1 | *Cunninghamella elegans* | EQ | $85.4 \pm 3.6^{a}$ | $91.5 \pm 0.7^{b}$ | $92.8 \pm 1.4^{b}$ | 8.0 |
| | | ST | $83.9 \pm 3.1^{a}$ | $88.1 \pm 4.1^{a}$ | $93.9 \pm 0.4^{b}$ | 10.6 |
| | *Rhizomucor pusillus* | EQ | $87.3 \pm 5.9^{a}$ | $91.7 \pm 2.0^{ab}$ | $93.4 \pm 0.5^{b}$ | 6.5 |
| | | ST | $89.6 \pm 2.3^{a}$ | $85.4 \pm 4.2^{a}$ | $78.4 \pm 7.9^{a}$ | – |
| | *Rhizopus stolonifer* | EQ | $89.1 \pm 7.3^{a}$ | $93.7 \pm 1.2^{a}$ | $92.3 \pm 2.9^{a}$ | 3.5 |
| | Chitosan | – | $80.6 \pm 3.3^{ab}$ | $83.3 \pm 0.0^{a}$ | $77.2 \pm 1.3^{b}$ | – |
| W2 | *Cunninghamella elegans* | EQ | $50.7 \pm 1.8^{a}$ | $56 \pm 3.1^{b}$ | $59.3 \pm 3.1^{b}$ | 14.5 |
| | | ST | $44.7 \pm 4^{a}$ | $53.2 \pm 0.5^{b}$ | $63.8 \pm 3.6^{c}$ | 29.9 |
| | *Rhizomucor pusillus* | EQ | $71.3 \pm 3.9^{a}$ | $69.6 \pm 4.6^{a}$ | $65.1 \pm 4.1^{a}$ | – |
| | | ST | $64.9 \pm 3.1^{a}$ | $64.5 \pm 3.3^{a}$ | $63.2 \pm 4.7^{a}$ | – |
| | *Rhizopus stolonifer* | EQ | $61.2 \pm 3^{a}$ | $61.7 \pm 2.0^{a}$ | $59.4 \pm 1.7^{a}$ | – |
| | Chitosan | – | $33.3 \pm 0.3^{a}$ | $46.6 \pm 1.7^{b}$ | $59.1 \pm 1.0^{c}$ | 46.3 |
| W3 | *Cunninghamella elegans* | EQ | $51.8 \pm 3.4^{a}$ | $66.5 \pm 2.8^{b}$ | $87.5 \pm 2.1^{c}$ | 40.8 |
| | | ST | $57.1 \pm 1.5^{a}$ | $74.9 \pm 3.0^{b}$ | $92.7 \pm 0.4^{c}$ | 38.4 |
| | *Rhizomucor pusillus* | EQ | $40.1 \pm 0.4^{a}$ | $56.7 \pm 2.8^{b}$ | $87.4 \pm 2.3^{c}$ | 54.1 |
| | | ST | $60.7 \pm 3^{a}$ | $72.8 \pm 4.1^{b}$ | $89.9 \pm 1.7^{c}$ | 32.5 |
| | *Rhizopus stolonifer* | EQ | $35.3 \pm 3.1^{a}$ | $51.1 \pm 3.2^{b}$ | $84.0 \pm 1.9^{c}$ | 58.0 |
| | Chitosan | – | $8.7 \pm 0.8^{a}$ | $28.0 \pm 0.4^{b}$ | $42.9 \pm 2.6^{c}$ | 79.7 |

$^{a,b,c}$Significant differences between decolourisation percentages at T2, T6 and T24.

**Table 4 – Sorption capacity (mg of dye $g^{-1}$ of biomass dry weight) of W1, W2 and W3 effluents by inactivated biomasses cultured on EQ (*Cunninghamella elegans*, *Rhizomucor pusillus* and *Rhizopus stolonifer*) and ST medium (*Cunninghamella elegans* and *Rhizomucor pusillus*) and by the chitosan**

| Biosorbent | Medium | Sorption capacity (mg of dye $g^{-1}$ of biomass dry weight) | | |
|---|---|---|---|---|
| | | W1 | W2 | W3 |
| *Cunninghamella elegans* | EQ | $20.1 \pm 2.2^{A,ab}$ | $169.5 \pm 3.5^{B,ac,*}$ | $204.0 \pm 12.1^{C,a,*}$ |
| | ST | $21.9 \pm 1.3^{A,ad}$ | $198.6 \pm 3.9^{B,b}$ | $235.8 \pm 6.3^{C,b}$ |
| *Rhizomucor pusillus* | EQ | $20.9 \pm 0.4^{A,a,*}$ | $187.2 \pm 14.4^{B,bc}$ | $203.5 \pm 7.2^{B,a}$ |
| | ST | $17.3 \pm 2.1^{A,b}$ | $188.1 \pm 19.2^{B,abc}$ | $226.9 \pm 15.1^{C,ab}$ |
| *Rhizopus stolonifer* | EQ | $23.4 \pm 1.3^{A,d}$ | $191.6 \pm 4.4^{B,b}$ | $200.8 \pm 34.6^{B,ab}$ |
| Chitosan | – | $13.5 \pm 0.3^{A,c}$ | $173.2 \pm 2.6^{B,c}$ | $75.5 \pm 4.3^{C,c}$ |

$^{a,b}$…Significant differences between the SC for the same effluent of different bisorbents.
$^{A,B}$…Significant differences between the SC for different effluents of the same biosorbent.
*Significant differences between the SC of a species cultured on different media.

method of biomass preparation, along with several physical–chemical factors such as temperature, pH, ionic concentration (Maurya et al., 2006). According to Aksu (2005), higher concentration of dyes may enhance the adsorption process by providing an important driving force to overcome the mass transfer resistance of the dye between the aqueous and solid phases. This is only partially true in our experiments. In fact, all the biomasses increased their SC passing from W1 (300 ppm) to W2 and W3 (5000 and 3000 ppm, respectively); however, the highest SC values have been recorded towards W3 (3000 ppm) and not towards W2 (5000 ppm). Probably, at very high concentration, dye–dye interactions become prevalent and this may result in decreased affinity of the dye binding sites on the biomass, as suggested by Bustard

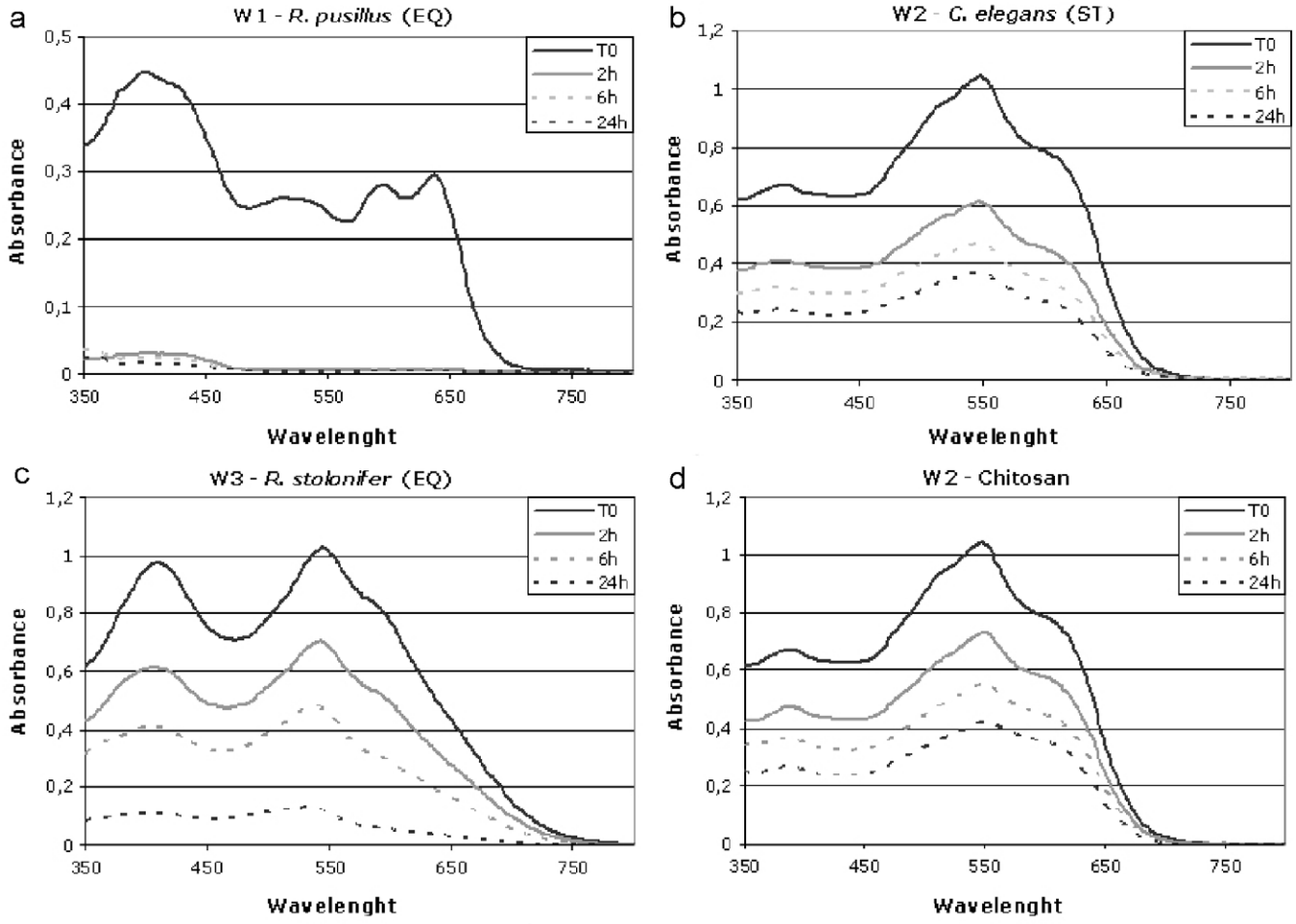



**Fig. 1 – Absorbance spectra of W1, W2 and W3 effluents at the beginning of the experiment (T0) and after 2, 6 and 24 h of incubation. (a) *Rhizomucor pusillus* biomass cultured on EQ medium, W1 effluent; (b) *Cunninghamella elegans* biomass cultured on ST medium, W2 effluent; (c) *Rhizopus stolonifer* biomass cultured on EQ medium, W3 effluent; (d) chitosan, W2 effluent.**

et al. (1998); besides other abiotic factors, i.e. ionic strength and pH, can play an important role. According to Shu et al. (2005) higher concentrations of dyes and/or salts promote the dye aggregation that, in turn, decrease the available ionic groups of the dye molecules, decreasing their solubility and their possibility to interact with the fungal biomass. Moreover, biosorption is also influenced by the structure of the dye molecules (Prigione et al., 2008). In our case, direct dyes were more easily removed than reactive dyes. This could be due to different steric conformations and charge distribution of the dye molecules that affect the interaction between the biomass binding sites and the dyes.

Finally, the influence of growing medium (EQ vs ST) was considered. It has been reported on several occasions that the cell wall of fungi responds to the culture medium and other physical–chemical properties of the environment by greatly changing its composition and chemical–physical properties (El-Mougith, 1999; Hefnawy et al., 1999; Nemcovic and Farkas, 1998; Znidarsic et al., 1999). Biomasses cultured on media containing starch as C source showed significantly higher SCs with respect to the biomasses cultured on glucose in two cases (*C. elegans* vs W2 and W3), whereas *R. pusillus* displayed significantly higher SC towards W1 when cultured in EQ. These results are of great significance for the application of the biosorption method to industry, since starch is a low-cost by-product of many manufacturing processes whose use as C source would reduce the generally very expensive production of biomasses.

Chitosan, the other biosorbent tested in our experiments, is an important by-product of the food industry, generally considered highly effective in biosorption (Crini, 2006). Besides, it is a characteristic component of the cell wall of Mucorales (where its content may exceed that of chitin), often indicated as the main responsible for dye biosorption by this group of fungi. The comparison between the SCs of fungal biomasses and those of chitosan point out two important results: (i) the selected biomasses are more efficient than purified chitosan in dye removal, especially towards effluents containing acid or direct dyes; (ii) other fungal cell wall components, in addition to chitosan, seem to play an essential role in biosorption process. Hence, further studies will be addressed to the understanding of the biosorption

**Table 5 – COD (mg $l^{-1}$) values for the untreated W1, W2 and W3 effluents (control) and after the treatment by the biomasses of the species cultured on EQ (*Cunninghamella elegans*, *Rhizomucor pusillus* and *Rhizopus stolonifer*) and ST medium (*Cunninghamella elegans* and *Rhizomucor pusillus*), and COD variation percentage**

| Effluent | Species | Medium | COD (mg $l^{-1}$) | COD variation (%) |
|---|---|---|---|---|
| W1 | Control | | $442.9 \pm 23.7^{a}$ | |
| | *Cunninghamella elegans* | EQ | $783.6 \pm 25.4^{b}$ | + (76.9 ± 5.7) |
| | | ST | $386.4 \pm 39.7^{c}$ | − (12.7 ± 8.9) |
| | *Rhizomucor pusillus* | EQ | $478 \pm 26.7^{a}$ | + (7.9 ± 6.0) |
| | | ST | $343.7 \pm 49.9^{c}$ | − (22.4 ± 11.3) |
| | *Rhizopus stolonifer* | EQ | $386.1 \pm 43.4^{c}$ | − (9.5 ± 10.2) |
| W2 | Control | | $2724.2 \pm 124.6^{a}$ | |
| | *Cunninghamella elegans* | EQ | $1696.7 \pm 53.5^{b}$ | − (37.7 ± 2.0) |
| | | ST | $1531.2 \pm 68.3^{cd}$ | − (43.8 ± 2.5) |
| | *Rhizomucor pusillus* | EQ | $1540.8 \pm 73.2^{cd}$ | − (43.4 ± 2.7) |
| | | ST | $1459.6 \pm 83.1^{c}$ | − (46.4 ± 3.0) |
| | *Rhizopus stolonifer* | EQ | $1591.2 \pm 51^{bd}$ | − (41.6 ± 1.9) |
| W3 | Control | | $1784.5 \pm 65.8^{a}$ | |
| | *Cunninghamella elegans* | EQ | $821.0 \pm 76.0^{b}$ | − (54.0 ± 4.3) |
| | | ST | $510.4 \pm 35.6^{c}$ | − (21.4 ± 2.0) |
| | *Rhizomucor pusillus* | EQ | $754.2 \pm 36.7^{b}$ | − (57.7 ± 2.1) |
| | | ST | $739.9 \pm 49.9^{b}$ | − (58.5 ± 2.8) |
| | *Rhizopus stolonifer* | EQ | $755.7 \pm 36.4^{b}$ | − (57.7 ± 2.0) |

[a,b,c]Significant differences among different treatments for the same effluent.

mechanisms and to the identification of the functional groups involved.

Despite the difficulties in comparing the SCs of various biosorbents, mainly due to the different experimental conditions (i.e. type of dye, biomass conditions, mathematical approach for the calculation of SC, etc.), our results confirm the high biosorption yields of the tested biomasses compared with other microbial biomasses (i.e. bacteria, yeasts and algae), as well as waste materials and by-products deriving from industry and agriculture (i.e. pinewood, corncob, bagasse, rice husk, chitosan, etc.), and mineral sorbents (i.e. dolomite, bentonite, zeolite, etc.) (Aksu, 2005; Crini, 2006, 2008; Ferrero 2007). Fungal biomasses, hence, can be considered very promising candidates for the replacement of activated carbons and organic resins, which have ensured their primacy for dye removal from industrial wastewaters due to their versatility and efficiency, but with important drawbacks (such as the high costs) hampering their use on large scale (Aksu, 2005; Crini, 2008).

The three mimmicked wastewaters displayed COD values from 443 to 2724 mg $l^{-1}$ confirming the high chemical oxygen demand generally pointed out by the literature for dyed effluents (Solmaz et al., 2006). In all cases, COD values exceeded the legal threshold value (160 mg $l^{-1}$) of the Italian law (D.Lgs. 3 aprile 2006, n. 152). In most cases a significant decrease in COD, although not proportional to DP, was obtained after the biosorption treatment. This datum could be explained by the preferential dye biosorption with respect to the organic molecules and/or by the release of organic matter from the fungal biomasses. This last phenomenon can also explain the increase of COD recorded in 2 cases. The release of organic matter from the biomasses was confirmed measuring a COD increase in experiments where the fungal biomasses were incubated in the same simulated effluents without dyes (data not shown). This datum shows that care must be taken in the rinsing during biomass preparation to remove accurately the residual medium and/or the cytoplasmatic content from the inactivated biomasses. To our knowledge this is the first report of COD monitoring in biosorption experiments. Actually, this parameter is particularly important since in the commercial products in addition to dyes many other molecules, i.e. additives and surfactants, are present which constitute up to 70% of the final dry weight and the removal of these molecules cannot be monitored by the effluent decolourisation.

The *L. minor* ecoxicity test has been often employed to assess the toxicity of several organic compounds, in particular it presents high sensitivity for dye-containing samples (Lytle and Lytle, 2001; Trivedi et al., 2004; Okay et al., 2005; Casieri et al., 2008). The test showed a significant reduction of toxicity after biosorption treatments, indicating that decolourisation corresponds to an actual detoxification of the treated wastewaters irrespective of the decrease of COD. This last datum indicates that the release of the organic matter from the fungal biomasses does not impact on the toxicity.

Although decolorisation is not always coupled to detoxification, as already demonstrated by several papers dealing with chemical–physical treatments (i.e. ultrasound and photochemical treatment) and biological degradation mainly of azo dyes (Vandevivere et al., 1998; Husain, 2006; Hai et al., 2007), detoxification of true or simulated industrial effluents by means of ecotoxicity tests has been verified in only very few studies (Gottlieb et al., 2003; Malachová et al., 2006). In decolorisation experiments, hence, the effluent toxicity before and after the treatment should be always monitored.

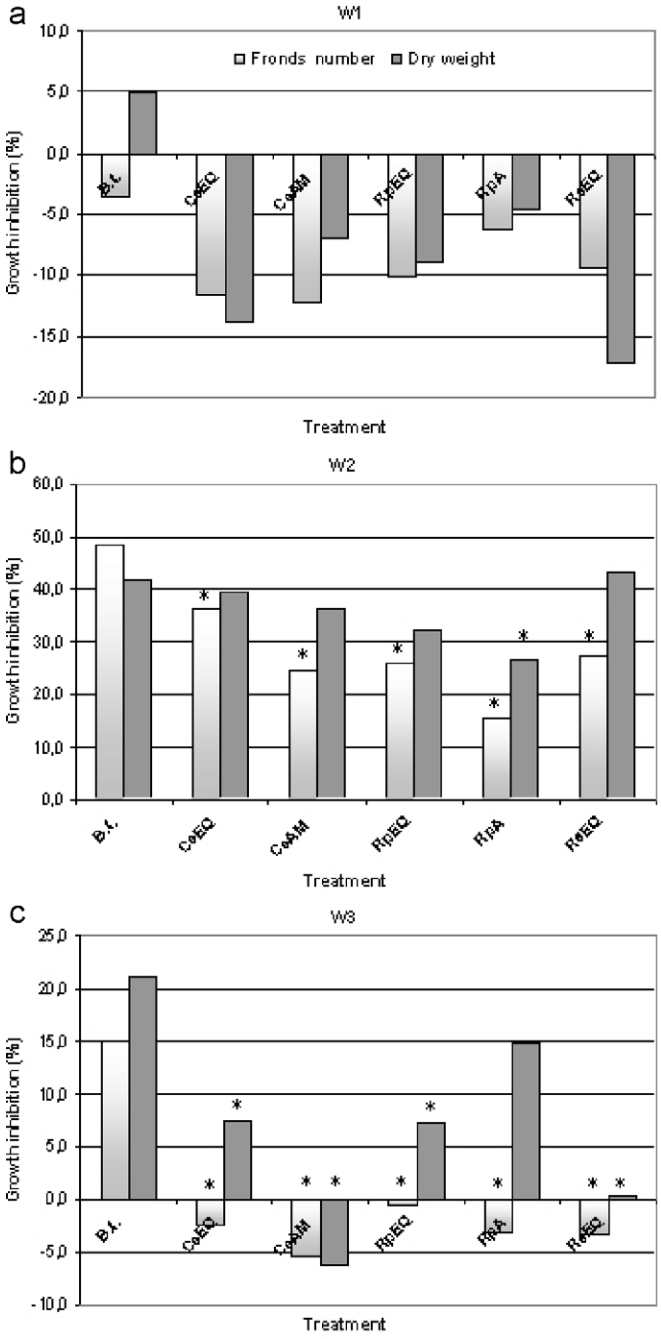



**Fig. 2 – Growth inhibition percentages measured by fronds number and plant dry-weight before treatment (B.t.) and after the treatment by the biomasses of *Cunninghamella elegans* (C.e.), *Rhizomucor pusillus* (R.p.) and *Rhizopus stolonifer* (R.s.) cultured on EQ and ST medium.**

## 5. Conclusion

In conclusion, biosorption by means of fungal biomasses could be really considered a valid alternative to other techniques for wastewater treatment aiming at water reuse, in terms of initial cost, flexibility and simplicity of design, ease of operation and insensitivity to toxic pollutants.

The biomasses of *Cunninghamella elegans*, *Rhizomucor pusillus* and *Rizopus stolonifer* seem to be very fast and effective in removing dyes characterized by different structures and/or different chemical reactivities irrespective of the composition of the wastewaters, exceeding in this way many drawbacks of biodegradation. Decolourisation corresponds to a substantial detoxification of wastewaters and is usually coupled to a significant decrease of COD.

The three selected biomasses can be regarded as powerful candidates for biotechnology of dye biosorption due to their high ability to remove industrial mixed dyes in high

concentration and in conditions that mimic those of real industrial effluents.

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## Section 1- Conclusions

The work described in Section1 has been aimed at evaluating the performances of several biological options for wastewater treatment. Two kind of processes have been specifically analysed: Biodegradation-based processes and Biosorption-based processes. In particular, the former alternative has been explored from a multiplicity of perspectives (whole-cell and enzymatic processes in free an immobilized forms).
Together with the powerful and promising results achievable by the application of the analysed biosystems, some constraints posed by each specific bioprocess have to be highlighted. Cost competitive biodegradation processes proved to be limited by the harsh conditions provided by the wastewaters (neither of the treatments was effective on Reactive model, for instance), and were often characterized by long process time (up to seven days of treatment for Direct model). Moreover, the tested microorganisms exhibited different decolourisation specificities, leading to the conclusion that a single strain/enzyme may be not sufficient to meet the highly variable nature of textile effluents, since there are too many factors that may affect the biodegradation rate of dyes (substrate specificity, pH and salt concentration). Thus, further work in this direction should be done by testing the performances of microbial consortia and enzymatic mixtures from different sources in order to exploit their potential synergistic effect.
On the other hand, biosorption based processes proved to be fast and effective in removing dyes characterized by different chemical reactivities, irrespective of the composition of the wastewaters. However, they pose the problem of disposal of spent biomasses.
Taken together, these observations indicate that each process alone might not represent a universally applicable solution to wastewater treatment. Combination of different techniques are generally required to devise a technically and economically feasible option. In light of this, the current research should be focused on both improving each single solution, as well as on proposing a wide range of hybrid decolourisation techniques in which the respective advantages of each methods would be exploited.
A comprehensive survey of potential combinations of different kind of processes was given by Hai and co-workers (see references chapter 3). The wide range of hybrid techniques proposed include chemical-biological, biological-chemical, chemical-physical, chemical-chemical as well as others alternatives.
An effective combined process for wastewater treatment could be developed as follows. The rapid wastewater decolourisation achievable by fungal biosorption may be followed by the physical treatment of the treated effluent. Such type of methods (i.e. nanofiltration, reverse osmosis) would be, in fact, much more cost-effective if applied on effluents with a low polluting load, producing purified water and allowing the reuse of water along with mineral salts. Subsequently, the spent biomasses may be treated by chemical oxidation methods or biodegradation processes to remove dye molecules. Solid state fermentation (SSF) of such biomasses by fungal strains could be exploited, for example, resulting in simultaneously dye degradation and enriching the nutritional quality of the solid substrates. Finally, these exhausted biomasses could be addressed to composting or biogas and energy productions, whilst the possible exhausted effluents deriving from this process may undergo conventional treatment by active sludge in order to reduce their COD loading.

Thus, the results provided by this section can be regarded as a preliminary key step in designing such combined approaches. However, further efforts will be required to confirm the results obtained on model wastewaters, by testing their potential application on real industrial wastewaters.

# Section 2

## Isolation of new members of *P. ostreatus* laccase family and their characterisation through heterologous expression

# *Chapter 4*

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RESEARCH ARTICLE

# The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members

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**Abstract** This work was aimed at identifying and at characterizing new *Pleurotus ostreatus* laccases, in order to individuate the most suitable biocatalysts for specific applications. The existence of a laccase gene clustering was demonstrated in this basidiomycete fungus, and three new laccase genes were cloned, taking advantage of their closely related spatial organization on the fungus genome. cDNAs coding for two of the new laccases were isolated and expressed in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, in order to optimize their production and to characterize the recombinant proteins. Analysis of the *P. ostreatus* laccase gene family allowed the identification of a "laccase subfamily" consisting of three genes. A peculiar intron–exon structure was revealed for the gene of one of the new laccases, along with a high instability of the recombinant enzyme due to lability of its copper ligand. This study allowed enlarging the assortment of *P. ostreatus* laccases and increasing knowledge to improve laccase production.

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**Keywords** Laccase gene · Fungus · Recombinant heterologous expression · Copper lability · Promoter · Phylogenetic analysis

## Introduction

Laccases are copper-containing polyphenol oxidases (EC 1\10\3\2), that have been found in fungi (Hatakka 1994), in plants (Mayer 1987; Mayer and Staples 2002) and in bacteria (Diamantidis et al. 2000; Hullo et al. 2001; Sanchez-Amat et al. 2001; Roberts et al. 2002; Enguita et al. 2003). These enzymes are, generally, monomeric, or, more rarely, homo- and hetero-dimeric or homo-tetrameric glyco-proteins, whose activity is dependent on four copper ions, distributed among three different highly conserved binding sites (Thurston 1994; Hoegger et al. 2006). Laccases are able of oxidizing a wide range of aromatic compounds, with concomitant reduction of molecular oxygen to water (Thurston 1994; Ullah et al. 2000). Thanks to their low substrate specificity, laccases can be used for several industrial applications, such as pulp bleaching in paper industry, textile dye decolorization and detoxification of environmental pollutants (Mayer and Staples 2002). For these applications, the most suitable laccase for each specific purpose should be found and produced. Therefore, many efforts are being focused on molecular characterization of fungal laccases as well as on improvement of the production levels by recombinant expression.

It has been reported that almost all the fungi examined secrete more than one laccase isozyme (Yaver et al. 1996; Yaver and Golightly 1996; Mansur et al. 1998; Soden and Dobson 2001; Hoshida et al. 2001; Palmieri et al. 2003; Rodrıguez et al. 2008). Even if a low similarity is revealed





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among some deduced laccase amino acid sequences, all laccases share the four copper-binding regions, as well as the highly conserved copper ligands 10 histidine and 1 cysteine residues (Thurston 1994).

Expression of different patterns of laccase isozymes coded by gene families is differentially regulated, depending on the growth conditions (Bollag and Leonowicz 1984; Rogalski and Leonowicz 1992) and physiological states (Mansur et al. 1998; Rogalski et al. 1993). Several studies indicated that the expression of most fungal laccase genes is affected by metal ions, such as copper and cadmium (Palmieri et al. 2000; Baldrian and Gabriel 2002), and by aromatic compounds (Galhaup et al. 2002).

The laccase family of the basidiomycete fungus *Pleurotus ostreatus* represents a variegated group of enzymes, some of which are endowed with peculiar properties. Besides the typical laccase isoenzyme POXC (Giardina et al. 1996), representing the most abundantly produced laccase from *P. ostreatus*, the fungus excretes the white laccase isoenzyme POXA1w, having a singular metal content (Palmieri et al. 1997), the heterodimeric laccase isoenzyme POXA3 (Palmieri et al. 2003; Giardina et al. 2007; Faraco et al. 2008a) and POXA1b, the notably stable at alkaline pH, with a $t_{1/2}$ of 30 days at pH 10 (Giardina et al. 1999). Studies of the genes coding for laccase isoenzymes in *P. ostreatus* have so far led to the identification of four different genes and the corresponding cDNAs, *poxc* (previously named *pox2*) (Giardina et al. 1996), *pox1* (coding for a laccase isoenzyme not yet identified) (Giardina et al. 1995), *poxa1b* (Giardina et al. 1999), and *poxa3* (Palmieri et al. 2003). Chromosomal location of the known laccase genes from *P. ostreatus* has been only partly investigated so far. Linkage analysis mapped both *poxc* and *pox1* on the distal end of chromosome VI at 5.1 centimorgans distance (Larraya et al. 2000).

The peculiar molecular aspects of *P. ostreatus* laccase isoenzymes along with their role in biotechnological applications of the fungus (Palmieri et al. 2005a, b; Faraco et al. 2008b, c) led us to further investigate the laccase family of *P. ostreatus*. The aim of this work was identification and characterization of new *P. ostreatus* laccases, in order to enlarge the assortment of these industrially useful enzymes. Three new laccase genes from *P. ostreatus* were cloned, thus enlarging the panel of these biocatalysts up to seven members. A "laccase subfamily" consisting of three laccase genes was identified, whilst a divergent intron–exon structure was revealed for the gene of one of the new laccases. Recombinant heterologous expression of one of the new laccases was also successfully performed and conditions to reconstitute the extremely instable enzymatic activity by copper addition were defined.

## Materials and methods

### Strains, media, and plasmids

*Pleurotus ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) liquid cultures were carried out in 2.4% potato dextrose broth in the presence of 0.5% yeast extract, supplemented when indicated with 150-μM copper sulphate with or without 2-mM ferulic acid. About 50 ml of a 5-day-old culture were transferred in 1-l flasks containing 450 ml broth at 28°C in the dark.

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

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 pSAL4 (copper-inducible CUP1 promoter) carrying *URA3* gene for auxotrophic selection. *S. cerevisiae* was grown on the selective medium [6.7 g $l^{-1}$ yeast nitrogen base without amino acids and ammonium sulphate, 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], named SD medium.

The *Kluyveromyces lactis* strain used for heterologous expression was CMK5 (a *thr lys pgi1 adh3 adh1URA3 adh2URA3*). The plasmid used for *K. lactis* expression, pYG132 (Piscitelli et al. 2005) contains an ethanol-inducible 1,200-nucleotides extended *KlADH4* promoter (Mazzoni et al. 1992) and the *S. cerevisiae* phosphoglycerate kinase terminator. *K. lactis* was grown in YPPG medium (10 g $l^{-1}$ yeast extract, 40 g $l^{-1}$ bacto tryptone, 20 g $l^{-1}$ glucose), supplemented with ethanol (0.5%), $CuSO_4$ (0.1 mM) and when required, with 100 μg $ml^{-1}$ of geneticin G418.

### Genomic DNA isolation and Southern analysis

Genomic DNA from *P. ostreatus* was isolated (Raeder and Broda 1988), digested with several restriction enzymes (Promega, Italy), and, after electrophoresis on 0.8% agarose gel, transferred to a Hybond-NX nylon membrane (GE Healthcare). The membrane was hybridized using the laccase cDNAs *poxc* (GenBank accession number **Z34848**), *pox1* (GenBank accession number **Z34847**), *poxa1b* (GenBank accession number **AJ005018**) and *poxa3* (EMBL accession number **AJ344434**) as radioactive probes, as previously described (Palmieri et al. 2000).







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## Isolation and sequencing of genes

A BAC (Bacterial Artificial Chromosome) library of the *P. ostreatus* genome was kindly made available by Prof Ramírez of Department of Agrarian Production, Public University of Navarre. The library, consisting of 150-kDa DNA fragments, was screened using *poxc*, *pox1*, *poxa1b* and *poxa3* cDNAs as probes. cDNAs were amplified and labelled by polymerization chain reaction (PCR) with dUTP digoxigenin conjugated, following manufacturer's instructions (Roche Applied Science). The screening was performed at 68°C in the buffer 5 × SSC, 0.5% SDS, 1 mM EDTA (where 1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate). The blots were washed at the same temperature; the final wash was performed in 0.1 × SSC, 0.1% SDS. Detection was performed by using DIG nucleic acid detection kit (Roche Applied Science), following manufacturer's instructions.

The positive bacterial clones were analyzed by PCR by means of Phusion polymerase (Invitrogen) according to the manufacturer's instructions, using both specific oligonucleotides, designed on *poxc*, *pox1*, *poxa1b* and *poxa3* cDNAs, and degenerate oligonucleotides, corresponding to consensus sequences of laccase copper-binding regions (Table 1), at the annealing temperatures reported in Table 1. The degenerate primers POXfw and POXrev were designed on the highly conserved copper-binding regions I (HWHGFFQ) and II (YHSHL) of POXC. Sequencing of PCR products was performed by PRIMM Sequencing Service (Milan, Italy) by using the dideoxy chain-termination method and specific oligonucleotide primers.

A sub-library of the positive BAC clone I24 was prepared by *Eco*RI partial digestion, giving ~6,000-bp extended fragments that were cloned in the plasmid pGEM3Zf (Promega, Italy). DNA plasmids from 80 independent clones were UV-linked to a Hybond-NX nylon membrane (GE Healthcare). The blots were hybridized at 70°C in the buffer 0.5 M sodium phosphate, 7% SDS, 1 mM EDTA (pH 8.0) and washed at the same temperature; the final wash was performed with 0.1 × SSC, 0.5% SDS. The sub-library was screened by using as probes the cDNAs *poxc*, *poxa1b, pox1* and *poxa3*, labelled with [$\alpha$-$^{32}$P]dATP (GE Healthcare) by random priming with a labelling kit (GE Healthcare). DNA preparation and restriction analyses were performed by standard methods according to Sambrook et al. (1989). Sequencing by PRIMM was performed using universal and specific oligonucleotide primers.

## Isolation and sequencing of cDNAs

Total RNAs were extracted from lyophilized mycelia, by using QIAGEN RNeasy Plant (QIAGEN, Italy), and following manufacturer's instructions. Reverse transcription reaction was performed using Super Script II Rnase H-Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and the oligonucleotide dT-NotI as primer. Products of the PCR experiments, performed using the gene-specific oligonucleotides (Table 1), were cloned into the p-GEM-T Easy Vector (Promega, Italy). DNA preparation was performed by standard methods (Sambrook et al. 1989). Sequencing

**Table 1** List of primers used in the amplification experiments

| Primer | Nucleotide sequences | Annealing temperature (°C) |
|---|---|---|
| POXfw | CAYTGGCAYGGNTTMTTYCAR | 54 |
| POXrev | MARRTGVGARTGRTA | 54 |
| poxcUp | **GCTGATACGCAGTACTGACC** | 60 |
| poxcDw | **CCGTCCTAAGGCCAGCCTC** | 60 |
| poxa1bUp | **CAACCTTGGCCCTCGACGG** | 62 |
| poxa1bDw | **GGCACCAATGCCATCGGCCG** | 62 |
| pox1Up | **GGTGCCCAAGACGCTCCCG** | 64 |
| pox1Dw | **GGTGGCATAGCTTAGGATATCTC** | 64 |
| poxa3Up | **GTTCTTGCTGCGACGAAGAAA** | 56 |
| poxa3Dw | **GCGCACGGATCCAGTAG** | 56 |
| RTpox3fw | **CCATTCACTTCGTTTCGAGCTTC** | 58 |
| RTpox3rev | **TGTGAGCTTGATTGTTTAGGTCGA** | 58 |
| RTpox4fw | **CGACTTCGAGTCGAGTCACTCACG** | 58 |
| RTpox4rev | **CCCATTCATGCCTTTCGTACTCAG** | 58 |
| HEpox4fw | TT*AAGCTTGAATTC***ATGCGCACATTCTCCCGC** | 62 |
| BHpox4rev | AA*GGATCCAAGCTT***CAGATACCTAGGTTGGG** | 62 |
| HEpox3fw | TT*AAGCTTGAATTC***ATGATTGCGCCTGTACTC** | 60 |
| BHpox3rev | AA*GGATCCAAGCTT***TTAGGTCGAAGGGTGCGT** | 60 |

Nucleotides in bold are complementary to the laccase gene/cDNA sequences. Nucleotide in italics are recognized by restriction enzymes. In the degenerate primers the following abbreviations were used Y = C, T; N = A, G, C, T; M = C, A; R = A, G; V = G, A, C





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was performed by PRIMM Sequencing Service using universal and specific oligonucleotide primers.

## Analysis of gene sequences

Positions of the ATG start codons and introns in the newly identified laccase genes were predicted from conserved intron positions, by alignment of deduced protein sequences with those of the previously defined *P. ostreatus* laccases and by comparison with the conserved sequences of intron splice junctions. Sequences of *pox3*, *pox4* and *pox5* genes and *pox3* and *pox4* cDNAs were submitted to EMBL Data Library and the assigned accession numbers were **FM202669**, **FM202670**, **FM202671**, **FM202672**, and **FM202673**, respectively.

Alignments of DNA and deduced amino acid sequences were generated with ClustalW2 (http://www.ebi.ac.uk/Tools/ clustalw2/index.html). Signal peptides were predicted with SignalP V2.0 (http://www.cbs.dtu.dk/services/). N-glycosylation sites (Asn-XXX-Ser/Thr) were identified with NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). The conservation scoring among the laccase signature sequences was performed by PRALINE (http://zeus.cs.vu.nl/programs/pralinewww/).

Phylogenetic analysis was carried out on *P. ostretaus* laccases using basidiomycete laccase sequences reported in Hoegger et al. (2006). These sequences, together with the newly isolated laccases, were aligned with ClustalW using default settings for multiple sequence alignments. Based on this alignment, a neighbor joining tree was constructed using MEGA version 4 (Tamura et al. 2007). Bootstrapping was carried out with 500 replications.

## Vector construction for heterologous expression in yeasts

*pox3* and *pox4* cDNAs were amplified (Phusion polymerase, Invitrogen) with HEFwpox3/BHRevpox3 and HEFwpox4/BHRevpox4 primers (Table 1), using as templates p-GEM-T Easy Vectors containing *pox3* or *pox4* cDNA, respectively, in order to insert restriction sites required for cloning in expression vectors. The PCR products were cloned in *Eco*RI-*Bam*HI digested pUC18 plasmid and checked by sequencing at PRIMM Sequencing Service. cDNAs, including signal peptides, were recovered by *Hin*dIII or *Eco*RI-*Hin*dIII hydrolysis and cloned in pYG132 or pSAL4, respectively.

## Yeast transformation, cultivation and laccase production

*Kluyveromyces lactis* transformation by electroporation, selection of transformed clones on agar plates and screening of selected clones for laccase production (in YPPD medium containing 100 μg $ml^{-1}$ geneticin and supplemented with 0.1 mM $CuSO_4$ and 2% ethanol) were performed as previously described (Piscitelli et al. 2005). To study laccase production, the best producing clones were grown in 250-ml flasks containing 50 ml of selective YPPD medium, starting from 0.06 $OD_{600}$. Cultures were grown at 28°C on a rotary shaker.

*Saccharomyces cerevisiae* transformation was done by using a lithium acetate protocol (Gietz et al. 1992). The cells were spread on the selective medium and the plates incubated upside down for 4 days at 28°C. Laccase production by transformants was first assayed on SD plates supplemented with 0.6 mM $CuSO_4$ and 0.2 mM ABTS or 0.1 mM DMP. Inocula grown on selective SD medium were used to seed flasks (250 ml) containing 50 ml of selective medium, starting from 0.5 OD600. Cultures were grown at 28°C on a rotary shaker at 150 rpm.

## Protein purification

Preparative cultures of *pox3* transformants of *S. cerevisiae* were performed in 1-l flasks containing 200 ml of SD medium. Culture media were harvested at about the optimal laccase production time, cells were sedimented by centrifugation at 1,600*g* at 4°C for 15 min. Secreted proteins were filtered through Whatmann sheet. The sample was concentrated using Amicon stirred cells with PM30 ultrafiltration membranes (Millipore, Billerica, MA), after addition of 2 mM $CuSO_4$. Then the sample was equilibrated in 50 mM MES pH 5.5, 20% glycerol and loaded onto a High Load 16/60 Superdex 200 prep grade (GE healthcare Bio-Sciences, AB, Uppsala, Sweden) column equilibrated in the same buffer. The active fractions were pooled and concentrated on an Amicon PM-30 membrane.

Protein concentrations were determined using the Bio-Rad Protein Assay (BioRad), with bovine serum albumin as standard.

Phenol oxidase activity was assayed at 25°C using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,6-dimethoxyphenol (DMP) as substrates, as previously described (Palmieri et al. 1997). Enzyme activity was expressed in IU.

## Homology modelling

The 3D-structure of POX3 was modelled selecting as template the crystal structure of *Trametes versicolor* (1GYC pdb entry) (Piontek et al. 2002), that shares 73% sequence identity with POX3. Aminoacid sequence alignment was generated by using the T-coffee programm that uses the Lalign algorithm from the FASTA package.

Several sets of models were generated, for each system, by using the program MODELLER 8v2 (Marti-Renom et al. 2000) In this program, the models are generated by







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satisfaction of spatial restraints. These restraints are expressed in terms of probability density functions, which are combined into an objective function. The optimization of the latter function allows the generation of several models Hundreds models were generated in each series and was selected ones on the basis of the lowest MODELLER objective function.

Hydrogen atoms were added to the model and the strain has to be removed by 100 steps of energy minimization of the structure using GROMACS package (Berendsen et al. 1995).

## Results

### Isolation of new members of *Pleurotus ostreatus* laccase gene family

Southern blot experiments using both *Xho*I and *Sac*I digested *P. ostreatus* genomic DNAs showed that *pox1* and *poxa3* genes are localized in the same 8.5-kb *Xho*I fragment, while *pox1* and *poxa1b* hybridized with the same 10-kb *Sac*I fragment (data not shown).

In order to investigate the chromosomal organization of the *P. ostreatus* laccase gene family, a library of the fungal genome in BAC (bacterial artificial chromosome) clones (made available by Prof Ramírez of Department of Agrarian Production, Public University of Navarre) was screened by colony hybridization using *poxc*, *pox1*, *poxa1b* and *poxa3* cDNAs as probes. Two BAC clones were selected, the clone C19 hybridizing with *poxa3* cDNA and the clone I24 with all the probes. Sequencing of PCR products amplified on these clones with specific oligonucleotides designed on *poxc*, *poxa1b*, *poxa3* and *pox1* genes showed presence of all these genes in the I24 clone and of *poxa3* gene in the C19 clone.

To identify new members of the *P. ostreatus* laccase gene family, PCR analyses on the BAC clones I24 and C19 were performed using degenerate oligonucleotides (Table 1), corresponding to the highly conserved copper-binding regions I and II of *poxc* gene, taking advantage of the high degree of similarity shared among laccase genes (D'Souza et al. 1996). PCR products of two different sizes (250 and 300 bp) were obtained from the clone I24, whereas only one 250-bp fragment was amplified from the clone C19. Sequence analyses showed that the clone I24 contains at least one new putative laccase gene, named *pox4*, in addition to the already known laccase genes. The entire DNA sequence of *pox4* (3,297 bp including 590 bp of 5′ and 130 bp of 3′ non-coding regions) was determined by walking on the clone I24. The *pox4* gene proved to share 63 and 64% of identity with the *pox1* and *poxc* genes, respectively.

Further investigations were performed on the clone I24, since the verified presence of at least five laccase genes in this clone strengthen the hypothesis of a clustered organization of these genes. A sub-library of the clone I24 was prepared and screened. A clone containing a ~7,000-bp fragment, hybridizing with all the probes *poxc*, *poxa1b*, *pox1* and *poxa3* cDNAs, was selected. By complete sequencing of this genomic fragment, two new putative laccase genes, named *pox3* and *pox5*, were found. The complete *pox3* gene sequence (4,400 bp including 1,400 bp of 5′ and 800 bp of 3′ non-coding regions) was determined on this fragment, being the gene entirely contained in the subclone. On the other hand, *pox5* gene is only partially contained in this fragment, and to complete its sequence (2,837 bp), sequencing on the clone I24 was required. *pox3* and *pox5* genes proved to have a tail to tail arrangement, with an opposite transcription orientation and a 800 bp distance between them. Despite their closeness, which could suggest a recent event of gene duplication, the *pox3* and *pox5* gene sequences proved to be quite divergent, sharing only 38% of identity.

### Isolation of new laccase cDNAs, analyses of gene structures and of deduced protein sequences

Expression of the three new putative laccase genes *pox3*, *pox4* and *pox5* in *P. ostreatus* was assessed by means of reverse transcriptase (RT)-PCR experiments with gene-specific primers (Table 1). Analyses were performed on 7-day-old fungus grown with or without addition of 150 μM $CuSO_4$ or 150 μM $CuSO_4$ plus 2 mM ferulic acid. *pox3* and *pox4* mature transcripts were isolated, while no RNA species coding for *pox5* could be detected in any tested conditions.

*pox3* proved to be expressed in all the analyzed culture conditions. The isolated *pox3* cDNA covers 1,569 bp, including an opening reading frame coding for a 501 aa long polypeptide. In all tested conditions two *pox3* cDNAs were isolated differing for only one nucleotide (at the position 1690 of the entered EMBL sequence), giving the aminoacidic substitution Ser/Ala at position 301 of the deduced proteins (POX3/Ser and POX3/Ala). The *pox3* coding sequence is interrupted by ten introns. An altered *pox3* transcript, due to missing excision of the intron IV and leading to an early stop in translation, was also amplified in the copper/ferulic acid growth condition. All the splicing junctions of *pox3* introns adhere to the GT-AG rule and most of the internal lariat sites are conform to the consensus CTRAY (Padgett et al. 1984). The intron size ranges from 47 to 67 bp, similarly to most of the fungal introns (Gurr et al. 1987).

*pox4* mature transcripts were isolated in the presence of both copper sulphate and ferulic acid. The 1,599-bp-long





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cDNA encompasses an opening reading frame coding for a protein of 509 aa. The *pox4* coding sequence is interrupted by 19 introns, with all splicing junctions adhering to the GT-AG rule.

No RNA species coding for *pox5* could be detected in the tested conditions. Moreover, identification of the splicing junctions, based both on the GT-AG rule and on homology with the other known laccase sequences, was uncertain in the 3′ region of the gene.

A comparison of *P. ostreatus* laccase gene structures is shown in Fig. 1. The coding regions for the L1 and L2 signature sequences, containing copper-binding regions I and II (Kumar et al. 2003), were located in analogous positions in *poxc*, *pox1* and *pox4*, whilst the same region is organized in a different way in *poxa1b* and *poxa3*. In the *pox3* gene only one intron instead of three (found in *poxc*, *pox1* and *pox4*) and two (found in *poxa1b* and *poxa3*) is included between the L1 and L2 signature sequences. The structure of the 3′ region is well conserved between *pox1*, *poxc*, *pox4* and *poxa1b* genes, that have the L3 and L4 signature coding sequences equally organized, whereas the 3′ regions of *poxa3* and *pox3* genes are more divergent.

Both POX3 and POX4 predicted protein sequences contain N-terminal secretion signals (20 and 22 amino acids, respectively), conform to the typical sequences of eukaryotic proteins (Nielsen et al. 1997), the conserved ten histidine and one cysteine residues of the copper-binding centers of laccases, the fungal laccase signature sequences L1-L4, as defined by Kumar et al. (2003), and the substrate binding loops (Fig. 2). Compared to the defined signature sequences, POX3 only showed a tyrosine to phenylalanine change in L1 and an alanine to serine change in L2 (Fig. 2). As in other laccases (Larrondo et al. 2003a), sequences of the substrate binding loops (loops I–IV in Fig. 2)—described from 3D structure analysis of crystallized laccases (Bertrand et al. 2002; Hakulinen et al. 2002; Larrondo et al. 2003a)—are relatively little conserved between the *P. ostreatus* proteins (Fig. 2).

Four potential N-glycosylation sites were identified in POX3 (Asn-212, Asn-297, Asn-436 and Asn-495) and two in POX4 (Asn-4214 and Asn-441).

Identity percentages shared among protein sequences of all the members of the *P. ostreatus* laccase family are reported in Table 2. Alignment of the deduced POX3 and POX4 amino acid sequences with other known fungal laccases shows for POX3 the highest overall identity (61%) with laccase 1 from *Pleurotus eryngii* (UniProt accession number **Q308C0**), whereas POX4 exhibits the maximum identity (88%) with laccase LCC2 from *Pleurotus sajor-caju* (UniProt accession number **Q7Z8S5**).

## Heterologous expression of rPOX3 and rPOX4 in *Saccharomyces cerevisiae* and in *Kluyveromyces lactis*

The heterologous expression of rPOX3 (rPOX3/Ala) and rPOX4 was evaluated in the yeasts *S. cerevisiae* and *K. lactis*, by using heterologous expression systems previously developed for the *P. ostreatus* laccases POXC, POXA1b and POXA3 (Piscitelli et al. 2005; Faraco et al. 2008a). Laccase native signal peptides were used.

*Saccharomyces cerevisiae* and *K. lactis pox4* transformants did not produce any activity neither toward ABTS nor toward DMP both in solid and in liquid cultures. The expression of rPOX4 was ascertained by Western Blotting with Anti-POXC antibodies, taking advantage of the high degree of identity shared by the two proteins (78%).

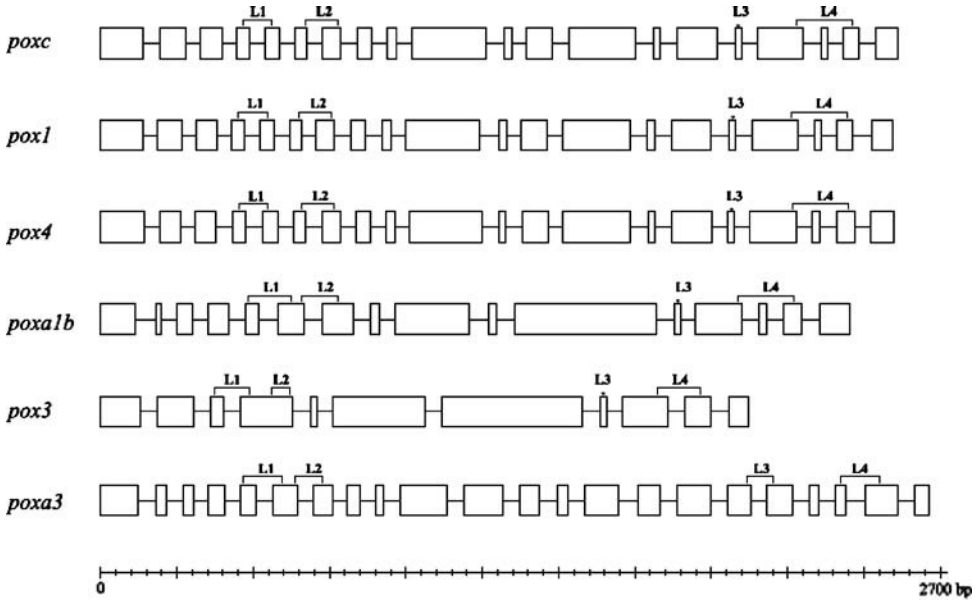


**Fig. 1** Comparison of *P. ostreatus* laccase gene structures. Exons are showed as *open boxes* and introns in *solid lines*. Regions coding for L1-L4 consensus motifs are displayed in brackets





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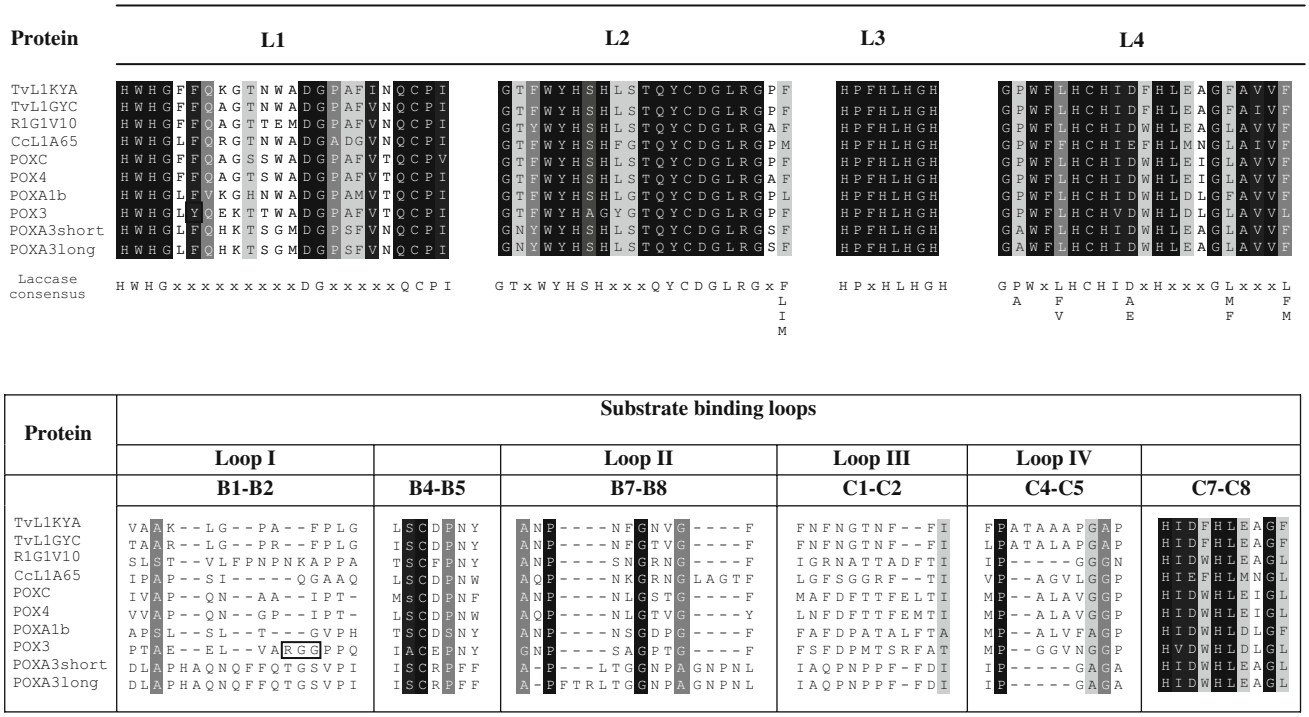


**Fig. 2** **a** Comparison of the laccase signature sequences from the *P. ostreatus* laccases POXC, POXA1b, POXA3, POX3 and POX4 with the corresponding sequences from laccases with known 3D structure: TvL1KYA, *T. versicolor* laccase III (Protein Data Bank [PDB] code 1KYA [Bertrand et al. 2002]), TvL1GYC, *T. versicolor* laccase I (PDB code 1GYC [Piontek et al. 2002]), R1G1V10, *Rigidoporus lignosus* Laccase (PDB code 1V10 [Garavaglia et al. 2004]), CcL1A65, *C. cinereus* laccase Lac-Cc (PDB code 1A65 [Ducros et al. 2001]). The fungal laccase signature sequences (L1-L4) are shown together with the fungal laccase consensus sequence (Laccase Cons.) according to Kumar et al. (2003). The conservation scoring is performed by PRALINE (http://zeus.cs.vu.nl/programs/pralinewww/); residue shading has been applied from scoring 10 (*black*) to 7 (*grey*). The Y69 of POX3 laccase is *boxed* .**b** Sequence alignment of the potential substrate binding loops of the *P. ostreatus* laccases and laccases with known 3D. The sequences of potential substrate binding loops of the laccases have been aligned according to Loops I–IV of *T. versicolor* 1KYA laccase given in Table 1 by Larrondo et al. (2003a, b) and according to the nomenclature of Hakulinen et al. (2002) for the each two $\beta$-strands flanking the respective loop in Lcc1. Loops B1-B2 locate in the primary POXC sequence at positions 165–175, B4-B5 at positions 213–219, B7-B8 at positions 271–280, C1-C2 at positions 339–350, C4-C5 at positions 395–403 and C7-C8 at positions 462–471. The stretch R162-G164 of POX3 laccase is boxed

**Table 2** Identity % among protein sequences of the members of *P. ostreatus* laccase family

| | POX3 | POX1 | POXA3 | POXA1b | POXC |
|---|---|---|---|---|---|
| POX4 | 57 | 75 | 47 | 57 | 78 |
| POX3 | | 56 | 48 | 62 | 57 |
| POX1 | | | 48 | 58 | 89 |
| POXA3 | | | | 45 | 47 |
| POXA1b | | | | | 59 |

A major band was detected in culture supernatants of *S. cerevisiae* transformants at about 70 KDa, whereas the predicted molecular mass of POX4 is about 60 KDa (data not shown). Two very faint signals were revealed by Western Blotting on culture supernatants of *K. lactis* transformants, one at a molecular mass lower than that expected (40 KDa), possibly indicating proteolytic degradation of the mature protein, the other one showing a slightly higher apparent molecular mass (about 65 KDa) (data not shown).

*pox3* transformants were found positive by plate activity assay with DMP for both yeasts, producing a colored halo in the presence of the chromogenic substrate. The enzyme production was further investigated in liquid cultures, by using ABTS as substrate. Time-course analysis of rPOX3 production was initially performed in the growth conditions previously optimized for rPOXA1b and rPOXC (Piscitelli et al. 2005). Maximum laccase activity production was reached in the early growth phase for both yeasts, with a higher level of production in *S. cerevisiae* (around 30 mU $ml^{-1}$ at the second day) than in *K. lactis* (around 12 mU $ml^{-1}$ at the third day). Because of the low level of rPOX3 activity production in *K. lactis*, further experiments were performed only in *S. cerevisiae*.

Unlike rPOXA1b production showing a continuous increase during the yeast growth at least until the seventh day, rPOX3 production exhibited a drop of activity on the third day. In order to improve laccase production, the effect of growth temperature and of induction conditions was evaluated. Decreasing the growth temperature to 20°C did







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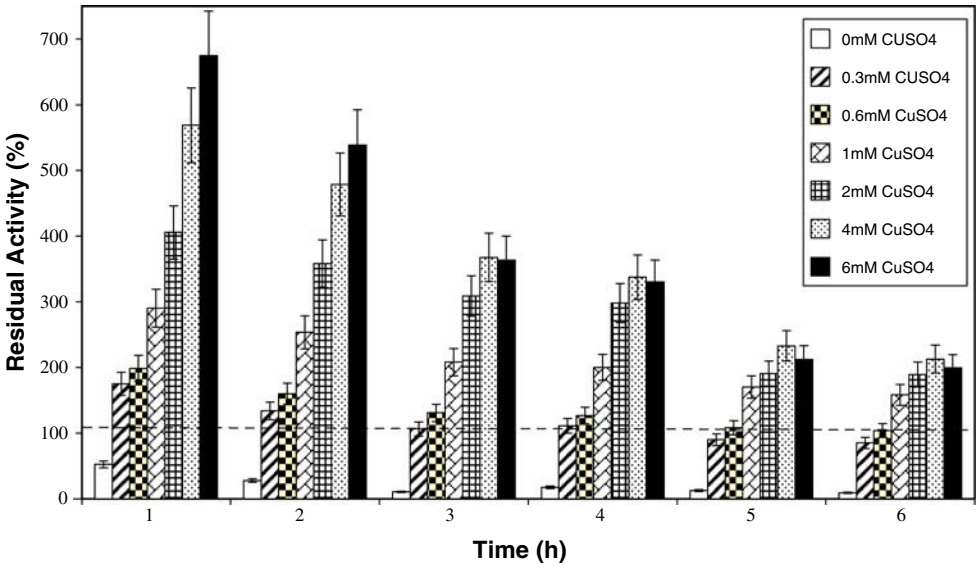


**Fig. 3** Reconstitution of laccase activity after incubation of POX3 culture supernatants with different amounts of copper sulphate (0.3–4 mM). Laccase activity was measured at the indicated times and data are expressed as ratio between the measured activity value and that measured at time zero



not significantly affect laccase activity maximum. Higher laccase activity production was obtained by adding the expression inducer (0.6 mM copper sulphate) later, 22 or 46 h after the inoculum time. In the latter condition a higher effect was observed with a laccase activity peak of 75 mU $ml^{-1}$ on the second day (data not shown).

### Stability and kinetic parameters of rPOX3

Further experiments were carried out in order to verify if the observed decrease of laccase activity in culture broth of *S. cerevisiae* transformed with *pox3* was due to enzyme instability. Supernatants of 42-h copper-induced cultures lost 60% of their laccase activity after only 1 h of incubation at room temperature, whilst culture supernatants of *S. cerevisiae* transformed with *poxa1b* retained 80% of their laccase activity even after 12 h, thus confirming the hypothesis of an unusual instability of rPOX3.

The addition of 20% glycerol to culture supernatants led to a total retention of rPOX3 laccase activity, after 2 h. Moreover, an even more relevant effect was observed by adding copper sulphate to the enzyme sample (Fig. 3). After 1 h incubation in the presence of the added copper, the enzyme did not lose its activity and showed a further increase, up to sevenfold the time zero value. This effect proved to be copper concentration dependent. However, loss of enzyme activity was observed at long times. When the same experiments were performed on culture supernatants of *S. cerevisiae* transformed with *poxa1b*, no effect on enzyme activity was observed after copper addition.

$K_M$ values of partially purified rPOX3 were determined toward the non-phenolic substrate ABTS and the phenolic substrate DMP and compared with those of rPOXA1b produced in the same host. rPOX3 showed a remarkable lower affinity toward ABTS exhibiting a $K_M$ value fourfold higher ($0.33 \pm 0.02$) than that of rPOXA1b ($0.08 \pm 0.01$), whereas the Michaelis constant toward DMP ($0.83 \pm 0.08$) is similar to that of rPOXA1b ($0.57 \pm 0.03$).

## Discussion

Three new members of the *P. ostreatus* laccase gene family, *pox3*, *pox4* and *pox5*, have been isolated. Hence, the panel of *P. ostreatus* laccase genes has been enlarged up to seven members, and their closely related spatial organization on the fungus genome has been demonstrated. All the *P. ostreatus* laccase genes, *pox1*, *poxc*, *poxa1b*, *poxa3*, *pox3*, *pox4* and *pox5* proved to be located within a 150-kb genomic region, with *pox3* and *pox5* situated at a 800-bp distance, *pox1* localized in the same 8.5-kb fragment with *poxa3* and in the same 10-kb fragment with *poxa1b*, whereas the closeness of *poxc* and *pox1* genes had been already previously demonstrated (Larraya et al. 2000).

The existence of such a gene clustering of laccase genes had previously been observed in the other fungi *P. chrysosporium* (Larrondo et al. 2004), *Agaricus bisporus* (Smith et al. 1998), *Rhizoctonia solani* (Wahleithner et al. 1996) and *Coprinopsis cinerea* (Kilaru et al. 2006), too. In many other basidiomycetes, the presence of several different laccase genes was reported (Yaver et al. 1996; Yaver and Golightly 1996; Mansur et al. 1997; Soden and Dobson 2001; Hoshida et al. 2001; Rodrıguez et al. 2008), and in this context the family of 17 laccase genes identified by Kilaru et al. (2006) in the released genome of *C. cinerea* is emblematic.

Comparison of *P. ostreatus* laccase gene structures revealed that *pox4* shows exactly the same gene organization of *pox1* and *poxc* genes. On the other hand, *pox3* and, even more, *poxa3* exhibit a structure very different from





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that of the other members. *pox3* is characterized by a shorter length (2,120 bp), related to a reduced number of introns. Only few intron positions are conserved in comparison to the other *P. ostreatus* laccase genes, with most of them common to the *poxa1b* gene. The heterogeneity of intron number between the L1 and L2 signature sequences allows us to explain the different lengths of fragments obtained in PCR experiments with degenerate primers and the absence of any amplified product for *pox3*. A similar variability in introns numbers and positions between the copper-binding regions I and II was found in *Morchellaceae* species (Kellner et al. 2007), in *lcc16* and *lcc17* of the basidiomycetes *C. cinerea* (Kilaru et al. 2006) and in *Piloderma byssinum* (Chen et al. 2003).

The high sequence similarity and the identical intron–exon structure shared by *poxc*, *pox1* and *pox4* genes along with the high degree of identity among the corresponding protein sequences indicate a close relationship between these genes and suggest the existence of a "laccase subfamily" consisting of these members. On the other hand, the high structural divergence of *poxa3* gene is in agreement with the evolutionary distance among POXA3 and all the other laccase family members. As a matter of fact, phylogenetic analysis of protein sequences of the *P. ostreatus* laccase family revealed that all the members, except for POXA3, fall in clades as close as similar intron–exon structure is shared by the corresponding genes (Hoegger et al. 2006). On the contrary, the atypical heterodimeric laccase POXA3 (Giardina et al. 2007) falls in the cluster with *Agaricus bisporus* Lac2, a protein found associated to smaller polypeptides (Perry et al. 1993; Wood 1980).

The nucleotide sequences extending 500-bp upstream of the start codons in *pox3* and *pox4* genes were analyzed and compared to those of *poxc*, *pox1*, *poxa3* and *poxa1b* (Faraco et al. 2003) (Fig. 4). This analysis allowed individuation of several putative response elements, differentially distributed among the promoter sequences. Two potential ARE motifs, involved in phenol antioxidant response in mammalian cells, and previously detected in *P. sajor-caju lac4* promoter (Soden and Dobson 2003), were found in *pox3* and one in *pox4*. Five putative Metal Responsive Elements (MRE), were identified in *pox3*, two of them matching the consensus sequence TGCRCNC, the others differing from the consensus for only one base pair, as already reported for the other *P. ostreatus* laccase promoters (Faraco et al. 2003). Only one MRE could be predicted in the *pox4* promoter region. A more complex pattern of putative response elements characterizes *poxc*, *pox1*, *poxa3* and *poxa1b* promoters, including several different putative regulative elements (Fig. 4), and among these all the putative MREs in the 400-bp extended *poxc* and *poxa1b* promoter regions are recognized by fungal proteins, except for the *poxc* MRE located downstream of the transcription-initiation site (Faraco et al. 2003).

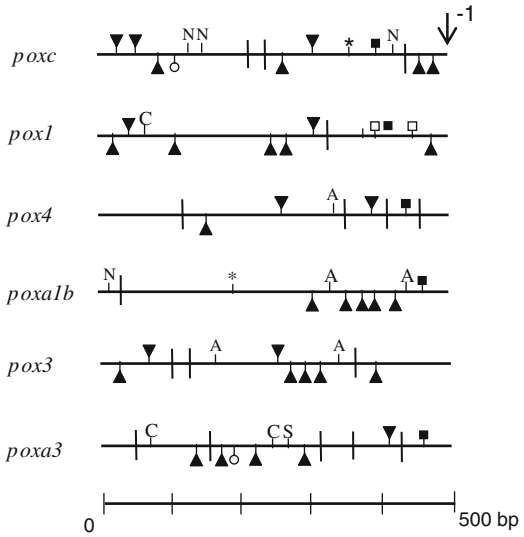


**Fig. 4** Distribution of putative *cis*-acting elements in the promoter regions of *P. ostreatus* laccase genes 500-bp upstream of the start codons (↓): (*filled square*) TATA box; (*vertical line*) CAAT box; (*filled inverted triangle*) GC box; (*) HSE (repeated NGAAN motif) (Mager and De Kruijff 1995); (N) NIT2 (TATCT) (Jarai et al. 1992); (*A*) ARE (TGACNNNGC) (Rushmore et al. 1991); (*open square*) Putative response elements PRE (ATATC and TGGGT motifs) (Soden and Dobson 2003); (*filled triangle*) MRE; (*open circle*) XRE (TNGCGTG) (Rushmore et al. 1991); (*C*) Cre-A-binding site (GCGGGG) (Arst and MacDonald 1975); (*S*) STRE Stress responsive element (CCCCT) (Galhaup et al. 2002)

The pairwise alignment of the promoters of the *P. ostreatus* laccase gene family reveals that they are very little conserved. The only exception comes from the comparison of *poxc* and *pox1* promoters, showing 81% identity, in agreement with their grouping in the same "laccase subfamily". No significant similarities were found, instead, between both *pox1* and *poxc* promoters when compared to *pox4*, despite the close relationship of these genes and of their corresponding proteins.

Interestingly, *pox1*, *poxc* and *pox4* promoters exhibit a very high degree of identity (95–97%) with the upstream regions of the laccase genes from *P. sajor-caju lac1*, *lac4* and *lac2* (Soden and Dobson 2003), respectively. Such relation among these genes is also confirmed by the high degree of similarity shared by their corresponding proteins: POX1 has a 99.5% identity with Psc Lac1, POXC shares a 99.1% identity with Psc Lac4, and POX4 shows a 91% identity with Psc Lac2. Transcripts levels of *P. sajor-caju* laccase isozyme genes have been shown to be differentially regulated in response to copper, manganese, nutrient nitrogen and several aromatic compound (Soden and Dobson 2001), suggesting that a similar mechanism could also occur for *P. ostreatus* laccases.







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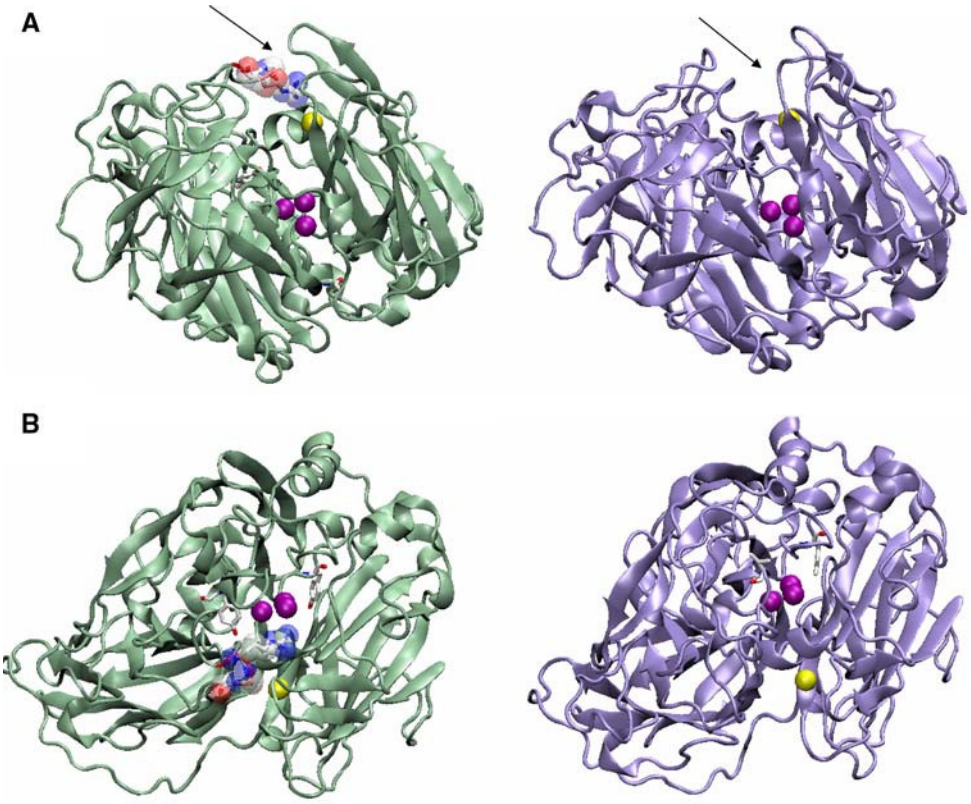


**Fig. 5** Comparison between POX3 (*in green*) and POXA1b (*in violet*) 3D structure models, with side (**a**) and front (**b**) views of substrate binding cavity (*indicated by arrow*). Cu (T1) is represented as a *yellow sphere*, whereas the (T2-T3) Cu trinuclear cluster is represented by means of *violet spheres*. The side chains of arginine and glycine residues forming the extra R162-G164 loop in POX3 are represented by means of *red and blue balls*, respectively



POX3 and POX4 production by *P. ostreatus* has not been detected so far. It could be due to the different conditions required to produce active forms of these isoenzymes that have not been explored yet. Therefore, to analyze properties of POX3 and POX4 isoenzymes their recombinant expression was needed. Previous attempts to obtain homologous expression of POXC isoenzyme had been unsuccessful because of the very low efficiency of fungal transformation (unpublished data). On the other hand, heterologous recombinant expression of POXA1b, POXC and POXA3 had been successfully performed in the yeasts *S. cerevisiae* and in *K. lactis* (Piscitelli et al. 2005; Faraco et al. 2008a). Therefore, recombinant heterologous expression of POX3 and POX4 was performed in these hosts, using the expression systems previously developed for the other *P. ostreatus* laccases. rPOX4 proved to be expressed at very low level both in *S. cerevisiae* and in *K. lactis*. Taken together, the results of laccase activity assays and Western blotting analyses on *pox4* transformants indicate either the expression of a not active form of the recombinant enzyme or expression of an active form at not detectable levels. Even when different culture conditions were explored or experiments of copper addition to culture supernatants of *pox4* transformants were performed no improvement of laccase activity was observed. A not correct folding and differences with codon usage of the hosts could explain the low *pox4* expression levels. On the other hand, an active rPOX3 was obtained in both hosts, even if at lower levels than those of the other *P. ostreatus* laccases (Piscitelli et al. 2005). The quick drop of rPOX3 activity production in *S. cerevisiae* culture broth was related to loss of copper and formation of an apo-laccase, that could be reconverted in holo-enzyme by copper addition to cell-free filtrates, thus demonstrating that metal depletion is reversible (Koroleva et al. 2001), and suggesting that the apo-enzyme retains at least a partially folded state (Wilson et al. 2004). Our results are in agreement with those of Larrondo et al. (2003b), showing that *Aspergillus nidulans* transformed with a laccase cDNA from *Ceriporiopsis subvermispora* secreted an apo-laccase that could be reconstituted by copper.

Analyzing the POX3 sequence, the only difference in the region surrounding the type II copper-binding site is a tyrosine residue at the position 69, unusual in basidiomycete laccases which have a conserved phenylalanine in the same position (Fig. 2). The corresponding position is occupied by a glutamic acid (Glu106) in CueO, and by an histidine in *Melanocarpus albomyces* laccase (His98), in which this residue was found as an oxidized form (2-oxohistidine) (Hakulinen et al. 2008). The polarity of this residue was found to influence solvent interaction and lability of the type II copper in CueO bacterial laccase (Li et al. 2007). A more hydrophobic residue would gate the tunnel connecting type II copper to the protein surface preventing its



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exchange with solvent. As a fact, mutation of this Glu to a Phe enhanced CueO oxidation activity lowering T2 copper deficiency (Li et al. 2007). In rPOX3, the addition of exogenous copper could counterbalance type II copper lability caused by the presence of a more hydrophilic residue (Y69), providing an overall enzyme stabilization.

The higher $K_M$ measured for rPOX3 toward the bulkier substrate ABTS compared with rPOXA1b, could be explained by analysis of the POX3 3D model, that revealed a more buried substrate interacting pocket with respect to POXA1b, due to an elongated loop B1-B2 (Fig. 2) produced by the insertion (R162-G164), that would hinder the access of the substrate (Fig. 5).

Although POX3 shares the highest closeness with POXA1b within the *P. ostreatus* laccase family in terms of both protein sequence similarity and conservation of some intron positions, the corresponding recombinant protein is revealed much less stable.

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# *Appendix*

This section deals with the work carried out in the Laboratory of BiosCiences at Paul Cezanne University in Marseille, under the supervision of Dr. T.Tron

**Development of molecular tools to improve laccase heterologous expression in yeasts**

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

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

Filamentous fungi are, at least so far, the best producers of recombinant laccases, with protein yields from 70 mg $l^{-1}$ [2] to 230 mg $l^{-1}$ [3]. However, they are more time consuming to work in comparison with yeasts. Besides possessing characteristics favourable for fermentation process, the yeasts can grow to high cell densities and secrete heterologous proteins efficiently, and also provide efficient post-translational modifications such as glycosylation. In addition, the genetic manipulations of yeasts are easy and well developed.

Regarding laccase production in yeasts, a considerable variability of production yields with respect both to cDNAs expressed (originating from the same organism) and to the host used, has been reported [4,5,6]. Such “selectivity” in expression may reflect the inability of yeast to process different laccases post-translationally with the same efficiency. Furthermore, these variable yields hinder forecast of the most suitable host, or of the most promising laccase to be expressed.

This appendix deals with the experimental work carried out in the Laboratory of BiosCiences at Paul Cezanne University in Marseille during my three months of foreign experience. The challenge was to design strategies to maximize the yield of recombinant laccases, in the attempts to raise production levels sufficient to enable deeper understanding of the structure/function relationships and, consequently, industrial applications of *P. ostreatus* laccases.

Two important criteria to be met in order to make recombinant protein production economically and industrially feasible are: 1) a high copy number of the gene introduced into the host organism in order to obtain a high-level expression and 2) a high mitotic stability of that gene, since the large-scale production of proteins often requires long-term culturing of the recombinant host in non-selective media.

These topics have been addressed by designing two suitable molecular strategies: 1) the development of autoselection system and 2) the construction of high-copy

number integrative vectors. Both approaches have been applied to *S. cerevisiae* expression system in attempt to optimize *P. ostreatus* laccase yields of production obtained in earlier experimental work [7].
Moreover, since the production of a functional protein can be intimately related to the cellular machinery of the host organisms, laccase expression was also investigated in an alternative host: *Pichia pastoris.*

## 1. Development of a *S. cerevisiae* autoselection system

The first key factor required for successful production of a gene product expressed from an episomal plasmid, is the stability of the vector. However, as the requirement for defined minimal medium for plasmid selection imposes sub-optimal growth conditions and reduces yields of protein production, so the use of selective drugs may be undesirable or expensive.
One strategy to avoid the limitations of selective medium is to use autoselection. Autoselection relies on the presence of a plasmid to provide an essential activity for an otherwise unviable host, without employing any external nutrient or antibiotic selection. In such systems, selection of the transformed population occurs independently of the growth medium composition, allowing the use of relatively inexpensive complex media, which are preferred in industry for commercial-scale fermentations. Different autoselection systems have been developed for *S. cerevisiae* host cells [8, 9, 10, 11]. Host cells may be unviable because they lack an essential gene, or because of a mutant gene which under certain conditions is essential, or because of additional lethal elements which are counteracted by a plasmid product.
Autoselection systems relying on the rescue of lethality of *fur1 ura3* mutants by *URA3* plasmids have been reported in *S. cerevisiae* and *Kluyveromyces lactis*. The *FUR1* gene of *S. cerevisiae* encodes uracil phosporibosyl-transferase, which catalyzes the conversion of uracil into uridine 5'-phosphate in the pyrimidine salvage pathway. If this gene were disrupted, *S. cerevisiae ura3* strains would therefore not be viable, unless it possessed a complementing functional *URA3* gene to synthesize uridine 5'-phosphate *de novo*. This autoselection system has found several applications in both host strains. Several works reported on an improved production of different expressed proteins by using autoselective strains grown in complex media as compared to minimal media. [12, 13]. β-xilanase activity, for example, increased up to 24 fold in autoselective *S. cerevisiae* strain when the cells were cultures in YPD medium instead of selective SC medium [13].

### Material and methods

*Construction of the disruptive plasmid*
Standard protocols were followed for DNA manipulation, accordingly to matherial and methods section of chapter 4.
The *FUR1* gene sequence was recovered from *S. cerevisiae* genome database (http://www.yeastgenome.org/) and the following oligonucleotide couple was designed for its amplification from genomic DNA: Fur1fw 5'-GCAGGTCATTGCAATAACAGAAAGGC-3' and Fur1rev 5'-GGTTCTATT TTAACGGGGTTATCTACC-3'. The resulting 1305bp fragment was cloned into pGEMT-Easy vector. A 250bp deletion was generated within the *FUR1* open reading frame by inverse PCR on the pGEMT-easy-*FUR1* vector: the reverse (Fur1int/rev CTGTTGCACAGGTAGATGG), and the forward primers (Fur1int/fw CTTCCTATTAGACCCAATGC) provide, respectively, a cleaved *PvuII* end and the

insertion of an *AgeI* restriction site on the amplified vector. *LEU2* gene was amplified using the *LEU2*-plasmid pYEP351 as template, with the following oligonucleotides: NarILeu2fw 5'-TTTGGCGCCGGTAAAATAGCGCTC TCG-3'; XmaILeu2rev 5'-AAACCCGGGTGCGGTATTTCACACCGC-3'. The amplified fragment was cloned into *SmaI* digested pUC19 and checked by sequencing. The *LEU2* gene was then isolated as a 2243bp *PvuII-XmaI* fragment and ligated to pGEMT-easy-Δ*fur1* vector produced by inverse PCR and digested with *AgeI*. The resulting disruptive allele *fur1*::*LEU2* can be recovered from the recombinant vector by *BstZI* digestion and used to promote gene replacement with the chromosomal *FUR1* gene.

**Results**

The aim of this study was to apply the *fur1*/*URA3* based autoselection system to laccase production by *S. cerevisiae*. As described in chapter 4, laccase heterologous expression in W303-1A *S. cerevisiae* strain (*MATade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100)* is linked to pSAL4 episomal vector (based on *URA3* as selection marker) and is carried out in a selective medium. The development of a reliable system which can provide *FUR1* deletion in the genomic background of whatever recombinant strain carrying an *URA3* based vector, would represent a valid tool to improve product yields by testing laccase production in rich media.

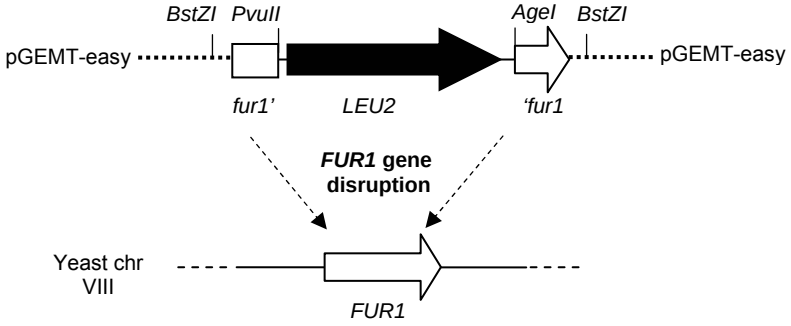


**Fig.1 Gene replacement of FUR1 gene**

In order to cause a genomic deletion in *FUR1* gene, a plasmid carrying a *fur1*::*LEU2* cassette was designed (Fig.1). The deletion cassette can be excised as a 3.3kb linear *BstZI* fragment and subsequently transformed into *S. cerevisiae* strains, to produce the mutated *fur1* phenotype by gene replacement with the chromosomal *FUR1* gene.

The results obtained so far, deals with the design and construction of the deleting plasmid, whilst further work will be addressed to achieve the deleted mutant and to analyze laccase production in the autoselective strains.

**2. Designing of expression vectors for high-copy number integration into the ribosomal DNA of *S. cerevisiae***

The potential instability of episomal vectors can pose problems for expression of some heterologous proteins, especially in large industrial applications where cells are often subjected to prolonged growth in the absence of selection. The use of integrative expression vectors would represent a valid alternative to overcome this limitation. Owing to their integration into the host genome by homologous recombination, this kind of expression vector assures an increased genetic stability of the transformed strain compared with episomal systems.

A number of integrative vectors have been developed for the yeasts most commonly applied in industrial processes such as *K. lactis* and *S. cerevisiae*. A widespread used integrative vector for *K. lactis*, utilizes the LAC4 strong promoter to both drive gene expression and to direct plasmid insertion into the homologous genomic locus.

[14]. In another example, plasmid integration into *S. cerevisiae* genome is obtained by directing the recombination at *ura3* locus [15]. The main drawback of this kind of integrative vector is the reduction in vector copy number and, consequently, in expression levels compared to episomal vectors. As a fact, the majority of transformants deriving form plasmid insertion at a specific genomic locus, contain a single copy of the integrated vector. Transformants containing several copies (2-10) of tandemly integrated vectors are generally obtained at very low frequencies (2-5% of the transformed population) [16].
One method devised to increase the copy number of integrative vectors, involves integration into the ribosomal DNA (rDNA) locus, a region of tandemly repeating DNA in the host chromosome, which frequently leads to multicopy integration.
The number and the length of these rDNA repeats can vary considerably between different species. In *S. cerevisiae*, about 150 copies of a 9-kb unit are localized at a single locus on chromosome XII. Each unit harbours the coding sequences for 25S, 18S, and 5.8S rRNA, which are processed from a 35S precursor transcript. The coding sequence for the 5S species is located between two precursor units and separated from them by non-transcribed spacers (NTS1 and 2) (Fig.2). The promoter elements are located in the NTS regions, whilst the functions of some other regulatory elements in the NTS or the ITS (intergenic spacer) have also been described [17].

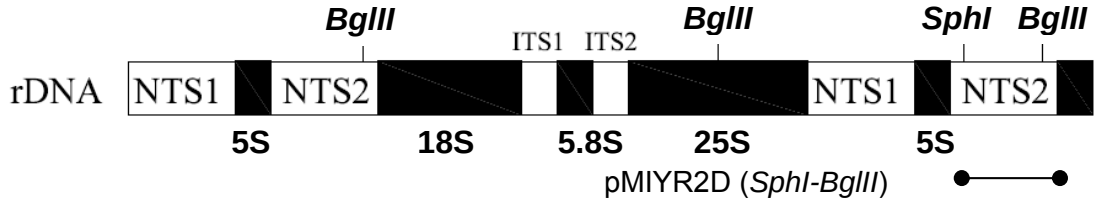


**Fig.2 Genetic organization of an rDNA unit from *S. cerevisiae.* The *SphI-BglII* fragment cloned in the designed integrative vector is also depicted.**

Integrative systems based on rDNA-targeting elements have been successfully applied in several yeasts, such as *Hansenula polymorpha* [18], *Arxula adeninivorans* [19], *K. lactis* [20], *S. cerevisiae* [21], *Yarrowia lipolytica* [22]. Besides being organized in repeated units, rDNA clusters contain highly conserved regions which can be exploited to direct plasmid integration into a broad range of heterologous hosts. Particular conserved rDNA sequences (generally derived from the portion ETS-18S-5.8S or covering the 25S rDNA region) have shown to be suitable for this purpose. Several “universal” vector based on rDNA targeting have been designed and applied for protein expression in different yeasts, providing a useful tool for the comparative assessment of several selected organisms for criteria like appropriate protein processing, modification or secretion [23]. rDNA integration approach also represents and interesting tool for the co-expression of more than one recombinant protein in the same host. Stable co-integration of up to three different expression plasmids by a single transformation step, followed by the efficient co-expression of the integrated genes, has been demonstrated in *Hansenula polymorpha* and *Arxula adeninivorans* [23, 24].

## Material and Methods

*Construction of the integrative vector*

Standard protocols were followed for DNA manipulation, accordingly to matherial and methods section of chapter 4.

The 1.1kb rDNA region was amplified from *S. cerevisiae* genomic DNA, by means of Taq polymerase (Promega) with the following couple of oligonucletides: rDNAPvuIIfw GACTAGGCAGCTGTGACGATCACC and rDNAPvuIIrev AAAGTTGGTCGGCAGCT GGCATGC. Both primers included an added *PvuII* site at their 5'-ends. The amplified fragment was cloned into pGEMT-easy vector and its sequence was checked. The rDNA containing fragment was then excised by *NotI* digestion and cloned into the pPICZαA (Invitrogen) vector, resulting in plasmid pPICrDNA. The DNA region including the elements required for propagation and selection of the integrative vector in *E. coli*, *Ori* and *bla* gene (conferring Ampicillin resistance, Ap), was amplified from pUC18 cloning vector using the following oligonucleotides: OriAmpSnaBIfw GGCGGTAATACGTATATCCACAG and OriAmpSnaBIrev TGGTTTCTTATACGTAA GGTGGC. This reaction allows the insertion of a *SnaBI* restriction site upstream and downstream the 1.8kb amplified fragment. Thus, the *SnaBI* digested Ori-Ap region was cloned into pPICrDNA, in the unique *SnaBI* contained in the rDNA cloned region, to obtain pPICrDNA-OriAp plasmid. The selection of recombinant clones was carried out in Zeocin containing LB medium, taking advantage of the presence of a *ble* gene (conferring Zeocin resistance) in pPICrDNA backbone. The same recombinant clones were also tested for their Ampicillin resistance in order to verify the correct amplification of *bla* gene.

The following couple of primers pSAL4PvuIIfw AAATTGCCCAGCTGTCTTAACC and pSAL4PvuIIrev CCTTTGAGTCAGCTGATACCGC, was used to amplify a 3.4kb fragment from B-pSAL4 expression vector (pSAL4 vector containing *poxa1b* cDNA described in Piscitelli et. al [7]) by the means of Takara polymerase (Lonza). The amplified fragment contains the CUP1-*poxa1b*-CYCterm expression cassette and the *URA3* selection marker. The latter has been amplified as an expression-deficient allele (*URA3d*), by providing a deletion of its promoter region: only 46 nucleotides upstream of its starting codon have been amplified, as previously reported in [25]. Both primers introduce a *PvuII* restriction site at both fragment ends. The 2.9kb cassette containing the *Ori*-Ap region flanked by the 250bp and 872bp rDNA fragments at 5' and 3' respectively, was excised by *PvuII* digestion from pPICrDNA-OriAp plasmid and ligated to the *PvuII* pSAL4-A1b-Ura3d fragment to get the 6.4kb integrative vector pYINA1b (Fig.3).

## Results

The aim of this study was to design a rDNA-targeting integrative vector for laccase expression in *S. cerevisiae*. Several elements have been considered to design such an integrative vector:

- The first step was the choice of a suitable rDNA region for genome targeting. Different rDNA portions have been used to direct vector integration in the rDNA locus of several yeast species. In all the reported examples, the number of plasmid copy integrations and its stability after long periods of growth under non selective conditions, turned out to be affected by the nature of the rDNA region.

The vector designed in this study is based on *S. cerevisiae* pMIRY2D (Multiple integration into ribosomal DNA in yeast) plasmid previously described by Lopes and co-workers [26]. This plasmid contains a 1.1kb *BglII-SphI* fragment of the rDNA unit (Fig.2). It was found to integrate into rDNA locus of *S. cerevisiae* with a high copy

number (100-200 copies per cell) and to be stably kept on yeast chromosome (80-100% of the plasmid copies retained over 70 generations of growth under non-selective conditions). This high mitotic stability has been ascribed to the nature of the rDNA elements contained in this region, being dependent on the presence of specific rDNA regulatory elements such as those contained in the ITS, HOT1 elements (Hot-spot for recombination) and the PolI transcription enhancer. Moreover plasmid size was found to be an important, determinant of mitotic stability, being pMIRY-type vectors stably maintained in the rDNA cluster only if their size is smaller than or at most equal to the size of the rDNA unit (9.1kb).

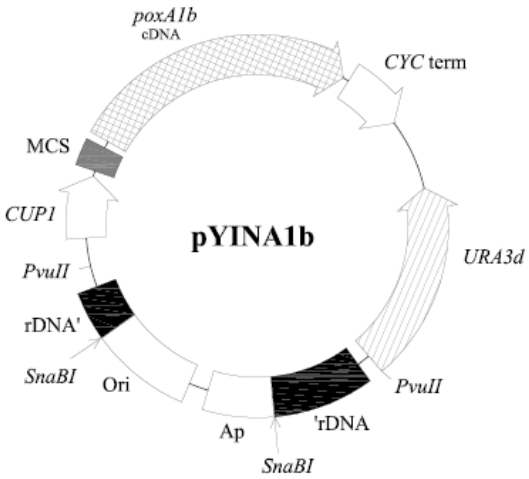


**Fig.3 Integrative vector designed for laccase expression**

- The construction of the integrative vector requires the presence of *ori* and *AmpR* gene for propagation and selection in *E. coli*. However, it has been reported previously that bacterial sequences are potentially toxic to other organisms and therefore may decrease vector stability in yeast [27]. Besides, bacterial DNA is not acceptable in a so-called "food-grade-vector", a status which is an advantage to gain clearance for use in the commercial biotechnological production of proteins. For these reasons the *Ori-Amp*$^R$ cassette was cloned inside the rDNA regions. In this way, by cutting the integrative vector with *SnaBI* enzyme, the bacterial sequences are removed and the linearized integrating fragment is produced as well. This strategy has the additional advantage that the final dimension of the integrating plasmid are shortened to 4.6 kb.
- High amplification of plasmid copy numbers can be obtained by applying a strong pressure for the selection of transformed clones. This can be achieved by using a selective marker gene that is expressed from a suboptimal promoter. To this aim, a promoter defective URA3 gene was created by providing a small deletion in its promoter region [26]. The defective URA3 gene (URA3d) was thus chosen for further construction of the integrative vector.
- The expression cassette of the integrative vector is derived from *S. cerevisiae* pSAL4 expression vector and consists of a CUP1 copper-inducible promoter, and a CUP1 terminator separated by multiple cloning sites for the insertion of foreign gene to express. *P. ostreatus* laccase POXA1b was chosen as reporter gene to validate the systems, since it has proved to be efficiently produced in *S. cerevisiae* when its expression was associated to the episomal pSAL4 vector [7].

The integrative vector for laccase heterologous expression in *S. cereivisiae*, named pYINA1b, was constructed following the above described criteria. Further work will be addressed to test the designed system for its copy number, mitotic stability and laccase production in *S. cerevisiae*.

### 3. Heterologous expression of *P. ostreatus* laccases in *Pichia pastoris*

*Pichia pastoris* has gained widespread attention as an expression system because of its ability to express high levels of heterologous proteins for both basic laboratory research and industrial manufacture. This methylotrophic yeast is particularly suited to foreign protein expression for a number of reasons, including ease of genetic

manipulation, high level of protein expression at the intra- or extracellular level, and the ability to perform higher eukaryotic protein modification, such as glycosylation, disulphide bond formation and proteolytic processing. Regarding its ability to perform post-translational modifications, the glycosylated gene products generally have much shorter glycosyl chains than those expressed in *S. cerevisiae*, thus making *P. pastoris* a much more attractive host [28]. Because of these characteristics, many proteins have been successfully produced in functionally active form in *P. pastoris* [29]. Several factors affecting the level of protein expression in *P. pastoris* have been investigated, ranging from molecular ones to those related to the production process.
Being a methylotrophic yeast, *P. pastoris* is able to utilize methanol as the sole carbon and energy source. A key enzyme for methanol metabolism is alcohol oxidase (AOX) whose expression is tightly regulated at transcription level, being repressed by glucose and induced by methanol to very high levels, typically ≥30% of the total soluble protein in cells grown with methanol. For this reason, the inducible AOX promoter has been the most widely utilized to regulate the foreign gene expression. Other molecular factors such has the choice of proper genetic background of a *Pichia* host strain, have been shown to influence the level of protein production. This is best illustrated by the use of protease-deficient strains to improve the quality and yields of various heterologous proteins. On the other hand, since *P. pastoris* has no native plasmids, expression vectors designed for chromosomal integration have been developed, thus ensuring the genetic stability of the recombinant strains even in continuous and large scale fermentation processes [29].
In addition to efficient expression of intracellular products, *P. pastoris* is also capable of secreting high levels of foreign proteins. This is particularly advantageous, since simple purification of secreted recombinant proteins is possible due to the relatively low levels of native extracellular proteins [29]. Subtle controlling of cultivation parameters has also shown to minimize proteolyitic instability in the culture medium. Lowering the pH of the medium to more acidic values results in inactivating many neutral pH proteases without affecting the growth significantly; the addition of amino acid-rich supplements (e.g., peptone, casamino acids) by possibly acting as alternative competing substrates for proteases or repressing protease induction caused by nitrogen limitation, has also shown to enhance product stability.
Further advantages of *Pichia* system include the fast growth in inexpensive chemically defined media, together with the ease of high-density growth and scale-up to higher volume fermentations. In this regard, shake-flasks conditions are normally sub-optimal, due to limitations of volume, oxygen transfer, substrate addition and an inability to monitor these factors efficiently. Among the other factors, monitoring methanol in *Pichia* process is extremely important, since high levels of methanol (above 5g/l) can be toxic to the cells [30] and low levels of this carbon source may not be enough to initiate transcription [31]. A large improvement in productivity is commonly achieved using bioreactors in which all these process parameters can be monitored and controlled simultaneously allowing more efficient production of the desired heterologous protein [29]
*Pichia* proved to be a useful system for the production of laccases from various sources, including *T. versicolor* [32], *Trametes sanguinea* [33], *P. cinnabarinus* [34], *Pleurotus sajor-caju* [35] and *Ganoderma lucidum* [36]. Generally, best results were obtained when AOX1 promoter was used, with levels of active protein concentrations ranging from 0.008 to 0.11g/L; however laccase expression by glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter system also turned out to be a potential option, achieving up to 0.1g/l of active protein production [37]. In most of the cases,

the use of controlled conditions in bioreactors instead of using shake-flasks produced higher laccase yields.

**Material and methods**

*Construction of expression vector*

Cloning and expression of *P. ostreatus* laccase have been performed using the "Easy Select *Pichia* Expression kit" (Invitrogen) following manufacturer's instructions. pPICZB was chosen as expression vector. Its expression cassette includes the AOX1 promoter that allows methanol-inducible high level expression and the AOX1 transcriptional terminator. POX3 and POX4 cDNAs have been amplified by means of *PrimeSTAR® HS* DNA polymerase (Takara) with the following couple of oligonucleotides: pox3EcoRIfw TTGAATT CATAATAATGATTGCGCCTGTAC and pox3XbaIPichREV AATCTAGATTAGGTCGAAGGGTGCGTG; pox4EcoRIfw TGAAT TCATAATAATGCGCACATTCTCCC;pox4XbaIPichREVATCTAGACTAGGTTGGGA GCAAACC respectively and using the corresponding pSAL4-POX3 and pSAL4-POX4 vectors (see chapter 4) as templates. These amplifications allow the insertion of *EcoRI* and *XbaI* cloning sites upstream and downstream the cDNAs. Moreover, the yeast consensus sequence AYAATA (Y= T, C) has been added before the starting ATG in order to promote laccase translation in the yeast. No tag has been added at 3' of each cDNA sequences, by preserving their native stop codons. The *EcoRI*/*XbaI* amplified laccase POX3 and POX4 sequences, including their native signal peptides, were cloned into pPICZB giving pPB3 and pPB4 respectively. Laccase sequences of both recombinant clones were checked to rule out any amplification error.

*Expression of POX3 and POX4 laccases in P. pastoris*

*P. pastoris* X-33 was transformed with pPB3 and pPB4 by electroporation. Both plasmids were digested with *PmeI*, whose cleavage site is located in the AOX1 promoter, to efficiently target plasmid intergration to the AOX1 locus of yeast genome. Transformants were selected on YPDS (1%Yeast extract, 2% peptone, 2% glucose with 1M sorbitol added) plates supplemented with 100 µg/ ml Zeocin. Mut (Methanol utilization) phenotype of transformants was tested by replica plating on MD (minimal medium plus glucose) and MM (minimal media plus 0.5% methanol) media. The same clones were screened for laccase production on MM solid medium containing ABTS (0,5mM final concentration) and $CuSO_4$ (0.6mM). Laccase-producing transformants were identified by the presence of a dark green colour around the clones. The best laccase producing transformants were inoculated into 5 ml of buffered glycerol complex medium BMGY (10g/l yeast extract, 20g/l peptone, 0.1 M potassium phosphate buffer, YNB 13.4g/l, biotin 400µg/l, 100 ml of 10% (v/v) glycerol) medium (Minimal glycerol medium buffered at pH6) and incubated at 28°C overnight in a shaking incubator (200rpm). Then the cells were harvested and resuspended into 25 ml of production medium (buffered minimal medium)BMMY (10g/l yeast extract, 20g/l peptone, 0.1 M potassium phosphate buffer, YNB 13.4g/l, biotin 400µg/l, 1.5% methanol, 0.6mM $CuSO_4$) in 250ml flasks, to an OD of ~1. Transformants were cultivated at 28°C or 20°C, shaking at 200 rpm and methanol was daily added to a concentration of 1.5% to replace that consumed by the cells or lost by evaporation. Samples were analysed daily for laccase activity using ABTS assay and cell growth was also measured by taking $OD_{600}$ readings.

**Results**

*Cloning and expression of POX3 and POX4 laccases*

The expression of *P. ostreatus* POX3 and POX4 coding cDNAs was carried out in the heterologous host *P. pastoris*. To this aim, both sequences, were cloned in the pPICZB expression vector. Laccase secretion in the extracellular medium is driven by its native signal peptide and is regulated by AOX1 methanol inducible promoter.

Both POX3 and POX4 expression vectors (pPB3 and pPB4) were successfully transformed into the yeast, and about 100 clones for each transformation were checked for their Mut phenotype by comparing their growth ability on MD and MM media. This step is required in order to properly optimize the conditions for protein production, since both $Mut^+$ (fast growth on methanol as the only carbon source) and $Mut^s$ (slow growth on methanol as the only carbon source) phenotypes can be produced by plasmid integration in AOX1 locus. As a fact, since the linearized plasmid is cut in the 5'AOX1 region, most of the transformants should be $Mut^+$ as the insertion results in an intact copy of AOX1 gene. However, with the presence of the AOX1 sequences in the plasmid, there is a chance that recombination will occur in the 3'AOX1 region also, disrupting the wild-type AOX1 gene and creating $Mut^s$ transformants. The latter ones rely on AOX2 gene expression, which yields 10-20 times less AOX activity than the AOX1 gene, and consequently their growth ability on methanol is reduced. Currently, the majority of researchers use the $Mut^+$ phenotype, although some reports have shown that low growth rate on methanol may be desirable for production of certain recombinant products. By performing this analysis, $Mut^+$ phenotype was confirmed for all the tested clones.

The same clones were also checked for their laccase activity production on solid medium. POX3 transformants produced a wide and intense green halo around each streaked colony after one day of incubation at 30°C. Otherwise, POX4 transformants didn't produce any activity after the same time of incubation. A very faint activity halo around POX4 transformants appeared only after prolonged incubation of the plates at 4°C. In both cases, a notable heterogeneity of laccase activity (evaluated by the size and intensity of the green halo produced) was noted. This effect is ascribed to the variable number of vector integrations events occurring into the yeast genome. Thus, the expression level of several recombinant clones was screened by plate assay in order to select the best producing ones. Three colonies were chosen for POX3 transformants as well as for POX4 and they were further tested for laccase production in liquid culture.

*Analysis of laccase production in liquid culture*

Preliminary results on laccase production in liquid medium were obtained performing *P. pastoris* cultures in shake-flasks. Two carbon sources are required for laccase production: growth on glycerol containing medium (BMGY) assures high-density growth, whilst the following switch to a methanol containing source (BMMY) is necessary to induce laccase expression. A time-course analysis of rPOX3 and rPOX4 culture supernatants was initially performed on the recombinant clones selected by the first screening on plate. A non-transformed *Pichia* clone was also analysed as a control. In non-transformed yeast control, laccase activity assays measured by monitoring the change in absorption at 420nm (to record the formation of a stable oxidized ABTS radical product) showed a constant negative slope. The same effect was observed in rPOX4 cultures until no activity was measured, indicating an interference in laccase assay when ABTS was used as substrate.

After eight days of culture, up to 4U/ml laccase levels were achieved for the best producing rPOX3 clone, whereas rPOX4 activity levels reached up to 0.015U/ml in

the clone showing the highest laccase production. These results indicate a significant improvement of rPOX3 laccase production compared to the levels reached in *S. cerevisiae* (around 0.03 U/ml at the 2nd day) and *K. lactis* (around 0.012 mU/ml at the 3rd day). Moreover, it is worth to note that rPOX4 laccase activity was not detected in neither of these two hosts, while it could be revealed, even if at low level, in *P. pastoris*.

These preliminary results indicate that *P. pastoris* could be a promising expression system for *P. ostreatus* laccase if compared to *S. cerevisiae* and *K. lactis*. However, the laccase production reported in these scouting experiments, could probably be enhanced by further optimization of medium and cultivation conditions. In this regard, the use of bioreactor would be preferable, since process parameters such as, oxygen transfer and methanol concentration would be more efficiently controlled than in shake-flask cultures, allowing, among the other factors, a better optimization of methanol-feeding strategies.

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## EXPERIENCES IN FOREIGN LABORATORIES

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**ORIGINAL ARTICLE**

# Evidence for a radical mechanism in biocatalytic degradation of synthetic dyes by fungal laccases mediated by violuric acid

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**Abstract**

The bleaching activity of the *Pleurotus ostreatus* POXC laccase isoenzyme has been tested against selected single textile acid dyes (two anthraquinonic and two azo dyes), as well as towards a solution mimicking a real acid dye waste-water for wool. The catalytic reaction of POXC has been investigated both in the presence and in the absence of the synthetic mediator violuric acid (VIO) (–NOH type of mediator). In all the cases tested, the presence of the mediator enhanced the reaction rate and the percentage of decolorization, apart from one of the dyes (Acid Blue 62), which is itself a good substrate for the laccase-catalyzed oxidation. Electron paramagnetic resonance (EPR) experiments, after the addition of an excess of VIO to the solution of laccase, showed the presence of a strong and stable radical signal that was assigned to a neutral radical form of VIO.

**Keywords:** *POXC laccase, mediator, violuric acid, radical species, textile dyes*

## Introduction

White-rot fungi produce several extracellular enzymes that are able to degrade natural polymers, such as lignin and cellulose, but can also degrade different synthetic chemicals which are usually recalcitrant to biodegradation. *Pleurotus ostreatus* produces laccases as major extracellular enzymes. Laccases (benzenediol: oxygen oxidoreductases; EC1.10.3.2) belong to a group of polyphenol oxidases containing copper atoms in the catalytic centers, and usually called multicopper oxidases. The active sites in these proteins include four Cu atoms that are classified into three types, according to their spectroscopic properties: type 1 (T1) or blue Cu, type 2 (T2), and type 3 (T3) or coupled binuclear Cu sites. Functionally, all multi-copper oxidases couple the four electron reduction of dioxygen to water with the concomitant oxidation of a wide variety of substrates, such as polyphenols, methoxy-substituted phenols, aromatic diamines, and also synthetic dyes. The main functional role of the T1 Cu site is to shuttle electrons from the substrate to the trinuclear Cu cluster, which is the site of oxygen reduction (Baldrian 2006). Several characteristics of laccases (i.e. broad substrate specificity and the use of molecular oxygen instead of hydrogen peroxide that is used by peroxidases) make this enzyme suitable to be investigated for industrial and environmental applications (Chivukula et al. 1995; Wong et al. 1999; Campos et al. 2001; Martins et al. 2001; Novotný et al. 2001; Mayer et al. 2002; Soares et al. 2002a,b; Martins et al. 2003; Wesenberg et al. 2003; Zille et al. 2004; Blánquez et al. 2004; Levin et al. 2004).

With respect to other ligninolytic enzymes, such as lignin peroxidase (LiP) and manganese peroxidase (MnP), which are more powerful oxidants ($E^\circ$ is slightly $<1$ V), laccases have a lower redox potential ($E^\circ$ from $\sim 0.5$ to $\sim 0.8$ V), allowing only the direct oxidation of phenolic compounds.

However, the activity of laccase can be expanded towards the oxidation of non-phenolic aromatic compounds by the use of appropriate mediators

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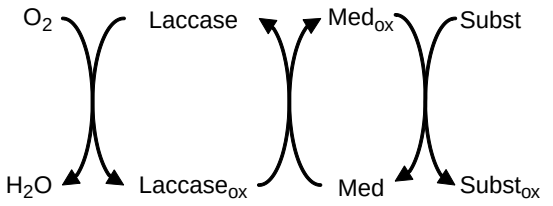



Scheme I. The role of mediator in the oxidation of substrate.

(Fabbrini et al. 2002a,b; Astolfi et al. 2005; Camarero et al. 2005).

The role of mediators in laccase oxidation is outlined in Scheme I.

Mediators are low molecular weight compounds that are easily oxidized by laccases (at the T1 center) producing, in some cases, cation radicals which diffuse in solution and can oxidize more complex substrates, that, because of their size, cannot get into the catalytic site. A large number of studies have been produced on the mechanism of oxidation of non-phenolic substrates by means of many mediators (Camarero et al. 2005). In many cases, it has been seen that the use of mediators during decolorization of industrial textile dyes results in a faster process, which often also produces more extensive decolorization. Therefore, the action of the laccase-mediator system has been extensively studied in recognition of its potential applications. An example of this is the laccase-mediator concept applied to pulp bleaching, which represents an important biotechnological application of a process based on a single ligninolytic enzyme (Camarero et al. 2004). Likewise, a laccase/redox mediator formulation is used in the textile industry in the finishing process for indigo-stained materials (Aly et al. 2004).

In this paper, the *P. ostreatus* POXC laccase (Giardina et al. 1996) bleaching activity towards selected textile acid dyes, two anthraquinonic and two azo dyes, in the presence and absence of violuric acid (VIO), a synthetic redox mediator of the –NOH type, is reported, as well as the bleaching activity towards a solution mimicking a real acid dye waste water for wool. The presence of a strong radical mediator has been detected by EPR.

## Materials and methods

*Organism, culture conditions and enzyme purification*

White-rot fungus, *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC No. MYA-2306) (PO 332 SOPHIED register) was maintained through periodic transfer at 4°C on potato dextrose agar plates (Difco Laboratories, Detroit, MI) in the presence of 0.5% yeast extract (Difco). Incubations were carried out as previously described (Palmieri et al. 2005a).

Secreted proteins were precipitated from the filtered medium by addition of $(NH_4)_2SO_4$ up to 80% saturation and, after extensive dialysis, loaded onto a DEAE Sepharose Fast Flow (Pharmacia Biotech Inc.) column, as previously described (Palmieri et al. 2005a). Fractions corresponding to POXC were pooled, equilibrated in buffer 50 mM Na phosphate pH 7, 1 M $(NH_4)_2SO_4$ (McIlvaine buffer) and loaded on Phenyl Sepharose High Performance 35/100 (Amersham Bioscience). POXC was eluted with a linear gradient $1 \rightarrow 0$ M $(NH_4)_2SO_4$ (500 mL).

The enzyme was assayed using ABTS as substrate, as previously described (Palmieri et al. 2005a).

*Decolorization experiments*

Four dyes were used: Acid Blue 62 (AB62), Acid Red 266 (AR266), Acid Yellow 49 (AY49) from Town End (Leeds, UK), and Reactive Blue 19 (RB19) from Sigma (St. Louis, USA). VIO was from Aldrich.

Each dye (80 μM) was incubated with 1 U $mL^{-1}$ (76 nM) of POXC in 0.1 M Na Citrate and 0.2 M Na phosphate buffer at pH 4.5 at room temperature. Control samples without enzyme were run in parallel under identical conditions. The effect of different VIO concentrations (80, 160 and 800 μM) was evaluated. The VIO stock solution (100×) was made in dimethylformamide (DMF), and the 1% DMF added to the reaction mixture had no effect on laccase activity.

Experiments in the pH range 3–6 using McIlvaine buffer, in the presence or absence of 160 μM VIO, were performed. The percentage of decolorization was determined monitoring the decrease in the absorbance maxima characteristic of AB62 (637 nm), AR266 (474 nm), AY49 (402 nm) and RB19 (592 nm).

A solution mimicking a real acid dye waste-water for wool was prepared by mixing the acid dyes (80 μM each) with 0.67 g $L^{-1}$ $Na_2SO_4$. The final pH was adjusted to pH 5 by adding acetic acid. Absorbance spectra were recorded in the range 280–800 nm after incubation with 1 U $mL^{-1}$ of POXC and 800 μM of VIO.

*EPR experiments*

CW X-band (9.4 GHz) EPR measurements were carried out with a Bruker E500 Elexsys Series using the Bruker ER 4122 SHQE cavity and an Oxford helium continuous flow cryostat (ESR900). EPR solutions were prepared with a final concentration of 0.34 mM enzyme and 7 mM VIO (molar ratio enzyme/VIO 1:20) in a 0.1 M phosphate buffer,

pH 6. All chemicals were of reagent grade and used without further purification. The reaction was stopped by rapid immersion of the EPR tube in liquid nitrogen after 30 s. The EPR spectrum of laccase was recorded at 120 K and laccase with VIO at 100 K. The EPR spectra were baseline-corrected and simulated using software for fitting EPR spectra systems with anisotropic g- and hf-tensors for copper complexes (COSMOS package) (Della Lunga et al. 2003) and for radicals (EasySpin program) (Stoll et al. 2006).

## Results and discussion

In this paper, three primary dyes from acid class used for wool dyeing (AB62 anthraquinonic, AY49 and AR266 azo dyes) (see Scheme II) were chosen to study the ability of the laccase POXC from *P. ostreatus* to decolorize textile dyestuffs, both individually and in mixtures, either in the presence or absence of the laccase mediator VIO. As the capability of POXC to decolorize the anthraquinonic dye RB19 (Scheme II) was already known (Palmieri et al. 2005b), the effect of the mediator on this reaction was tested to verify whether or not an improvement of the decolorization process is obtained. Synthetic dyes represent one of the major sources of pollutants due to their extensive use in industrial coloring, such as textile, paper, food and pharmaceuticals. Inappropriate treatment of industrial wastes has resulted in critical environmental damage. Since some of the compounds contained in these effluents are either toxic and mutagenic or may be modified under anaerobic conditions to become carcinogenic, there is great interest in the resolution of the waste problem (Wong et al. 1999). Azo and anthraquinone groups are the major chromophores present in commercial dyes and because of the large chemical differences among the structures, there are no general methods for detoxification of colored wastes. Moreover, conventional methods are usually limited by high costs and the production of secondary toxic metabolites. Enzyme-based methods have a minimal impact on ecosystems, so the new enzyme-based methods can be employed in the degradation of a wide range of chemically different compounds (Forgacs et al. 2004; Whiteley et al. 2006).

In Figure 1, the residual color, measured at the dye absorbance maxima, after 15 min incubation with POXC, in the absence and in the presence of different mediator concentrations are shown. It is worth noting that AB62 is directly decolorized by POXC, while only 29% decolorization at 800 μM VIO was obtained for AY49, a highly recalcitrant dye. In the latter case, apparent enzyme inhibition can be observed at low VIO concentration and short reaction times, while after 2 h incubation the percentage of AY49 decolorization increased proportionally with the VIO concentration. In the case of AR266 and RB19, the presence of the synthetic mediator increased the reaction rate and a higher VIO concentration led to a higher percentage of decolorization.

Analyses of the spectra recorded during incubation of AB62 and RB19 with POXC and mediator showed an early increase in the absorbance at 425 nm, followed by the decrease of this peak at longer reaction times (data not shown). This phenomenon is noticeable only in the presence of the mediator, and is more marked at higher VIO concentration. This event is probably related to the formation of a transient species that decays with time. The optimal pH of the VIO mediated decolorization was in the range 4.5–5.0 for all the tested dyes.

The ability of POXC to decolorize a dye mixture mimicking a real acid dye waste-water for wool was examined both in the presence and absence of VIO. The optimal pH determined for the VIO-mediated decolorization process corresponds to that of waste-water arising from the wool dyeing process (pH 5.0). Figure 2 shows the positive effect of VIO on the laccase treatment of acid waste-water. The high absorbance in the wavelength range 280–350 nm

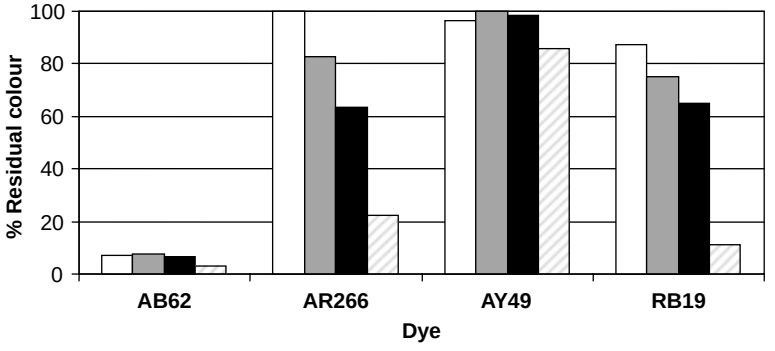



Figure 1. Percentage of residual color after treatment with POXC in the absence and presence of violuric acid (VIO) after 15 min of incubation. Symbols: no VIO (□); VIO 80 μM (▩); VIO 160 μM (■); VIO 800 μM (▨).

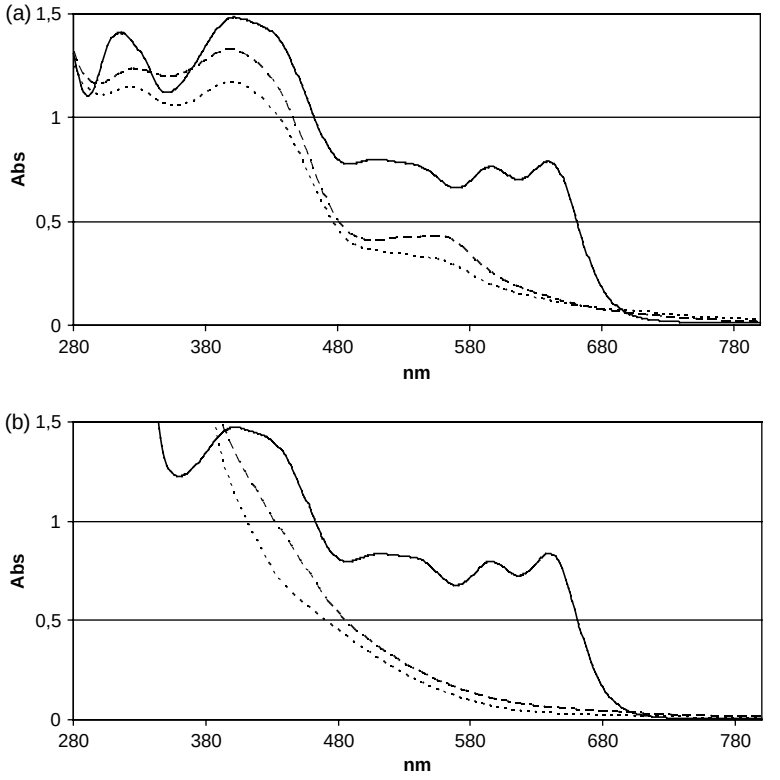



Figure 2. Model waste-water decolorization by POXC in the absence (a) and in the presence (b) of 800 μM violuric acid. Symbols: control (—); 1 h incubation (– –); 3 h incubation (. . . .).

in Figure 2(b) is due to the high concentration (800 μM) of VIO whose $\lambda_{max}$ is 312 nm. The presence of VIO in the laccase reaction mixture led to a significant absorbance reduction in the wavelength range 400–680 nm.

The catalytic mechanism of the POXC/VIO system was investigated by EPR. In Figure 3(a) the EPR spectrum of *P. ostreatus* POXC laccase paired with the simulation for the Cu T1 site is reported. Magnetic parameters for the T1 center are $A_{||} = 88*10^{-4}$ cm$^{-1}$ and $A_{\perp} = 7.5*10^{-4}$ cm$^{-1}$ and $g_{||} = 2.191$, $g_{\perp} = 2.046$, and for the T2 center $A_{||} = 178*10^{-4}$ cm$^{-1}$ and $g_{||} = 2.257$. The values of magnetic parameters for the T1 center are in agreement with those reported in the literature for other laccases with a redox potential comparable with that reported for this enzyme (Xu et al. 1996). The redox potential E° for the *P. ostreatus* POXC laccase was reported as 740 mV *vs*. NHE at pH = 5.0 (Garzillo et al. 2001). Although the E° values of

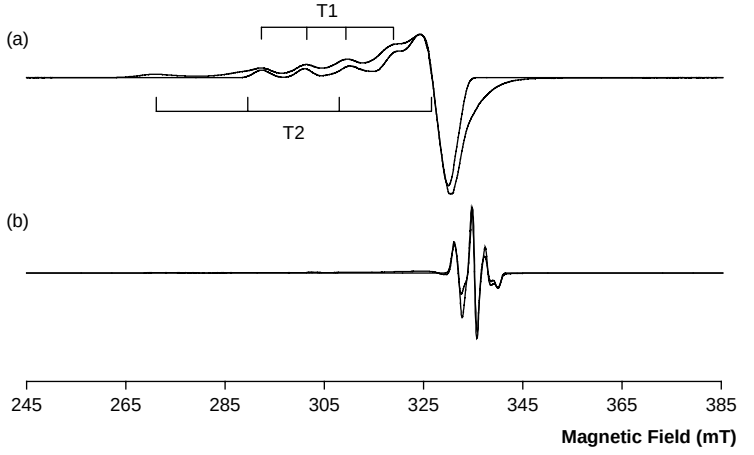
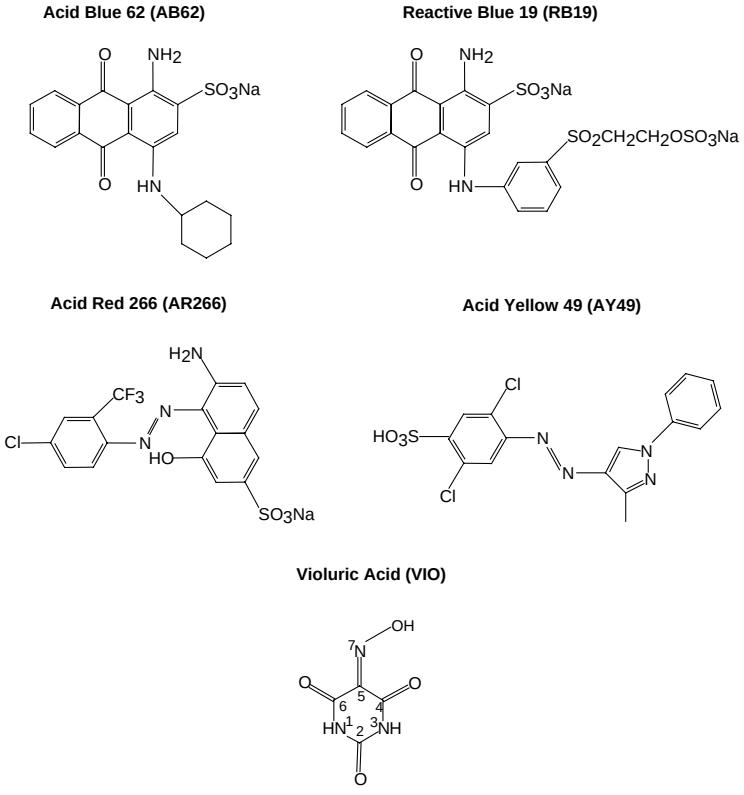



Figure 3. EPR spectrum of *P. ostreatus* POXC laccase, paired with the simulation for the T1 site, in the absence (a) and presence of an excess (1:20 enzyme:mediator molar ratio) of VIO (b). The radical spectrum of violuric acid is paired with the simulation obtained with the parameters reported in the text (ν = 9.3937 GHz).




Scheme II. Structures of dyes and mediator.

fungal laccases span the range from 0.5 to 0.8 V, the EPR spectra of their T1 centers are very similar. In the case of a lower value of $E^\circ$ ($\sim$500 mV) the $A_{\|}$ value of the T1 center can decrease to values $\sim 40*10^{-4}$ $cm^{-1}$ with a $g_{\|}$ value around 2.3 (Guckert et al. 1995; Xu et al. 1996).

After addition of an excess (1:20 enzyme:VIO molar ratio) of VIO mediator, the appearance of a strong and stable radical signal was evident. In Figure 3(b), the EPR spectrum is compared with its simulation obtained using the following magnetic parameters: $g_{iso} = 2.0042$, $A_{N7} = 30.8*10^{-4}$ $cm^{-1}$ and $A_{N3} = 2.5*10^{-4}$ $cm^{-1}$. The higher coupling constant is primarily due to the nitrogen in position 7 and, to a lesser extent, to the nitrogen in position 3 (see Scheme II). From analysis of the isotropic coupling constants it is not possible to define the nature of the radical (cationic or neutral), but preliminary DFT calculations have shown that the VIO radical is in the neutral form (data not shown). This confirms the suggested mechanism for the synthetic –NOH mediators (Baiocco et al. 2003) which follow a radical hydrogen atom transfer route. In this case, the laccase-catalyzed –NOH oxidation proceeds through the reaction $-NOH \rightarrow -N-O^\bullet + e^- + H^+$ as the rate limiting step, similar to the reaction $-COH \rightarrow -C-O^\bullet + e^- + H^+$ which is involved in laccase oxidation of phenolic compounds (Xu 1996).

Many factors contribute to the activity of laccase for the oxidation of non-phenolic substrates; first is the redox potential that is essentially determined by the structure of the T1 site. It has been proposed that, in blue copper proteins, the nature of the axial ligand affects $E^\circ$; stronger axial ligands help in stabilizing Cu(II), thereby lowering the $E^\circ$; weaker axial ligands tend to destabilize Cu(II), thus raising the $E^\circ$ (Solomon et al. 1996). In the case of *P. ostreatus* POXC laccase, a Leu instead of a Met is present as axial ligand giving $E^\circ = 0.79$ V at pH = 5 and 0.69 V at pH = 7 *vs.* NHE (Garzillo et al. 2001). Other factors can contribute to $E^\circ$: the protein environment can also affect $E^\circ$ through H-bonding, charged residues, etc. (Stephens et al. 1996). However, laccase is able to oxidize substrate-mediators with redox potential higher than that of the enzyme itself, such as VIO ($E^\circ = 0.90$V *vs.* NHE at pH = 5) (Xu et al. 2000). There is no direct correlation between the redox potential of laccases and the oxidation rate of specific substrates. Nevertheless, the redox potential of laccase has to be high enough to ensure the oxidation of a mediator with high redox potential which will then be able, in turn, to oxidize high redox potential non-phenolic aromatic compounds.

The pH value is also a key factor. Optimum pH for the oxidation of acid dyes is around pH = 5 in the presence of VIO, and lower (pH = 3) in the absence.

Furthermore, a basic pH could affect laccase activity. However, the majority of laccases shows optimal pH changing with the substrate used (Garzillo et al. 2001).

In conclusion, a neutral radical species is formed and detected during the reaction of *P. ostreatus* POXC laccase in the presence of an excess of VIO. The bleaching activity towards single textile dyes with different structure, as well as towards a model waste-water, is greatly enhanced by the presence of the mediator. In this context, the oxidized species of mediator has a dominant role in the oxidation of non-phenolic aromatic compounds. Understanding the mechanism of electron and proton transfer and insight on the interactions of laccases with mediators can be of great help in designing more active laccase-mediator systems for biotechnological applications.

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# Decolourization of textile dyes by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus*

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## Abstract

**BACKGROUND: The ability of the fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium* to decolourize and detoxify 11 (mono-, dis-, poly- azo, and anthraquinonic type) dyes, widely used across the textile and leather industries, was tested.**

**RESULTS: Different substrate specificities were revealed between *P. ostreatus* and *P. chrysosporium* in decolourization experiments. The latter fungus provided almost complete decolourization of the tested azo dyes up to 600 ppm and dis-azo dyes up to 1000 ppm, and 80% decolourization of the tris-azo dye DBU1L38 at 1000 ppm, after 6 days. *P. ostreatus* provided almost total decolourization of the anthraquinone type dye ABU62 (1000 ppm) after just 1 day. *P. ostreatus* also showed the ability to decolourize the tested dis-azo and tris-azo dyes, giving the best performances against the dis-azo DBU1U1 (600 ppm) dye, which was 100% decolourized after 6 days. Laccases proved to be the main enzymatic activities acting in *P. ostreatus* decolourization.**

**CONCLUSION: The potential of the fungi *P. ostreatus and P. chrysosporium* as efficient bio-systems for decolourization and detoxification of several toxic industrial dyes was demonstrated. The role of laccases in the decolourization of dis-azo dyes by *P. ostreatus* was demonstrated for the first time.**


**Keywords:** textile dyes degradation; white rot fungi; ligninolytic enzymes; colour industry wastewater

## INTRODUCTION

Over 100,000 commercially available dyes exist and more than $7 \times 10^5$ tonnes of dyestuff are produced annually and used within the food, pharmaceutical, cosmetic, textile and leather industries.[1,2]

In order to meet the criteria necessary for industrial applications, a huge variety of dyestuffs characterized by different colours, properties and molecular structures is produced. Existing dyes can be classified either according to their origin, or according to their chemical and/or physical properties and characteristics related to the application process. The colouristic approach characterizes dyes according to the dyeing method, and classifies them as acid or basic dyes, direct, reactive, azoic, mordant dyes, or vat dyes. The chemical structure of the chromophoric group allows classification of synthetic dyes as azo dyes, anthraquinonic dyes, xanthene dyes, triphenylmethane dyes, etc.[3] Depending on their mode of binding to the fibre the release of dyes into the effluent may vary greatly, leading to a total annual discharge between 30,000 and 150,000 tons.[4]

In addition to their visual effect and their adverse impact in terms of chemical oxygen demand (COD) and biological oxygen demand (BOD), many synthetic dyes are also toxic.[5–9]

Moreover, the frequently high volumetric rate of industrial coloured effluent discharged, in combination with increasingly stringent legislation, makes the search for appropriate treatment technologies an important priority.[10] Unfortunately, all dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents, and microbial attack. Chemical or physico-chemical methods for the treatment of dye-containing wastewaters are generally costly, not very efficient, of limited applicability, and produce wastes, which are difficult to dispose of.

Brightly coloured, water soluble reactive and acid dyes are the most problematic, as they tend to remain unaffected by conventional treatment systems,[11] and are also not removed by municipal aerobic treatment systems.[12] Concern arises as many dyes are made from known carcinogens such as benzidine and other aromatic compounds.[13] Weber and Wolfe[14] demonstrated

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that azo- and nitro-compounds are reduced in sediments, resulting in the formation of toxic amines. Anthroquinone-based dyes are very resistant to degradation due to their fused aromatic ring structure. The ability of some disperse dyes to bio-accumulate has also been demonstrated.[13]

As a viable alternative for remediation of textile industry effluents, biological processes have received increasing interest since they can offer a low-cost and environmentally friendly solution applying publicly acceptable treatment technology and producing less sludge.[15]

By far the most widely studied dye-decolourizing microorganisms are the white-rot fungi (WRF). In addition to their natural substrates, white-rot fungi have been found to be capable of mineralizing a diverse range of persistent organic pollutants including synthetic dyes, unlike bio-degradative bacteria that tend to be rather substrate-specific.[16] The ability of these fungi to degrade such a range of organic compounds results from the relatively non-specific nature of their ligninolytic enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. Certain WRF strongly decolourize particular dyes but not others.[17,18]

Small structural differences in dye mixtures can markedly affect decolourization, and this may be due to electron distribution and charge density, although steric factors may also contribute[19].

In this report the ability of the WRF *Pleurotus ostreatus* and *Phanerochaete chrysosporium* to decolourize 11 dyes endowed with different chemical structure (mono-, dis-, tris-azo and anthraquinone) and different colours and belonging to four different classes (reactive, disperse, direct and acid) has been tested.

## MATERIALS AND METHODS

### Microorganisms

Strains of the WRF, *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) and *P. chrysosporium* Burdsall M1 (DSM 13 583) were maintained through periodic transfer at 4 °C on agar (1.5% w/v) plates containing undiluted and ten-fold diluted PDY medium (24 g $L^{-1}$ potato dextrose (Difco, Detroit, Michigan, USA) and 5 g $L^{-1}$ yeast extract (Difco)), respectively.

### Dyes

All the dyes used in this work (Table 1) were purchased from Sigma (St Louis, USA), except for the acid dyes, which were supplied by Yorkshire Europe. Before adding to each of the autoclaved growth media, dyes were sterilized by tyndallization, consisting of three 1 h cycles at 60 °C with a 24 h interval between cycles.

### Decolourization experiments

Decolourization of dyes in agar (1.5% w/v) plates was performed using two different media: PDY medium, and 20 g $L^{-1}$ malt extract medium (Difco). Solid cultures were supplemented with dye (Table 1) at a final concentration of 30 ppm. Higher dye concentrations (150, 300, 600 and 1000 ppm) were added to solid media only when fungal strains proved to effectively decolourize at the lower dye concentration. For these experiments, the fungus mycelium (a 5 mm diameter agar plug from the edge of a 7 day old agar culture) was grown on agar plates (diameter 100 mm) containing the above described media supplemented with dye, and incubated in the dark at 28 °C for 2 weeks. Controls consisting of un-inoculated dye supplemented media were simultaneously incubated at 28 °C.

Decolourization in liquid cultures was performed in micro-titre plates with U bottom wells, using malt extract broth with different dye concentrations chosen among 30, 150, 300, 600, 1000 ppm, on the basis of the ability of fungal strains to decolourize the dyes. In the experiments performed in micro-titre plates,[18] the fungus mycelium (a 3 mm diameter agar plug from the edge of a 7 day old agar culture) was grown in four inside columns of eight-well columns plates. Dyes were distributed in six-well columns, filled with 200 µL of 20 g $L^{-1}$ malt extract broth containing dye, the two extreme columns remaining un-inoculated, representing the control. External wells were filled with water to limit evaporation. Cultures were incubated in the dark at 28 °C for 7 days on a rotary shaker at 120 rpm.

Decolourization abilities by liquid cultures of fungi were also investigated in 500 ml flasks containing 250 ml of 20 g $L^{-1}$ malt extract broth at dye concentrations that had been decolourized with an efficiency of at least 50% of decolourization in micro-titre plates.

Liquid cultures in flasks were prepared by pre-inoculating 300 mL of 20 g $L^{-1}$ malt extract broth in 1 L shaken flasks with *P. ostreatus* mycelia (5 agar plugs of 12–15 mm diameter from the edge of a 7 day agar culture) or *P. chrysosporium* mycelia (three agar plugs of 12–15 mm diameter from the edge of a 7 day agar culture) in a temperature-controlled incubator at 28 °C. 25 mL of a 7-day-old pre-culture were then inoculated in 500 mL flasks containing 225 mL of 20 g $L^{-1}$ malt extract broth, supplemented

**Table 1.** Dyes used in the study: respective wavelength of maximum absorption ($\lambda_{max}$) and classes

| Class | Dye | Abbreviation | $\lambda_{max}$ (nm) | Chemical structure class |
|---|---|---|---|---|
| Acid | Acid blue 62 | ABu62 | 590 | anthraquinone |
| | Acid red 299 | AR299 | 540 | dis-azo |
| Reactive | Reactive blue 19(Remazol Brilliant Blue R) | RBu19 | 590 | anthraquinone |
| | Reactive black 5 | RBl5 | 590 | dis-azo |
| | Reactive red 4 | RR4 | 540 | mono-azo |
| | Reactive yellow 81 | RY81 | 405 | mono-azo |
| Direct | Direct red 81(Congo Red) | DR81 | 492 | dis-azo |
| | Direct blue 1 (Chicago Sky Blue) | CSB | 620 | dis-azo |
| | Direct black 38(Chlorazol Black) | DBl38 | 540 | tris-azo |
| Disperse | Disperse blue 1 | DBu1 | 590 | anthraquinone |
| | Disperse yellow 3 | DY3 | 405 | mono-azo |

  

with dyes as above described. Cultures were incubated in the dark at 28 °C for 2 weeks on a rotary shaker at 120 rpm. At the same time, controls consisting of 250 mL of either un-inoculated liquid culture broth with or without added dyes or inoculated medium without dye addition were incubated in 500 mL flasks at 28 °C. From time to time, supernatant samples were collected from liquid cultures. The pH was measured and samples were used for decolourization assays and for the determination of enzyme activities.

### Assays of dye decolourization by *P. chrysosporium* and *P. ostreatus*

Decolourization in solid media was assessed by visual disappearance of colour from the plate and by measuring diameters of decolourization halo and mycelia growth.

Dye decolourization in liquid cultures in micro-titre plates was monitored spectrophotometrically, measuring, at the third, fourth and seventh day of fungal growth, the variation in intensity of absorption at the wavelength of maximum absorption of each dye ($\lambda_{max}$ in Table 1) compared with un-inoculated controls, using a multi-well plate reader (Benchmark Plus, Bio-Rad, UK).

Dye decolourization in liquid cultures in flasks was daily measured spectrophotometrically (Jasco spectrophotometer V530, Tokyo, Japan) recording the light absorption spectra between 350 and 750 nm and measuring the decrease in absorption intensity at the wavelength of maximum absorption of each dye compared with that of the un-inoculated control. Experiments were performed in triplicate and values shown are representative of at least two experiments.

To evaluate the extent of colour absorption on mycelium, mycelium was filtered, re-suspended in methanol, extracted and centrifuged, and after this treatment, intensity of absorption at the wavelength of maximum absorption of the dye in extracted supernatants was measured.[20] When adsorption of dyes on mycelium occurred, decolourization due to the fungal dye transformation was evaluated by subtracting the colour adsorbed on mycelium from the measured decolourization.

### Enzyme assays

Laccase activity was assayed using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. The assay mixture contained 0.4 mmol $L^{-1}$ ABTS in 0.1 mol $L^{-1}$ sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 414 nm ($\varepsilon = 36,000\ M^{-1}\ cm^{-1}$).

Dye-decolourizing peroxidase (DYP) activity was assayed using RBU19 as substrate. The assay mixture contained 50 μmol $L^{-1}$ RBU19 and 0.1 mmol $L^{-1}$ $H_2O_2$ in 20mmol $L^{-1}$ sodium acetate buffer, pH 4.0. RBU19 degradation was followed by absorbance decrease at 592 nm ($\varepsilon = 9,000\ M^{-1}\ cm^{-1}$).

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

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

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

All the enzyme activities were measured at 25 °C and expressed in international units (IU).

### Assays of dye detoxification by *P. chrysosporium*

*P. chrysosporium* (five plugs of 5 mm diameter from the edge of a 7 days old mycelium plate) were inoculated in 25 mL of 20 g $L^{-1}$ malt extract broth in 100 mL flasks in the presence of the lowest dye concentration efficiently decolourized by the fungus in liquid cultures in flasks, and incubated at 28 °C under shaking (120 rpm) for 7 days. Toxicity assays were performed using Lumistox 300 system (Hach Lange, S.*r.l.* Milan – Italy) and performing all measurements in the colour correction mode.

At these concentrations, all the dyes, except DBu1 and DR1, showed OD values >1800 mE; this high OD values did not allow Lumistox toxicity assay to be performed. Therefore, for these dyes, dilutions at final OD values <1800 mE were prepared and used for toxicity tests. Toxicity values (percentage inhibition of bacterial bioluminescence) of dyes at the new defined concentrations were measured.

## RESULTS AND DISCUSSION

### Dye decolourization by *P. chrysosporium* and *P. ostreatus* whole cells on solid media

For decolourization assays 11 dyes were chosen in the frame of the European Union Sixth Framework Program funded project SOPHIED (contract NMP2-CT2004-505 899) (Table 1). These dyes form a representative group including a variety of mono-, dis- and poly- azo dyes, and three anthraquinone dyes. At least one azo dye representative of each primary colour was chosen. They are all commercially important dyes, with a wide range of applications across the textile and leather industries. Decolourization of these dyes by the WRF *P. ostreatus* and *P. chrysosporium* was initially evaluated in solid media, to establish the optimal conditions for dye decolourization and to verify substrate specificities of the fungi. Decolourization was assessed as colour disappearance during fungal growth. Extensive growth of the mycelia occurred in both the analyzed growth media: potato dextrose plus yeast extract containing medium and malt extract containing medium. Nevertheless, both fungi were shown to be more effective in decolourization on malt extract medium, which was selected for further decolourization tests at different dye concentrations up to 1000 ppm. *P. chrysosporium* was shown to display a higher decolourization efficiency than that of *P. ostreatus*. In fact for the reactive, direct and acid dyes the former fungus provided complete disappearance of colour after 4–5 days at 30 ppm, 4–7 days at 150 ppm, 5–10 days at higher concentrations. *P. chrysosporium* proved to almost completely decolourize all the reactive dyes tested up to a concentration of 1,000 ppm after 7 days. On the other hand, decolourization by *P. ostreatus* of reactive, direct and acid tested dyes at minimum concentration took at least 7 days, and a longer time (8–13 days) was required for decolourization at higher concentrations. The colour disappearance observed for the above mentioned dyes suggested dye degradation following fungi growth. In fig. 1 the maximum dye concentration decolourized by

  

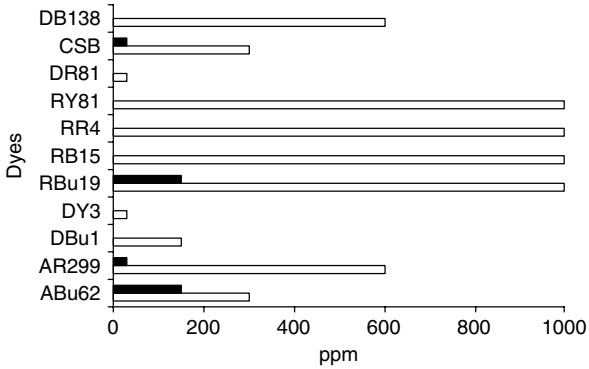


**Figure 1.** Maximum dye concentrations decolourized in agar plates by *P. chrysosporium* (□) and *P. ostreatus* (■), giving a decolourization diameter higher than 70 mm after 7 days.

each fungus – giving a decolourization diameter greater than 70 mm – after 7 days is reported.

### Dye decolourization by *P. chrysosporium* and *P. ostreatus* whole cells in liquid media

Decolourization experiments using *P. chrysosporium* and *P. ostreatus* liquid cultures were initially performed in micro-titre plates using malt extract broth, containing dyes (Table 1) at different concentrations in the range 30 – 1,000 ppm. Results of dye concentrations that were at least 50% decolourized after 4 days are reported in Fig. 2. *P. ostreatus* was found to be more effective in decolourizing acid and disperse dyes than *P. chrysosporium*, the latter fungus being more active against direct and reactive dyes. However, both fungi provided reduced of absorbance of RBU19 up to 1000 ppm after only 4 days, and decolourized nearly all the tested dyes more efficiently than in solid media. The different performances that each fungus displayed during decolourization experiments in solid media and micro-titre plates could be due to a greater adsorption on mycelia in micro-titre plates, because of the higher ratio biomass/culture medium.

The decolourization abilities of fungal liquid cultures were then investigated in flasks at dye concentrations decolourized with an efficiency of at least 50% in the micro-titre plate tests. For almost all the tested dyes and for both fungi, an initial delay in decolourization was observed, probably due to delayed production of the involved enzymatic activities. Table 2 summarizes the percentage decolourization of the selected dyes by the two fungi. The reported percentage decolourization values are related to the fungal dye transformation, since they have been calculated by subtracting the extent of colour absorption on mycelium from the measured decolourization.

It is worth noting that *P. ostreatus* decolourized some dyes either through enzymatic oxidation or through biomass adsorption on mycelium.[17] The mono-azo dyes tested were decolourized mainly

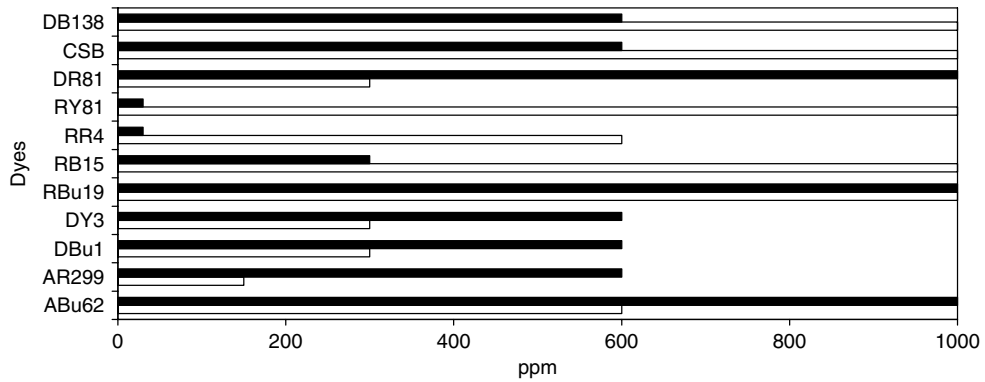


**Figure 2.** Dye concentrations that were at least 50% decolourized after 4 days by liquid cultures of *P. chrysosporium* (□) and *P. ostreatus* (■) in micro-titre plates.

**Table 2.** Percentage decolourization of the tested dyes by *P. ostreatus* and *P. chrysosporium* in shaken flask. Note that *P. ostreatus* achieved 40% and 90% decolorization of AR299 and DY3, respectively, after 2 days

| | *P. ostreatus* | | | *P. chrysosporium* | | |
|---|---|---|---|---|---|---|
| Dye | Conc. (ppm) | Dec (%) | Time (d) | Conc. (ppm) | Dec (%) | Time (d) |
| Acid blue 62 | 1000 | 80 | 1 | 600 | 90 | 8 |
| Acid red 299 | 600 | 70 | 13 | 150 | 90 | 2 |
| Reactive blue 19 (Remazol Brilliant Blue R) | 1000 | 20 | 10 | 1000 | 30 | 2 |
| Reactive black 5 | 300 | 90 | 10 | 1000 | 90 | 3 |
| Reactive red 4 | 30 | 20 | 13 | 600 | 100 | 6 |
| Reactive yellow 81 | 30 | 5 | 10 | 1000 | 60 | 6 |
| Direct red 81(Congo Red) | 1000 | 50 | 8 | 300 | 90 | 3 |
| Direct blue 1 (Chicago Sky Blue) | 600 | 100 | 6 | 1000 | 90 | 3 |
| Direct black 38 (Chlorazol Black) | 600 | 80 | 14 | 1000 | 100 | 10 |
| Disperse blue 1 | 600 | 80 | 10 | 300 | 80 | 6 |
| Disperse yellow 3 | 600 | 20 | 10 | 300 | 100 | 2 |

  

by absorption on mycelium. In contrast, the fungus was shown to be able to decolourize by transforming dis- and tris-azo dyes. Among these, the dis-azo dye AR299 was 40% decolourized in just 2 days, reaching 70% decolourization in 13 days. Some dyes were decolourized after transformation in an intermediate differently coloured. Indeed, after only 1 day of fungal treatment, reduction in the absorption peak of the blue dye ABU62 was observed together with a shift of wavelength of maximum absorption due to transformation of the dye in a red product, that is then completely degraded after 6 days. After 3 days of treatment the blue CSB dye was transformed to a violet product, and completely decolourized after 3 days. After 6 days of treatment the black dye RBL5 was transformed to a violet product, further transformed to a pink product in other 7 days, reaching colour disappearance one day later. The tris-azo black dye DBL38 was decolourized after 8 days of treatment, through production of a grey compound observed at the sixth day.

*P. chrysosporium* was shown to be more effective than *P. ostreatus* in decolourizing azo, dis-azo and tris-azo dyes. In most dye decolourization experiments a colour change was observed before final colour disappearance, the fungus transforming the blue dyes ABu62 and CSB into violet products and DBu1 to a yellow product, the red dyes AR299 and RR4 into yellow products and the red CR into an orange product; the black dyes RBL5 and DBL38 were converted into red products.

The disappearance or variation of colour was not due to protonation/deprotonation of dye, since the pH of dye-containing cultures was not altered during fungal growth, remaining almost constant in the range 5.0–6.0, depending on the dye.

In conclusion *P. ostreatus* and *P. chrysosporium* showed different substrate specificities in dye degradation, which can be related to production of different patterns of oxidative enzyme activities by the fungi during dye decolourization.

### Analysis of oxidative enzyme activities production by the fungi during dye treatment

The oxidative enzyme activities mainly responsible for dye decolourization were investigated.

A preliminary analysis of enzyme patterns produced by the fungi was performed by assaying oxidative enzyme activities of 50% decolourized samples (from experiments at dye concentrations reported in Table 2). The assays showed that *P. ostreatus* secreted laccase ($2-5 \times 10^{-1}$ U mL$^{-1}$), manganese peroxidase ($1-10 \times 10^{-2}$ U mL$^{-1}$) and dye decolourizing peroxidase ($10^{-3}$ U mL$^{-1}$) activities, whereas only manganese peroxidase ($1-10 \times 10^{-2}$ U mL$^{-1}$) and dye decolourizing peroxidase ($10^{-3}$ U mL$^{-1}$) activities were found in *P. chrysosporium* cultures.

To further investigate the role of fungal enzymes in dye decolourization, the time course of oxidative enzyme activity production during fungi treatment of the dyes at different concentrations was analyzed. DyP activity was detected in a few dye decolourization experiments, and for both the fungi very low activity levels were measured for this enzyme suggesting a negligible role in dye decolourization. On the other hand, laccase and MnP were the enzymes produced most abundantly by *P. ostreatus* and *P. chrysosporium*, respectively. Therefore laccase activity seems to be the oxidative activity mainly responsible for *P. ostreatus* dye decolourization, while manganese peroxidase acts as the most effective enzyme in dye decolourization by *P. chrysosporium*.

As a main difference in dye decolourization specificity between the fungi, *P. ostreatus* showed lower efficiency of dye degradation against the azo dyes compared with *P. chrysosporium*. Reactive Red 4 and Reactive Yellow 81 were not enzymatically transformed in *P. ostreatus* cultures, probably due to the low specificity of fungal enzymes toward these dyes.

In the case of Direct Red 81, a maximum of 50% dye transformation was reached and laccase activity was the only enzyme activity detected (0.24 U mL$^{-1}$) among those measured.

For Disperse Yellow 3, 90% dye decolourization was observed on the second day of fungal treatment, with 80% due to enzyme transformation (and 10% to dye absorption) and 0.5 U mL$^{-1}$ laccase activity were detected. However, while the total decolourization did not change during the following days, dye absorption increased to 30 and 70% on the third and sixth day of fungal treatment, respectively. At the same time, a reduction of enzyme activity levels was observed. These results indicated that laccases are involved in dye transformation in the early stages. However, to explain the later increase in color absorption on mycelium, we hypothesize the production of some other coloured compounds and their absorption on mycelium.

### Dye detoxification by *P. chrysosporium*

$EC_{20}$ (concentration of a sample that causes 20% inhibition in the luminescent bacteria test) values were determined for ABU62, DBu1, RBu19, RBl5 and RY81 , but not for AR299, RR4, DR81, CSB, DBL38, since these dyes were not toxic at the required dilution, neither before nor after fungal treatment. Residual toxicity values measured were expressed as percentage inhibition of bacterial bioluminescence at $EC_{20}$. After treatment with *P. chrysosporium*, the most efficient decolourizing fungus, residual toxicity of the dyes DBu1, RBu19, RBl5 and to a lesser of RY81 was reduced by fungal treatment, decreasing percentage of inhibition by 37-, 57-, 119- and 27-fold, respectively.

## CONCLUSIONS

This study showed the efficiency of *P. ostreatus* and *P. chrysopsorium* to decolourize a wide range of dyes. *P. chrysopsorium* proved to be the most efficient in decolourizing all the dyes tested. Decolourization of mono-azo and dis-azo dyes had previously been demonstrated using *P. chrysosporium*,[21–23] but the reported systems required longer decolourization times, typically several days, or required lower dye concentrations. In this work higher efficiency and rate of decolourization were demonstrated for the strain Burdsall M1 (DSM 13 583) of *P. chrysosporium* which proved capable of almost complete decolourization of the azo dyes tested up to 600 ppm, and of the dis-azo dyes up to 1000 ppm, after only 6 days, and of at least 80% decolourization of the tris-azo dye DBl38 at 1000 ppm after 7 days. The hypothesis that MnP represents the main oxidative enzyme activity responsible for decolourization of dyes by *P. chrysosporium* is consistent with the reported involvement of this enzyme, with or without LiP cooperation, in the decolourization of dyes by this fungus.[24–25]

*P. ostreatus* performed best in decolourizing the anthraquinone type dyes. The suggested crucial role of laccases in the decolourization of these dyes by the fungus is in agreement with the observation reported in the literature that anthraquinonic dyes are good substrates for laccases.[26,27,19]

The strain of *P. ostreatus* was also capable of transforming dis-azo dyes but not mono-azo dyes. *P. ostreatus* mediated decolourization of the latter dyes was mainly due to mycelium absorption. Knapp *et al*.[17] and Novotny *et al*.[28] reported the ability of a natural isolate

  

of *P. ostreatus* to decolourize various azo and anthraquinone dyes at 500 and 200 ppm, respectively, contrary to the work of Swamy and Ramsay,[29] where no ability to decolourize various azo dyes on mineral agar medium was detected with this fungus. *P. ostreatus* was also reported by Wesenberg *et al*.[30] to be a WRF able to decolourize a wide range of dyes, such as reactive azo dyes, (poly) and antraquinone dyes. Variations among strains might be responsible for these differences.

Recent studies reported the oxidation of mono and dis-azo dyes by purified laccases from *Pycnoporus cinnabarinus* [31], *Pycnoporus sanguineus* [32] and *Pyricularia oryzae*[33]. Chivukula and Renganathan[33] demonstrated that degradability of azo dyes by laccases from *P. oryzae* depends on their different aromatic substitution patterns, and that phenol and aniline azo dyes can be degraded. This work suggests, for the first time, the involvement of laccases from *P. ostreatus* in the decolourization of dis-azo dyes. The ability of *P. ostreatus* to degrade dis-azo dyes CSB and DR81 with the involvement of laccases could be explained by the presence of amino/hydroxy/methoxy and amino substituting groups, respectively, on the aromatic rings of the dyes, similarly to that proposed by Chivukula and Renganathan[33] for the degradation of mono-azo dyes by laccases from *P. oryzae*.

Finally, the potential of *P. chrysosporium* for use as an effective dye-detoxifying microorganism has been demonstrated.

## ACKNOWLEDGEMENT

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# Possibilities and limitations of the investigation of colored samples in the luminescent bacteria test

New methods for wastewater purification and for the production of new dyes were investigated. Besides decolorization of wastewater and industrial applicability, toxicity testing was essential. As a screening, the luminescent bacteria test is suitable to evaluate the toxicity of colored samples quickly, and with low costs.

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In the framework of the European Research Project "SOPHIED – Novel Sustainable Bioprocesses for the European Color Industries", three main goals were pursued:

- The biotechnological detoxification and decolorization of textile wastewaters
- The environmentally-friendly biotechnological production of classical dyes and
- The biotechnological development and synthesis of new dyes.

For this purpose and in a project-spanning work package an evaluation was made of the newly developed wastewater purification technologies with regard to detoxification and decolorization of the dyes with regard to (eco)toxic properties and industrial quality. Suitable test methods were chosen to test the toxicity and ecotoxicity of the dyes and wastewaters involved. Thereby the test methods had to satisfy the following requirements:

- Valid methods, which where possible are oriented on standardized procedures.
- Rapid and economical procedures, as the numbers of samples are large.
- Procedures which require little material, as only limited amounts of sample material are available.
- The procedures must be suitable for the evaluation of heavily-dyed samples.
- Participating partners must be technically able to establish the procedure in their laboratories.

As the first screening test for the investigation of the ecotoxic properties the luminescent bacteria test was chosen. The luminescent bacteria test is a biotest for the investigation of the toxicity of environmental samples. Its application to wastewater is standardized according to ISO 11348-2 (2007). The test is generally recognized and is also recommended by the OSPAR working group "Whole effluent assessment" (WEA) as a bacterial test for the investigation of wastewaters (OSPAR, 2000, 2005, 2007). The test has been used for many years in standardized form in various European countries, (e.g. Belgium, Ireland, Portugal, Germany, the Netherlands, Sweden, the UK; OSPAR, 2007) in the framework of wastewater. Its execution is simple after brief instruction, and it can be carried out rapidly and economically, and requires only small sample volumes (≥10 ml or 50 mg). Colored samples can be measured using color correction cuvettes or, in newer instruments, through integrated automatic measurement and calculation of the absorption.

## Methods

The luminescent bacteria test is a biotest procedure. In this test the cumulative effects of toxic substances in water can be measured without any knowledge of the exact composition or the ecotoxicity of the individual substances (EC50 – effect concentration or IC50 – inhibition concentration). The test is based on the fact that toxins can reduce the normal luminescence of the marine bacterium Vibrio fischeri. A reduction of the emitted light is proportional to the concentration of the toxins and is measured in a luminometer photometrically.

### Colored samples

For colored samples a color correction must be made, otherwise false positive results may be obtained, because the dye molecules physically absorb the light emitted by the bacteria. The color correction can be done manually with relatively cumbersome color correction cuvettes or with a color correction integrated in the instrument and thus an automatic color correction. Thereby the absorption of the sample is first measured in the wavelength range of the light emitted by the bacteria (490 nm) before the luminescence of the bacteria is determined. Absorption and emission are automatically calculated. In the framework of the project the luminometer "LUMIStox 300" (Hach Lange GmbH LPV 321) was used. The color correction here is made automatically.

### Preparation of the samples

Wastewater samples can be stored up to 24 hours in the refrigerator at 2-5 °C and two months at -18 °C (EN ISO 5667-16, 1999). For shipping, samples must be transported in insulated packages (e.g. Styrofoam boxes) with cooling elements or dry ice via an overnight express courier. The samples must not be allowed to thaw during transport.
Frozen samples are thawed at room temperature before the beginning of the test. Conductivity, pH and optical density (OD) are measured. If the pH is between 6 and 8.5, an adjustment is not necessary. In the other cases, the pH is adjusted with HCl or NaOH to 7.0 ± 0.2. Since the bacteria used are marine bacteria sodium chloride is added to 2 %, when the conductivity of the sample is less than 35 mS/cm. Since the possibility for making a color correction is limited, the optical density of the sample (490 nm) must be maximally 1.8. Otherwise this must be adjusted appropriately through the concentration of the stock solution or with 2 % NaCl.

### Test performance

The test is carried out with the marine luminescent bacterium *Vibrio fisheri*. The bacteria were purchased lyophilized (Nr. Hach Lange GmbH LCK 482). The sensitivity of each bacterial batch had already been controlled by the manufacturer with dichlorophenol, zinc

**Table 1**
**Examined model wastewater**

| Model wastewater | Treatment with |
|---|---|
| Acid dye bath (wool) | strain PS344, GS333, PO332, and PC374; activated sludge; laccase |
| Acid dye bath (leather) | strains PS344 and GS333 |
| Direct dye bath | strains PS344 and GS333; activated sludge |
| Reactive dye bath | strains PS344 and GS333; activated sludge |

**Table 2**
**Examined dyes**

| Name | CAS-No. |
|---|---|
| Disperse Dyes | |
| Disperse Red 1 | 2872-52-8 |
| Disperse Blue 1 | 2475-45-8 |
| Disperse Yellow 1 | 2832-40-8 |
| Reactive Dyes | |
| Reactive Blue 19 | 2580-78-1 |
| Reactive Black 5 | 17059-24-8 |
| Reactive Red 4 | 17681-50-4 |
| Reactive Yellow 81 | 59112-78-6 |
| Direct Dyes | |
| Direct Red R | 573-58-0 |
| Direct Blue 1 | 2610-05-1 |
| Direct Black 38 | 1937-37-7 |
| Acid Dyes | |
| Acid Blue 62 | 4368-56-3 |
| Acid Red 299 | 57741-47-6 |

**Table 3**
**Results model wastewaters**

| Model wastewater | Treatment with | Residual toxicity in % in comparison to the control |
|---|---|---|
| Acid dye bath (wool) | strain PS344 | 108.4 |
| Acid dye bath (wool) | strain GS333 | 100.2 |
| Acid dye bath (wool) | strain PO332 | 6.3 |
| Acid dye bath (wool) | strain P0332 EM | 1.1 |
| Acid dye bath (wool) | Activated sludge | 52.5 |
| Acid dye bath (wool) | Laccase | 36.2 |
| Acid dye bath (leather) | strain PS344 | 54.8 |
| Acid dye bath (leather) | strain GS333 | 98.0 |
| Direct dye bath | strain PS344 | 93.6 |
| Direct dye bath | strain GS333 | 88.4 |
| Direct dye bath | Activated sludge | 21.8 |
| Reactive dye bath | strain PS344 | 100.6 |
| Reactive dye bath | strain GS333 | 101.2 |
| Reactive dye bath | Activated sludge | 102.8 |

**Table 4**
**Results model dyes**

| Name | CAS-No. | Concentration [mg/l] | OD | pH | cond [µS cm$^{-1}$] | Inhibition in % |
|---|---|---|---|---|---|---|
| Disperse Dyes | | | | | | |
| Disperse Red 1 | 2872-52-8 | Limited solubility Saturated solution | 23 | 5.4 | 3.6 | 61.3 |
| Disperse Blue 1 | 2475-45-8 | 145 | 1867 | 7.4 | 540 | 66.1 |
| Disperse Yellow 3 | 2832-40-8 | 260 | 1800 | 6.6 | 364 | 48.2 |
| Reactive Dyes | | | | | | |
| Reactive Blue 19 | 2580-78-1 | 1002 | 1865 | 5.7 | 860 | 32.1 |
| Reactive Black 5 | 17059-24-8 | 170 | 1862 | 4.8 | 1513 | 9.2 |
| Reactive Red 4 | 17681-50-4 | 252 | 1526 | 7.2 | 818 | 25.9 |
| Reactive Yellow 81 | 59112-78-6 | 801 | 1794 | 4.4 | 1986 | 24.5 |
| Direct Dyes | | | | | | |
| Direct Red R | 573-58-0 | 40 | 1794 | 9.3 | 259 | 3.4 |
| Direct Blue 1 | 2610-05-1 | 1106 | 1626 | 7.0 | 624 | 31.4 |
| Direct Black 38 | 1937-37-7 | 160 | 1594 | 8.4 | 1774 | 20.6 |
| Acid Dyes | | | | | | |
| Acid Blue 62 | 4368-56-3 | 680 | 1895 | 5.6 | 540 | 57.5 |
| Acid Red 299 | 57741-47-6 | 504 | 902 | 6.8 | 288 | 50.2 |

sulfate heptahydrate and potassium dichromate. The inhibition is measured at predetermined concentrations and is given in the quality certificate.

In a first screening the inhibitory effect of the dyes was measured at the maximal possible test concentration (corresponding to an optical density of 1.8). To determine the EC50 the samples were diluted in a geometric series with a 2% NaCl solution. 12 ml of reconstitution solution (Hach Lange GmbH: LCX047) was thawed and maintained at 15 °C in a LUMISTherm Block (Hach Lange GmbH LZV 093). The liquid-dried bacteria, which had been stored at -20 °C, were dissolved in approx. 500 µl of the reconstitution solution and then transferred to the remaining volume of approx. 11.5 ml. After adaptation for 15 min at 15 °C 500 µl bacterial suspension were transferred to the cuvettes (Hach Lange GmbH LZP 187).

After another 15 min adaptation time the measurements were begun. Each concentration was investigated in two replicates. First the light emission without sample was determined. Then the sample was added and incubated for 30 min at 15 °C. Then the light emission $t_{30}$ was determined. From this, using the LUMIStox Software (Hach Lange GmbH LZV 093), the %-inhibition was automatically calculated for each sample, taking into account the color correction as necessary.

## Evaluation of results

In the evaluation of the results a correction factor is determined, which demonstrates the validity of the measurements. This must lie between 0.6 and 1.8; otherwise the measurement is invalid and may not be included in the evaluation of the results. In addition, the variation between the two parallel measurements must be under 3%.

From the %-inhibition data a dose-effect curve is made and after a probit transformation using a Maximum Likelihood Approximation the EC50 is calculated with a confidence interval. In the framework of the investigations the statistics software ToxRat (ToxRat-Solutions, 2004) was used.

## Validation of test method

Since the test was performed in the framework of the SOPHIED project in several different laboratories, an internal quality control was developed in order to ensure the validity and the comparability of the results. For this, each laboratory made a dose-effect curve with $K_2Cr_2O_7$ in a 2 % NaCl solution with the

Table 5
Results newly developed dyes

| Name | Maximum tested concentration (mg/l) | OD | EC 50 (mg/l) |
|---|---|---|---|
| SR1 | 1000 | 1800 | 642.7 |
| SO25 | 230 | 1680 | Not toxic |
| SG26 | 850 | 1800 | 10.3 |

following test concentrations: 14, 9.3, 7, 4.6, 3.5, 2.3, 1.8 1.75 and 1.16 mg/l. Additionally, a curve was measured with the addition of 25 mg/l Direct Red R (CAS No. 573-58-0) in the same concentration range of $K_2Cr_2O_7$, in order to test the correctness of the color correction.

### Wastewater

Four model wastewater samples were differently treated with microorganisms or enzymes, in order to obtain a decolorization and detoxification of the samples (Table 1). The toxicity of the samples in the luminescent bacteria test was given as a %-inhibition. Thereby the untreated sample was measured in parallel as a control, i.e. samples were tested before and after the treatment.

### Dyestuffs

Twelve different commercially available as well as new textile dyestuffs which were developed during the project were investigated in the luminescent bacteria test. The dyestuffs were chosen so as to include the four most important chemical groups of dyes: dispersion dyes, reactive dyes, acid dyestuffs and direct dyestuffs (Table 2). They represent the most important and most common chemical structures such as mono-, di- and tri-azo dyestuffs, anthraquinone and chromium complex dyestuffs. The dyestuffs were dissolved in de-ionized water and adjusted to an OD of approx. 1.8 for an initial screening. For the chosen dyestuffs the EC50 was determined.

## Results

### Validation of method

For validation, different concentrations of potassium dichromate were measured and in addition with the same concentrations each fortified with 25 mg/l direct red R. The results of two exemplary investigations with potassium dichromate and one investigation with potassium dichromate and direct red R are presented in figure 1. In both cases the EC50 should lie in the range of 1-10 mg/l.

### Wastewater

Four selected model wastewaters were treated with microorganisms or enzymes. The wastewaters were each tested in the lumi-

Fig. 1
EC50 determination of potassium dichromate

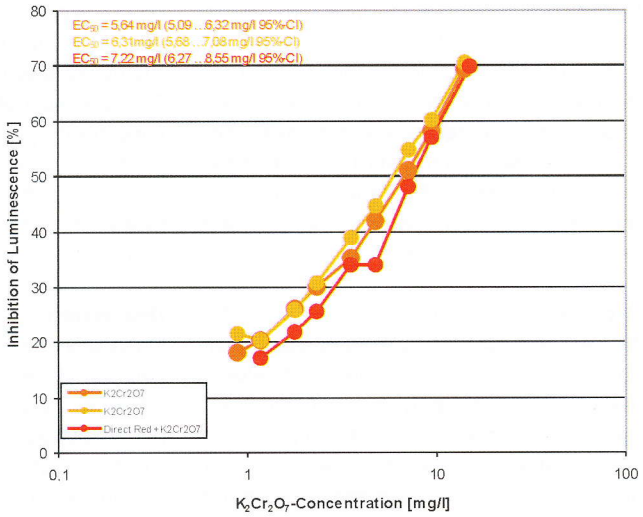
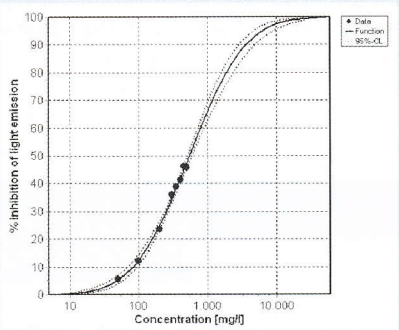
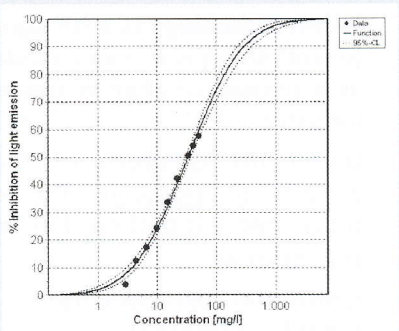



Table 6
Dose response curves

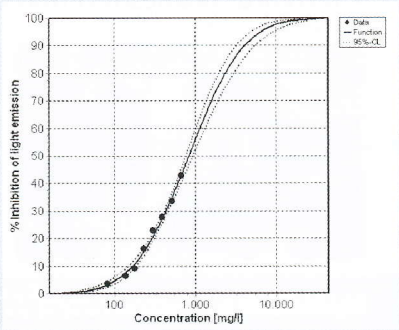
Acid Red 299 (NY1)
CAS-No.: 57741-47-6
stock solution: 1006 mg/l
highest concentration tested: 504 mg/l
EC10: 83.2 mg/l ( 69.2 ... 96.5 mg/l 95 % CI)
EC20: 159.2 mg/l (142.9 ... 174.1 mg/l 95 % CI)
EC50: 547.6 mg/l (511.7 ... 593.2 mg/l 95 % CI)
(probit analysis using linear max. Likelihood regression)
Slope of the curve: 1.562
Chi²: 0.006
Degr. of freedom: 6
r²: 0,988




Disperse Blue 1
CAS-No.: 2475-45-8
stock solution: 2212 mg/l
highest concentration tested: 1106 mg/l
EC10: 3.7 mg/l ( 3.0 ... 4.4 mg/l 95 % CI)
EC20: 7.9 mg/l ( 6.9 ... 8.9 mg/l 95 % CI)
EC50: 33.1 mg/l (30.4 ... 36.4 mg/l 95 % CI)
(probit analysis using linear max. Likelihood regression)
Slope of the curve: 1.349
Chi²: 0.016
Degr. of freedom: 7
r²: 0,987




Acid Blue 62
CAS-No.: 4368-56-3
stock solution: 1336 mg/l
highest concentration tested: 666 mg/l
EC10: 169.7 mg/l (150.1 ... 187.7 mg/l 95 % CI)
EC20: 294.1 mg/l (274.9 ... 312.1 mg/l 95 % CI)
EC50: 837.1 mg/l (765.3 ... 933.3 mg/l 95 % CI)
(probit analysis using linear max. Likelihood regression)
Slope of the curve: 1.847
Chi²: 0.007
Degr. of freedom: 6
r²: 0,987



nescent bacteria test both before and after the treatment. It could be clearly seen that there were differences in the effectiveness of the different treatment methods with respect to the toxicity in the luminescent bacteria test. Thus, for example for the treatment of the model wastewater "acid dyestuffs" with P0332 EM, a nearly complete detoxification is obtained (residual toxicity 1.1% in comparison to the control). For other samples, e.g. the reactive dyestuffs, in contrast there was no detoxification (102.8%).

### Dyestuffs

Altogether a total of 12 commercially available dyestuffs and three newly developed dyes were investigated in the luminescent bacteria test. Initially in a screening the maximal testable concentration at an OD of approx. 1.8 was tested and the % inhibition was measured. Concentrations higher than 1 g/l were not tested. Some of the dyestuffs were poorly soluble under the test conditions. In these cases saturated solutions were made. Undissolved material was removed by centrifugation.

For the poorly soluble dyes it was not always possible to work in the range of the maximal measurable optical density, because this value could not always be reached. The measured inhibition of the dyestuffs in the luminescent bacteria test lay in the range of 9.2 to 61.3 % (Table 4). In table 5 the EC50s of the newly developed dyes can be found.

For selected dyestuffs, several suitable test concentrations were investigated in order to produce a dose-effect curve and to calculate the EC 50 with the statistics program ToxRat. Examples of these results are presented in table 6.

## Discussion

Principally the luminescent bacteria test is well-suited as a rapid and economical screening test. Despite the photometric measurement method it is also applicable for the investigation of colored environmental samples and substances, when an instrument with color correction is used. Also the comparison of wastewater samples before and after a biological treatment is possible. The investigation with color correction can be performed with an optical density of up to 1.8. Thereby the concentration range which can be tested has an upper limit. With darkly colored samples it is quite possible that the toxicities cannot be determined, because the respective concentrations can no longer be measured at an optical density > 1.8.

Three of the tested dyestuffs showed no toxicity at the highest testable concentration. In these cases it is advisable to include another screening test. Here a Daphnia test could be chosen, for which the coloration of the samples plays no role. However the Daphnia test requires considerable experience, above all in the cultivation and handling and thus it cannot be established as simply as the luminescent bacteria test.

For the evaluation of the success of a treatment of wastewater samples the reporting of the results in %-reduction relative to the control (wastewater before the treatment) is appropriate. Even when the wastewater does not react toxically in the luminescent bacteria test before the treatment, it can show a toxic effect thereafter because through degradation processes toxic substances can also be formed. For the comparison of different methods the determination of the EC X with a 95 % confidence interval is recommended. Thereby the differences in the toxicity can be determined statistically.