DEVELOPMENT OF BIOSISTEMS FOR THE DETECTION AND DEGRADATION OF ENDOCRINE DISRUPTING CHEMICALS (EDCS)

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A great discovery solves a great problem,
but the solution of any problem there is a hint of discovery.
Your problem may be modest, but if it stimulates your curiosity,
it brings up your creativity and solve it with your means,
you may experience the tension and
enjoy the triumph of discovery

(George Pólya)

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SUMMARY

In recent years, a large number of materials and compounds have been produced without adequate knowledge on their interaction with the environment and of their influence on human health. This resulted in a continuous pollution by a wide array of hazardous chemicals with different structures and toxicity levels. These chemicals have deleterious effects on the reproductive systems of various animals, including humans. This dangerous situation has required the formulation of strict environmental regulations in order to reduce the emissions and to ban the production of the incriminated compounds.

Furthermore, the development of news efficient and cost-effective technologies became necessary to resolve the problems of effluents treatment. To this aim physical and chemical processing were initially tested, but they resulted expensive and may generate a large volume of sludge.

In the last years, biological wastewater treatments have represented an attractive alternative as they could be cost-effective and environmentally friendly. For these reasons, many biodegradation processes were encouraged to resolve this problem.

In this context, the present research explores various methods for biodegradation of a class of above-mentioned chemicals: endocrine disrupting chemicals (EDCs). EDCs are able to interfere with endocrine function in a number of wildlife species, leading to adverse effects particularly in relation to reproduction.

Two kinds of processes have been specifically analysed:

- oxidation of these compounds using laccase enzymes from the white-rot fungus *Pleurotus ostreatus;*
- biodegradation process based on the use of different mushrooms.

As regard as laccases, their degradability performances, against each EDC, were studied in the presence and in the absence of mediators. Two different mediators were chosen, a natural and synthetic one. Mediator addition enhanced laccase activity, favouring oxidation of recalcitrant xenobiotics. Degradation capabilities of best enzyme were also studied against EDCs mixture. In order to increase oxidative capabilities of selected enzyme, its immobilization on glass beads was performed.

As concern as various mushrooms, white-rot and aquatic fungi were chosen to analyse their degradative capabilities towards each xenobiotic. Moreover, to mimic real condition, their capabilities were studied also in the presence of EDCs mixture.

In the end, considering the great amount of these micropollutants in the environment, a screening of mushrooms able to growth on EDCs was performed using ground sample.

RIASSUNTO

Negli ultimi decenni la comunità scientifica ha assistito con crescente preoccupazione all'incremento di sostanze tossiche nell'ambiente. L'esposizione incontrollata a tale gruppo di sostanze comporta l'insorgenza di potenziali effetti negativi sia nell'ambiente sia alterazioni nel normale funzionamento del sistema endocrino degli animali e di quello umano. Tali sostanze sono state identificate come interferenti endocrini (IE) o *endocrine disrupting chemicals* (EDCs) e sono quindi riconosciuti come "sostanze esogene che interferiscono con la sintesi, la secrezione, il trasporto degli ormoni naturali, alterando quindi la funzione del sistema endocrino, provocando effetti avversi sulla salute in un organismo intatto, o sulla sua progenie". Gli alteratori endocrini comprendono una vasta gamma di sostanze che possono essere sia di origine naturale (fitoestrogeni, micoestrogeni, etc) che sintetica (ftalati, bisfenoli, etc.); nella fattispecie questi ultimi sono stati largamente utilizzati per la produzione di plastiche, detergenti e prodotti di largo consumo.
Gli IE scelti in questo progetto sono rappresentativi di differenti classi di sostanze xenobiotiche e sono stati selezionati considerando sia la loro disponibilità commerciale sia la loro presenza nell'ambiente. Ciò ha permesso di selezionare: il bisfenolo A (BPA), il nonilfenolo (NP), il metilparabene (MTPRB) ed il butilparabene (BTPRB), il dimetilftalato (DMPTL). Questi composti sono impiegati nella produzione di plastiche, prodotti per l'igiene personale e prodotti utilizzati nel settore agricolo e possono determinare l'insorgenza di patologie differenti, sia nell'essere umano sia in altre specie animali. In base al tipo di interazione con il sistema endocrino, queste sostanze possono indurre la formazione di tumori, causare sterilità e provocare danni al sistema nervoso centrale. Considerato quindi l'elevato fattore di rischio a cui siamo costantemente esposti, negli ultimi anni è stata definita e approvata una normativa sempre più stringente per l'emissione e l'utilizzo di tali sostanze in prodotti di largo consumo.
L'ubiquitarietà di utilizzo di tali composti ne ha rappresentato la principale causa di diffusione nell'ambiente. Inoltre molte di queste sostanze risultano essere persistenti alla degradazione e solubili in acqua. In tale contesto, negli ultimi anni molti studi sono stati volti all'utilizzo di differenti metodi per la rimozione e/o degradazione di tali inquinanti dalle acque di scarico. I convenzionali metodi di trattamento chimico-fisico si sono rivelati estremamente costosi e inadeguati se rapportati alle differenti di strutture chimiche e agli ingenti volumi di reflui da trattare. Il biorisanamento, d'altra parte, rappresenta un'efficace alternativa ai metodi convenzionali, mostrandosi come una tecnologia valida, economicamente sostenibile, e a basso impatto ambientale. Il biorisanamento può sfruttare due differenti processi: il bioassorbimento e la biodegradazione. Il bioassorbimento consiste nella rimozione delle sostanze inquinanti presenti nei reflui da parte di biomasse microbiche, vive o inattivate. 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 inquinanti. In particolare, i funghi *white-rot* trovano numerose applicazioni nel biorisanamento in virtù della loro capacità di degradare un ampio *range* di composti xenobiotici. Tali funghi, inoltre, sono in grado di secernere 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 microinquinanti.

Processi di degradazione basati sia sull'utilizzo di enzimi ossidativi che sull'utilizzo di funghi sono stati esaminati in questo progetto di tesi. In tale contesto, è stato possibile affrontare la suddetta problematica con due differenti approcci:

1. Utilizzo di **enzimi ossidativi** per il trattamento di alteratori endocrini;
2. Sviluppo e applicazione di **funghi** per la degradazione di alteratori endocrini.

1. Enzimi ossidativi.

L'approccio basato sull'utilizzo di enzimi ossidativi per il trattamento di alteratori endocrini mira alla messa a punto di sistemi di sviluppo per l'ossidazione di composti inquinanti, sfruttando le naturali caratteristiche dei catalizzatori enzimatici selezionati. Comune denominatore di questa sezione è l'analisi delle *performances* degradative degli enzimi selezionati in presenza dei vari IE. 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. In particolare, 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 e caratterizzato da un elevato potenziale redox; 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.

In questo progetto di tesi sono state analizzate le capacità ossidative di due laccasi secrete da *P. ostreatus:* POXC e POXA1b, quelle di 1H6C, un mutante dell'enzima POXA1b, ed una laccasi commerciale disponibile sul mercato, Novoprime Base 268. In particolare, la laccasi POXA1b e il suo mutante 1H6C sono state prodotte in maniera ricombinante nel fungo filamentoso *Aspergillus niger,* ciò ha permesso di ottenere una overespressione delle proteine di interesse fino a 100 volte maggiore rispetto alla produzione delle stesse in altri sistemi di espressione (quali i lieviti *Saccharomyces cerevisiae* e *Klyveromyces lactis*). Questi enzimi sono stati purificati all'omogeneità e caratterizzati sia da un punto di vista chimico-fisico sia strutturale. Gli studi effettuati hanno evidenziato un maggiore potenziale redox del mutante rispetto all'enzima *wild-type* (770 mV *vs* 650 mV), rendendolo un adeguato candidato per l'ossidazione di composti recalcitranti alla degradazione. POXC è stata ugualmente purificata all'omogeneità da *P. ostreatus* e utilizzata per le analisi di degradazione. Le *performances* ossidative di tutti gli enzimi sono state studiate nei confronti di tutti gli IE selezionati. Gli enzimi hanno mostrato avere attività ossidativa solo nei confronti del bisfenolo A, mentre gli altri substrati si sono rivelati recalcitranti all'ossidazione. Per incrementare le *performances* di questi enzimi, sono stati utilizzati due differenti mediatori, ovvero molecole a basso peso molecolare che ampliano l'attività catalitica delle laccasi rendendole capaci di ossidare substrati recalcitranti alla degradazione. In tale contesto, sono stati utilizzati uno mediatore sintetico, ABTS, e uno naturale, acetosiringone (AS), a due differenti concentrazioni (20 µM e 200 µM). È stato interessante notare che, a basse concentrazioni

dell'ABTS, le laccasi POXC, POXA1b e 1H6C presentano un incremento di attività ossidativa nei confronti del BPA. L'utilizzo dell'ABTS per l'ossidazione del nonilfenolo ha implementato le *performances* degli enzimi. Per quanto riguarda gli altri tre IE selezionati, nessuno è stato suscettibile alla degradazione da parte di questi enzimi in presenza dell'ABTS. Per tale motivo, le analisi effettuate successivamente hanno previsto l'utilizzo di una maggiore quantità di mediatore per la degradazione di questi contaminanti. I risultati ottenuti hanno mostrato che POXC e 1H6C sono in grado di degradare il BTPRB in presenza di ABTS.
Le stesse analisi sono state effettuate in presenza di acetosiringone. Anche in questo caso, a basse concentrazioni di AS, il mediatore utilizzato incrementa le *performances* degli enzimi prodotti da *P. ostreatus*, ma decrementa quelle dell'enzima commerciale nei confronti del BPA. Le laccasi selezionate presentato attività ossidativa implementata in presenza di AS nei confronti del NP, anche se con *performances* inferiori a quelle ottenute in presenza di ABTS. Anche in questo caso, come già osservato in precedenza, gli altri IE si rivelano estremamente recalcitranti all'ossidazione. Incrementando la concentrazione di mediatore naturale utilizzata, sono stati ottenuti interessanti risultati, in quanto tutti gli enzimi mostrano avere attività ossidativa nei confronti de due parabeni selezionati.
Dai risultati ottenuti, si evince che le *performances* migliori sono state ottenute da POXC, per tale motivo questa laccasi è stata scelta per studiare le sue capacità ossidative in presenza di una miscela di IE. Tali analisi sono state effettuate sia in presenza dell'enzima in forma libera che in forma immobilizzata. La possibilità di produrre biosistemi enzimatici immobilizzati costituisce un'opportunità interessante da applicare al trattamento dei microinquinanti. 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. La miscela di IE utilizzata è stata composta da BPA, NP e i due parabeni; il dimetilftalato non è stato utilizzato considerata la sua refrattarietà all'ossidazione da parte delle laccasi.
Analizzando le *performances* di POXC in forma libera contro la miscela di IE, è stato possibile monitorare la scomparsa dell'80% di NP dopo un'ora di incubazione, mentre, nello stesso intervallo di tempo il 35% di BPA risulta essere ossidato. Per quanto riguarda in parabeni, questi sono risultati essere recalcitranti alla degradazione. Anche in questo caso, le analisi sono state effettuate in presenza dei due mediatori precedentemente selezionati. L'unico IE verso cui POXC mostra implementate attività ossidative in presenza dei mediatori è il BPA, infatti, in presenza di ABTS e AS, POXC mostra il 75% e il 100% di degradazione, rispettivamente. Per incrementare le *performances* di POXC, questo enzima è stato immobilizzato covalentemente su sfere di vetro. Le analisi sono state monitorate per un'ora e hanno evidenziato un incremento della capacità ossidativa solo per il NP, che risulta essere totalmente degradato dopo 15 min. Gli altri IE mostrano le stesse velocità di degradazione ottenute dall'enzima in forma libera.
I risultati indicano che l'immobilizzazione dell'enzima comporta un incremento di attività nei confronti di uno dei quattro IE, rispetto alla controparte in fase libera.

2. Funghi.

Sviluppo e applicazione di funghi per la degradazione di alteratori endocrini.

L'approccio basato sull'utilizzo di funghi per il trattamento di alteratori endocrini mira alla messa a punto di sistemi per la degradazione di composti inquinanti, sfruttando

le potenzialità applicative di differenti funghi. In tale sezione sono state analizzate le capacità degradative di funghi basidiomiceti e di funghi acquatici.
-*Biodegradazione degli alteratori endocrini mediante funghi white-rot:* le potenzialità applicative dei funghi *white-rot P. ostreatus, Phanerochaete chrysosporium* e *Trametes versicolor* sono state analizzate impiegandoli come catalizzatori del processo di decontaminazione. Colture in liquido dei funghi sono state allestite con ciascuno dei microinquinanti selezionati, monitorando giornalmente la degradazione di ogni composto. I dati ottenuti sono schematizzati nella seguente tabella:

Velocità di degradazione di ogni IE in funzione del tempo (giorni) per ogni fungo.

Funghi	**BPA**		**NP**		**MTPRB**		**BTPRB**		**DMPTL**	
	$t_{50\%}$	$t_{100\%}$	$t_{50\%}$	$t_{100\%}$	$t_{50\%}$	$t_{100\%}$	$t_{50\%}$	$t_{100\%}$	$t_{50\%}$	$t_{100\%}$
P.ostreatus	3.5	8	0.5	>8	3	>8	>8	>8	2	>8
T.versicolor	1	2	1	8	1.5	3	1.5	2	1	>8
P.chrysporium	3	>8	1	>8	2.5	4	1	4	7	>8

Come si evince dalla tabella, i risultati migliori sono ottenuti in presenza del fungo *T. versicolor*, che risulta essere in grado di degradare il 50% di tutti gli inquinanti in 24 h. Questo fungo è stato quindi scelto per analizzare le sue capacità degradative minimizzando sia la fonte di carbonio che di azoto in presenza di un microinquinante modello, il BPA. Dopo 24 h di incubazione, tutto il BPA presente nel mezzo di coltura è stato totalmente degradato. Lo stesso esperimento è stato quindi allestito in presenza di solo BPA, senza alcuna fonte di carbonio e azoto. Anche in questo caso, dopo 24 h tutto il BPA è stato degradato. Lo step successivo ha riguardato l'analisi delle *performances* di degradazione del BPA da parte della stessa coltura di *T. versicolor*, in seguito ad aggiunte giornaliere dell'inquinante. Tali analisi sono state monitorate per 15 cicli, mostrando la giornaliera scomparsa del BPA e la capacità di tale fungo di degradare in maniera continua questo inquinante mantenendo inalterata la velocità di degradazione.
Per mimare al meglio le condizioni di un refluo reale, le capacità degradative di questo fungo sono state studiate in presenza della mix di alteratori endocrini. È stato interessante notare che tra i 2 e i 4 giorni di crescita, tutti gli IE selezionati e utilizzati per ottenere la miscela, sono stati completamente degradati. Anche in questo caso, sono state effettuate analisi della degradazione della miscela eliminando la fonte di azoto e di carbonio. Dopo 24 h, si è registrata la totale degradazione della miscela. Questo importante risultato ha portato all'analisi delle *performances* di degradazione della stessa coltura in seguito ad aggiunte giornaliere della mix di IE. In questa fase è stato possibile monitorare la scomparsa di tutti i componenti della miscela dopo il primo ciclo, ma successivamente solo la parziale degradazione di alcuni composti è stata evidenziata. Nella fattispecie, il BPA e il BTPRB sono stati degradati in maniera continua, senza registrare il loro accumulo all'interno del brodo. Invece NP e MTPRB hanno mostrato un comportamento atipico, evidenziando un profilo di concentrazione di tipo gaussiano, determinato dall'alternarsi di totale degradazione, parziale accumulo, completa degradazione. Il DMPTL è stato totalmente degradato solo

durante il 1° ciclo, successivamente una parziale degradazione dello stesso è stata monitorata, determinandone quindi il progressivo accumulo nel brodo di coltura.
Da tale studio si evince che i funghi *white-rot* selezionati presentano buone rese di degradazione nei confronti di questi alteratori endocrini. Nella fattispecie, le migliori *performances* sono state ottenute da *T. versicolor*, che risulta essere in grado di degradare in maniera continua molti microinquinanti, sia in miscela sia come composti singoli, senza l'utilizzo di alcuna fonte di carbonio e di azoto. Se riportati su scala industriale, questi risultati rappresentano un ottimo punto di partenza su cui basare processi di decontaminazione *in situ*.
-*Biodegradazione degli alteratori endocrini mediante funghi acquatici:* in collaborazione con il Helmholtz Centre for Environmental Research, Leipzig (Germany), sono state esplorate le capacità degradative di differenti funghi acquatici nei confronti degli alteratori endocrini selezionati. In tale contesto, le *performances* di sei differenti funghi acquatici sono state studiate in *multiwell* in presenza dei differenti IE, e due di questi funghi sono stati scelti per il successivo *scale-up* in beuta. *Cladosporium herbarum* AP2-2009-7 e *Phoma* sp. UHH 5-1-03 hanno mostrato avere buone capacità degradative nei confronti dei due parabeni, esibendo una totale degradazione degli stessi in sei giorni. Gli altri IE sono risultati recalcitranti alla degradazione. Il successivo passaggio in beuta di *C. herbarum* AP2-2009-7 ha permesso di monitorare la totale degradazione del MTPRB in 2 giorni e del DMPTL in 7 giorni. Il BTPRB invece si è rivelato essere recalcitrante alla degradazione; stesse *performance* sono state osservate per il BPA e il NP. Per valutare se effettivamente questo fungo fosse in grado di crescere in presenza di questi microinquinanti, nuove colture sono state allestite utilizzando come inoculo una parte della biomassa sviluppatasi durante la prima crescita in beuta. Questa seconda crescita ha evidenziato che le capacità degradative di *C. herbarum* AP2-2009-7 nei confronti del MTPRB restano invariate, in quanto, dopo due giorni, è stata mostrata la totale scomparsa dell'inquinante. Stesse analisi sono state effettuate in presenza del DMTPL, ed in questo caso un incremento delle capacità degradative è stato rilevato, in quanto il DMPTL è stato totalmente degradato dopo 4 giorni. Analizzando le *performances* di tale fungo in presenza della mix, i due parabeni sono stati totalmente degradati dopo due giorni, il NP dopo 15 giorni, mentre il BPA e il DMPTL sono stati degradati solo parzialmente. Una parte della biomassa proveniente dalla prima crescita, è stata utilizzata per inoculare una seconda coltura, La degradazione del NP è avvenuta dopo 11 giorni, mentre le *performance* nei confronti degli altri substrati sono rimaste invariate.
Stesse analisi sono state condotte in presenza di *Phoma* sp. UHH 5-1-03. Questo fungo è stato in grado di degradare il DMPTL in 7 giorni, e, rinoculando una parte della biomassa in colture contente lo stesso inquinante, si è monitorata una totale scomparsa dell'IE dopo 3 giorni. In presenza dei parabeni, invece, *Phoma* sp. UHH 5-1-03 ha evidenziato un processo di biodegradazione associato ad un processo di bioassorbimento. Infatti, in presenza di MTPRB, è stato possibile notare che una parte del microinquinante era stata adsorbita sulla superficie del fungo dopo tre giorni di crescita. Dopo 4 giorni, il MTPRB presente nel brodo di coltura era stato totalmente degradato, mentre una parte dello stesso era adsorbita sulla membrana cellulare del fungo. Dopo 7 giorni, il MTPRB è stato totalmente degradato, sia in coltura che sulla superficie del fungo. L'utilizzo di una parte di questa biomassa per una nuova coltura contente MTPRB ha evidenziato la totale degradazione del composto inquinante in 3 giorni, senza evidenziare un processo di bioadsorbimento.

In presenza del BTPRB è stato possibile monitorare solo un processo di bioadsorbimento, senza però registrare l'effettiva degradazione dello xenobiotico.
Analizzando infine le *performances* di *Phoma* sp. UHH 5-1-03 in presenza della mix di IE, è stato possibile notare la scomparsa di tutti i componenti della miscela dopo 2 giorni di crescita. Successivamente, reinoculando una nuova coltura con parte di questa biomassa, è stata monitorata la totale degradazione solo dei due parabeni dopo tre giorni di crescita, mentre per gli altri IE è stata monitorata una parziale degradazione.
-*Biodegradazione degli alteratori endocrini mediante nuovi funghi:* campioni di terreno provenienti da Cospudener See, un lago a sud di Lipsia, sono stati utilizzati per l'isolamento di nuovi ceppi fungini capaci di crescere su una miscela di IE. Questo *screening* ha permesso di isolare un ceppo fungino, le cui *performances*, in presenza dei vari IE, sono state valutate in colture liquide. Tale fungo ha mostrato attività degradativa solo nei confronti del NP e del MTPRB. In presenza della miscela di IE, le *performances* di degradazione sono state differenti, in quanto dopo 2 giorni, tutti i componenti della mix sono stati degradati del 50%, ma sono il NP risulta essere totalmente degradato dopo 10 giorni di crescita. Successivamente si è proceduto all'identificazione del fungo, analizzando le sequenze ITS (Internal transcribed spacer), ovvero sequenze specie-specifiche di DNA non codificante. Un'identità del 99% è stata riscontrata con le ITS di *Trichoderma virens.* Analizzando il genoma di questo fungo, si è evidenziata la presenza di geni potenzialmente responsabili della produzione di enzimi ad attività ossidativa e idrolitica.

I risultati ottenuti con questo lavoro di tesi hanno consentito di analizzare diverse alternative da applicare al trattamento di microiquinanti. In particolare, l'utilizzo di organismi per processi di biorisanamento ha evidenziato notevoli caratteristiche che li rendono una valida e competitiva alternativa ai metodi di degradazione convenzionali.

Introduction

1. Endocrine disruptors: background information

In the last years valuation and conservation of environmental quality has represented an interesting field of technologic applications. Concerning the industrialized states, the main problem is represented by a constant and continuous pollution of soil, water-bearing stratum, surface water and air. This has been due mainly to the introduction in the environment of contaminants toxic for many organisms, including humans. In this contest, **endocrine disrupting chemicals** (**EDCs**) play a significant role. Since the early 1990s, the presence in the environment of EDCs displaying oestrogen-like activities has become a major issue in environmental research and policy [1]. Various natural and synthetic chemical compounds induce estrogen-like responses, including pharmaceuticals, pesticides, industrial chemicals, and heavy metals [2]. EDCs are defined as "*An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour."* [3-7]. These substances interfere with the endocrine system of humans and wildlife leading to adverse effects particularly in relation to reproduction and development of secondary sexual characteristics. Exposure to EDCs causes many different anomalies, as diminished fertility and reproduction, altered sex differentiation, changes in behaviour, abnormal growth, altered immune function, neurological impairment, altered hormonal levels and deformed organ histology among other effects [8-11]. For the first time, Sharpe and Skakkebæk hypothesized the existence of a correlation between EDCs and reproductive health in 1993. This study showed a decreased of sperm count/quality and an increased incidence of testicular cancer, testicular maldescent and male reproductive tract malformations [12]. Moreover, all animals, from humans to invertebrates, are particularly sensitive to EDCs exposure during their sexual differentiation. Any hormonal variation or exposure to EDCs during this critical period may induce permanent gonadal and/or phenotypic sex reversal [13]. Obviously, the effects of EDCs may vary between species and between males and females, showing higher susceptibility of one of the sexes to a specific EDC. Estrogenic activity of these xenobiotics is correlated to phenolic ring para-substitution and polycyclic structure capable of assuming planar configuration. This phenolic moiety mimics natural steroid hormones and enable EDCs to interact with steroid hormone receptors as analogs or antagonists [14; 15].

The high toxicity at low dosage of these substances and the long-term exposure - especially during crucial periods of life-cycle development- has caused alarm in political and scientific research communities. These substances are found in many products derived from cosmetic industries and working environment. Table 1 gives some examples of cases of endocrine disruptors observed in human and wildlife across the world. These xenobiotics represent latent crisis to humans and the environment. This crisis could be easily controlled by applying regulatory policies in order to reduce the emissions and to ban the production of the incriminated compounds and by their total removal from wastewater before final release into the environment.

2. Regulation of EDCs

In the European Union, the absence of regulation on EDCs until the beginning of the new millennium has been due to the lack of knowledge about their impact on human health. In 2001, the Commission released a chemical regulation proposal to harmonize the existing and new substances and close the knowledge gap (safety data were lacking for existing substances). Considering that the endocrine disruptors show different mechanisms of action that may lead adverse consequences on humans and ecosystems, the first reaction of EU was the establishment of a priority list of substances for further evaluation of their endocrine disrupting effects. This

Substance category	Name	Source/uses	Reported properties
Dioxins and dibenzofurans	2,3,7,8-TCDD, 2,3,7,8-p-TCDD, 2,3,7,8-PCDF, 2,3,4,7,8- PeCDF, 1,2,3,7,8 (9)-PeCDF	Arise as unwanted by-products from certain incineration and industrial chemical process	Anti-estrogenic
Industrial chemicals and related	Phthalates (DEHP, BBP, DBP, DPP, DPrP)	Used as plasticisers in the production of flexible plastics	Estrogenic
Substances	Alkylphenols (penta- to nonylphenols)	Degradation products of Alkylphenol polyethoxylates	Estrogenic
	Alkylphenols polyethoxylates	Non-ionic surfactants used extensively	Estrogenic
	Bi-phenolic compounds (bisphenol A, bisphenol F)	Used in the production of PVC, rubber, epoxy and polycarbonate resins, and plastics	Estrogenic
	PCB	No longer used manufactured or used, but some equipment (electrical) containing PCBs remains in use	Anti-estrogenic
Pesticides	DDT, DDD, DDE, alachlor, aldicarb, amitrole, atrazine, beta-HCH, dieldrin, endosulfn, lindane, heptachlor, kepone, toxaphene, ziram and so on	Many pesticides used throughout the world today and present in aquatic environment in significant concentration. Organochlorins are highly lipophilc and bioaccumulate.	Influence the endocrine system in more than one way
Synthetic estrogens	Diethylstilbestrol	Prescribed to women to prevent miscarriages (banned in present)	Estrogenic
	Ethinylestradiol	Oral contraceptive pill	Estrogenic
Phyto-estrogens	Isoflavones, coumestol, Lignans and stilbens	Synthesized in plants	Estrogenic
Myco-estrogens	beta-zearalenol, zearalenone	Found in fungi	Estrogenic

Table 1. Toxic substances found in many products of daily use.

prioritization work started in the year 2000. Somewhere, around 600 chemical substances were screened, evaluated and a preliminary priority list was established. Between 2000 and 2006, the Commission contracted three studies on identification and evaluation of this class of substances. In 2004 the first EU Directive -67/548/EEC- for hazardous chemicals was drawn up, and today it is recognized as the backbone for chemical regulation in the EU [16]. Over the past six years, 575 substances were investigated as endocrine disruptors. Among these chemicals, **320** showed evidence or potential evidence for ED effects. In 2002, another study was carried out and 435 substances were analysed. Investigation was focused on candidate substances identified as **High Production Volume Chemicals** (HPVC), persistent in the environment and to which human- or wildlife-exposure could be expected. According to these criteria, 204 substances were selected and their endocrine disrupting effects were evaluated. **147** of them were identified, showing either clear and/or potential effects on endocrine system. In the last year, other investigations were carried out, increasing this list. Indeed, **1372** substances are prohibited in cosmetic products; furthermore, **256** substances are allowed only at low concentrations [17]. Considering the complexity of endocrine system, it is not surprising that a wide range of substances cause endocrine disruption and these include both natural and synthetic chemicals.

2.1. The different groups of EDCs

The group of molecules identified as endocrine disruptors is highly heterogeneous and includes synthetic chemicals used as industrial solvents/lubricants and their by-products, plastics, pesticides, fungicides, pharmaceutical agents, cosmetics and other personal care products [18]. The molecular structures of several EDCs, with accompanying varying functionalities, are summarized in Table 2.

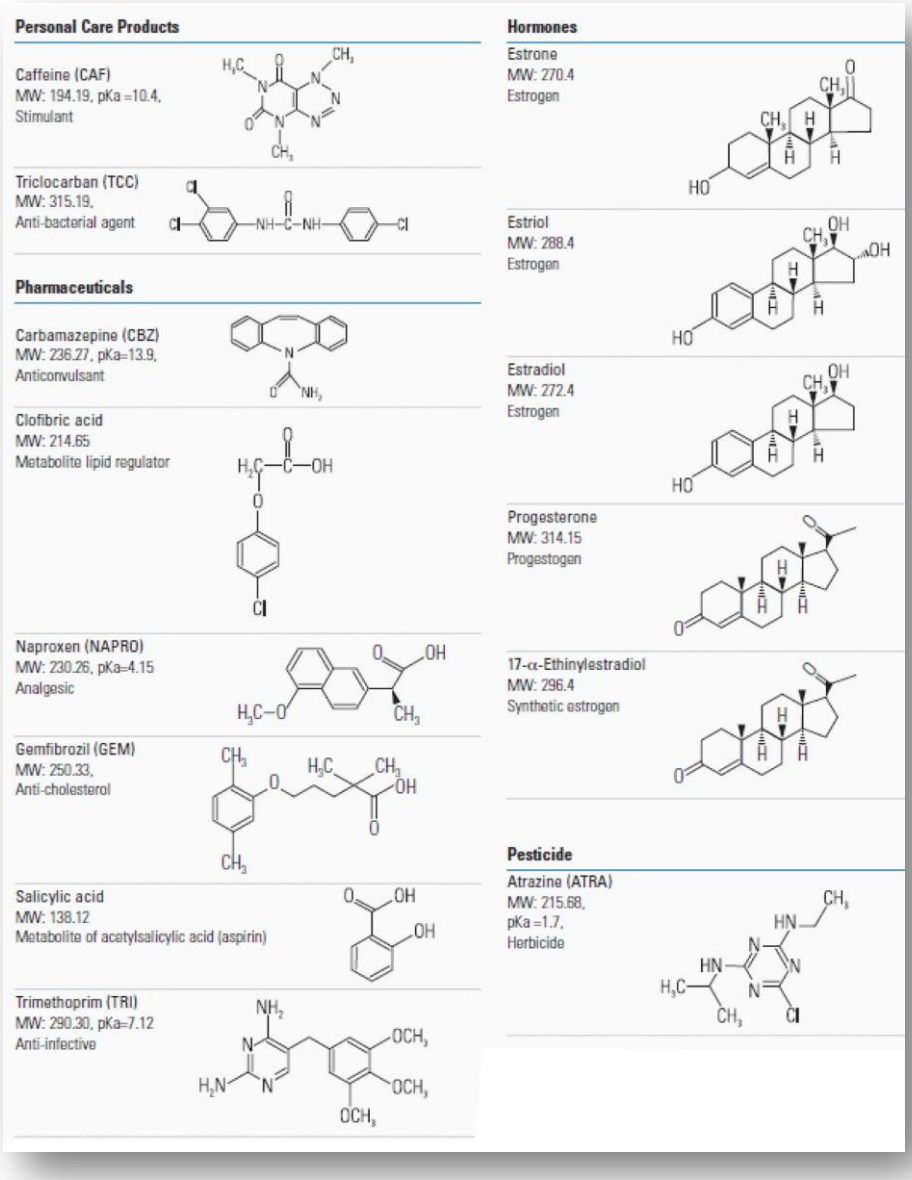

Table 2. Examples of various types of EDCs classified.

They are broadly classified into several categories, such as hormones (natural and synthetic estrogens or steroids), pharmaceuticals and personal care products (PPCPs), industrial chemicals, pesticides, combustion by-products, and surfactants [2; 11]. Moreover, natural chemicals found in human and animal food (e.g., phytoestrogens, including genistein and coumestrol) can also act as endocrine disruptors.

Among various synthetic EDCs, **bisphenol A, nonylphenol, parabens and phthalate** have been investigated in this project.

Bisphenol A (BPA) (CAS No: 80-05-7) is high production volume chemical used as an intermediate in the fabrication of polycarbonate plastic and epoxy resins which can be used in eyeglass lenses, medical equipment, water bottles, cell phones, electronics, baby bottles, water cooler jugs, drink, food packaging and plastic dental fillings [19-22]. Bisphenol A is one of the most widely used chemicals in the world with production of 650,000 tonnes per year [20]. The Environmental Protection Agency (EPA) identified Bisphenol A as EDC, World Wide Fund for Nature (WWF) declared this compound as a social, environmental and global issue. Due to the daily use of these products, high concentrations of BPA are observed in wastewater (WW) and in wastewater sludge (WWS) (0.004-1.36 mg/kg) [23]. The exposure to these chemicals varies from regions and lifestyle. According to the National Health and Nutrition Examination Survey (NHANES), BPA concentration is higher than 12 parts per billion (ppb) (12 nM) in North American waters [24]; this endocrine disruptor is present at a concentration of 43 ppb in European waters [25] and 191 ng/L in sediments [26]. Moreover, BPA is present at high concentration (1.16 µg/L) in urine of Canadians aged 6-79 [27], and -considering the short half-life of orally ingested BPA and the high detection frequency- the obtained data suggest continual and widespread exposure to this endocrine disruptor.

Human exposure to bisphenol A may occur when this chemical leaches plastic because of the breakdown of the polymer upon heating [28]. In fact, after heating, a migration of free BPA has been displayed from various consumer products, such as baby bottles, into the food or beverage [29]. For this reason, in 2010 Canada became the first country to declare BPA to be a toxic compound and required its removal from infant formula bottles. In 2011, European Union banned BPA in infant formula bottles [30].

Toxic effects of BPA influenced reproductive system of different species [31-34], for this reason, it is classified as a reproductive substance category three, which means that there is concern for human fertility. Animal studies provide sufficient evidence to reveal strong suspicions of reduced fertility in presence of toxic compound, but insufficient data were presented to place the substance in category two [29].

Nonylphenol (NP) (CAS No: 25154-52-3) is a mixture of para-, ortho-, and meta-isomers, the most prevalent of them is para-NP (4-NP). It is viscous, colourless liquid and it is subjected to ethoxylation to give alkylphenol ethoxylates (APEs) [35]. It is used in the production of “nonylphenol ethoxylates, a non-ionic surfactants applied as emulsifying, wetting, dispersing or stabilizing agent in industrial, agricultural and domestic consumer products” [36-38]. Nonylphenol is present in polystyrene plastics, cleaning supplies, leather auxiliaries; it is used in paints, agricultural products and photographic equipment [1; 36]. In the last 50 years, NP was extensively used as surfactants with an annual world production estimated at 360,000 tonnes in 1988 [39]. In Europe, in 1997 the total production was 73,500 tonnes. In China is about 50,000 tonnes per year; approximately 70% of the NP is used for production of synthetic detergent [40]. Since 2000, 4-NP was included in the list of priority hazardous substances by Directive 2000/60/EC [41]. Nowadays only a few countries (e.g. Asian countries) continue to use APEOs [42].

Because of the widespread use of NP, the potential exposure for human is high. Nonylphenol is valuated to be very toxic for the aquatic environment and may cause long-term harm as it is not biodegradable and because it shows high potential to bio-concentrate [43]. Bounding to the soil, NP is unlikely to enter groundwater, for this reason contamination is found only in surface water. Toxicological studies on laboratory animals have proved that the exposure to NP is associated with

morphologic, functional, and behavioural anomalies related to reproduction [44-46]. The main sources of exposure for humans are food (fish and root crops) and drinking water. NP has also deleterious effects on central nervous system (CNS), causing alteration on neuroendocrine homeostasis, altering cognitive function, and neurotoxicity of tissues, etc. [335].

Parabens (CAS No: 99-76-3) are esters of p-hydroxybenzoic acid. Since the 1930s, they are widely used as preservatives in food, pharmaceutical and cosmetic industries in order to prevent bacterial growth [48-50]. According to Ingerslev and co-workers [50], approximately 12 parabens are used commercially as preservatives in cosmetics, food, and pharmaceutical products. In the EU, since 1976, cosmetic legislation has been harmonized through the Cosmetic Directive [51]. Parabens are the most predominant group of used preservatives; more than 35% of cosmetic products registered in the USA contain one or more parabens [52], and more than 28% of Danish products contain at least one paraben [53]. In personal care products, parabens are used at relatively low concentrations. In the EU, for example, the maximal concentration is 0.4% for methyl- (MTPRB) or ethylparaben (ETPRB), or 0.19% for propyl- (PP) or buthylparaben (BTPRB). Denmark has banned the use of these preservatives in cosmetic products for children up to three years old. Danish cosmetic regulations permit the preservations of cosmetics products with methylparaben, ethylparaben, propylparaben and buthylparaben up to a maximum combined concentration of 0.8% (w/w) [54]. Harvey and co-workers suggest a correlation between the increasing breast cancer and the heavy usage of parabens. This correlation is due to fast "*parabens absorption through the skin as intact esters, their hormonal activity and their reproductive toxicity*" [49]. The only paraben that has been associated with endocrine disruption and reproductive adverse effects is buthyl paraben [54; 55].

Parabens are also present in many natural products (additives E 214-219), such as some fruits and vegetables, strawberries, grape juice, yeast extracts, barley, vinegar, cheeses, royal jelly, propolis. As concern this study, methylparaben and buthylparaben were chosen for degradative analyses.

Phthalates are a group of persistent, high production volume chemicals, primarily used as additives in plastics, in order to make them more flexible. They are a family of industrial compounds with a common chemical structure, dialkyl or alkyl/aryl esters of 1,2- benzenedicarboxylic acid. Since the 1930s phthalates have been used for a variety of products, including personal care products (e.g. perfumes, lotions, cosmetics), varnish, medical devices, pharmaceuticals, solvents, additives and insect repellents; but phthalates are primarily used as plasticizers to impart flexibility to an otherwise rigid polyvinylchloride (PVC) [56-62]. The use of certain phthalates in many consumer products is banned in various countries, including Europe by EEC directive 2005/84/EEC [63]. Phthalates may constitute up to 50% of the total weight of PVC plastics, and their worldwide annual production is approximately 2.7 million metric tonnes [64]. Phthalates are not classified as persistent compounds [65], but their occurrence in the environment has been widely reported, possibly arguing against a rapid biodegradation in some environments [66-69].

Depending on the alkyl chain, phthalates have different properties and can be used in diverse applications. The long-chain phthalates, such as di(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), diisodecyl phthalate (DIDP), and di(2-propylheptyl) phthalate (DPHP), are primarily used in PVC polymers and plastisol applications. Short-chain phthalates, such as dimethyl phthalate (DMPTL), diethyl phthalate (DEP), buthylbenzyl phthalate (BBP), and di-buthyl phthalate (DBP), are

often used in non-PVC products such as personal care products, paints, adhesives, and enteric-coated tablets [70]. Phthalates are easily released from plastics into the environment via direct release, migration, evaporation, leaching, and abrasion [69] because they are not chemically bound [71]. Analytical surveys of the presence of phthalate ester metabolites in urine, serum, and other body fluids have confirmed the ubiquitous nature of exposure to multiple phthalates [72]. Ingestion is the major route of exposure, although other ways, including inhalation, dermal, and parenteral routes, have also been recognized [72]. These substances have a low order of acute toxicity but they are embryo-toxic and teratogenic in mice and rats. They also present low propensity for bioaccumulation. Among various phthalates, this study is focused on dimethyl-phthalate (CAS No: 131-11-3).

2.2. Applicable treatment options to remove EDCs

Endocrine disrupting chemicals are present in large amount in environment and are recalcitrant to degradation, for these reasons, many initiatives have been tested for the degradation of these micropollutants. EDCs removal methods fall into three categories: physical removal, chemical advanced oxidation (CAO) and bioremediation.

2.2.1. Physical removal

Various separation or oxidation techniques have been considered as potential treatment options for the effective removal of EDCs from water. Unfortunately, the chosen treatment did not consistently conform to the desired removal efficiency level. Among physical removal, coagulation, flocculation and precipitation processes, are not effective in removing EDCs, especially for low molecular weight compounds ranging from 100 to 500 Da [73-75]. On the other hand, adsorption and membrane filtration normally show superior removal efficiencies (up to 95%), depending on the compounds tested [76]. Absorption can take place using both granular activated carbon (AC) and various matrices. The former removes most organic contaminants, including EDCs, by artificial and real wastewater in the laboratory and pilot and full-scale plants [77-83]. Many factors influence physicochemical properties of AC determining strict control of adsorption parameters and operational factors, such as kinetic and equilibrium constants, contact time, solubility, carbon type, competition with natural organic matter, etc [73; 84-86]. On the other hand, membrane separation is efficient in removing micro-contaminants, such as EDCs [77; 87-89], but elimination is incomplete with fluctuations in the range of 10-95%. Indeed, hydrophobicity and surface charges play a significant role in the retention of such compounds [89-91]. Filtration and its performances are strictly dependent by the used matrix.

2.2.2. Chemical advanced oxidation (CAO)

Chemical advanced oxidation (CAO) is mechanisms of mineralization of pollutants in wastewater to CO_2 or transfer of pollutants to other products through oxidation-reduction reactions. To increase the removal effect, some combinations such as UV/O_3, UV/H_2O_2, UV/Fenton are widely applied to the removal of EDCs. In this way, generation of the hydroxyl radical is obtained (redox potential 2.80 V) [92]. Thus, CAO uses generally the combination of two methods for the removal of EDCs.

2.2.3. Bioremediation

Bioremediation is the use of biosystems (microorganisms and/or organisms able to degrade hazardous contaminants) to remove pollutants; environmental impact due to the use of biosystem is very low, water and sludges being the only products of the treatment. This technology utilizes the metabolic potential of microorganism for degradation of toxic compounds [93]. In this frame, bacteria, fungi and enzymes play a major role.

- **Bacteria** are able to mineralize a wide range of aromatic compounds, using them as carbon source for microbial growth [94]. Indeed, they are used for activated sludge process. This process is widely used in the world and it shows high removal efficiency of EDCs [95-97].
- **Fungi** also represent a vastly bio-diverse group of organism showing degradation abilities towards several pollutant compounds [96].
- Oxidative **enzymes** secreted by white-rot fungi are also able to degrade a large range of toxic aromatic pollutants. This is due to their broad substrate specificity and to the strong structure similarity between pollutants and their natural substrates. Oxidative enzymes are tyrosinases, peroxidases and laccases. The particularity of tyrosinases (EC 1.14.18.1) is their ability to catalyse two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), which, in turn, polymerize to brown, red, or black pigments [97-99], both reactions use molecular oxygen [100]. Peroxidases (EC 1.11.1.7) are oxidoreductases that catalyse the reduction of peroxides, such as hydrogen peroxide (H_2O_2) and the concomitant oxidation of a variety of organic and inorganic compounds [101]. Laccases (EC 1.10.3.2) are blue multicopper oxidases, catalysing the oxidation of an array of aromatic substrates concomitantly with the reduction of molecular oxygen to water [102].

For the reason previously described, this project is focused on study of degradative capabilities of enzymes and organisms in the presence of selected EDCs. In this frame, white-rot fungi and laccases produced by these mushrooms were studied.

3. Laccases: general features

-*Distribution*: Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are a large group of multicopper oxidases [103] belonging to the family of blue oxidases produced by plants (*Rhus vernicifrera*), insects (*Bombix sp.*) and bacteria (*Azospirillum lipoferum*), and widely occuring in several species of filamentous fungi [104]. Laccases were first described 120 years ago, and then enzymes from plants and fungi have been extensively studied. Among the several different biological roles so far ascribed to fungal laccases [105], they have a main role in lignin degradation, together with lignin and manganese peroxidase [106; 107].

-*Biochemical features*: laccases are cuproproteins that couple the four single-electron oxidations of the reducing substrate to the four electron reductive cleavage of the dioxygen bond, using four Cu atoms distributed against three sites, defined according to their spectroscopic properties [102]. Typical metal content of laccases includes one type-1 (T1) copper (Cu1) (where the reducing substrate place is), and one type-2 (T2) and two type-3 (T3) copper ions (Cu2 and Cu3), with Cu2 and Cu3 arranged in a trinuclear cluster (TNC) (where oxygen binds and it is reduced to water) [108] (fig. 1).

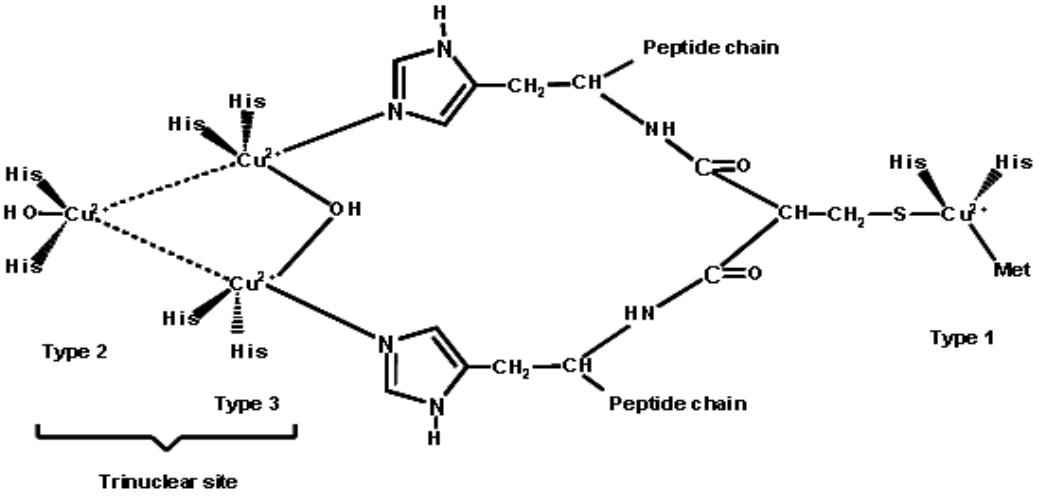

Fig. 1. Laccase active site. The Type 1 copper is coordinated with two histidine ligands and two sulphurs one of methionine and the other of cysteine. The Type 2 center is 3-coordinate with two histidine ligands and water as ligands. The Type 3 coppers are each 4-coordinate, having three histidines ligands and bridging hydroxide [109].

-*Structural properties*: Laccases are thought to be nearly ubiquitous among fungi, and their presence has been documented in virtually every fungus examined so far. Current knowledge about the structure and physico-chemical properties of fungal proteins is based on the study of purified proteins. Up to now, more than 100 laccases have been purified and characterized from fungi. Based on the literature, general characteristics of these enzymes can be drawn out. In fungi, laccases carry out a variety of physiological roles including morphogenesis, fungal plant-pathogen/host interaction, stress defence and lignin degradation. In general, fungal laccases are monomeric globular proteins of approximately 60–70 kDa with acidic isoelectric point (pI) around pH 4.0, although several exceptions exist [110]. The majority of fungal laccases are extracellular enzymes generally glycosylated, with an extent of glycosylation usually ranging between 10 and 25% and only in few cases higher than 30% [111]. Laccases secreted from native sources are usually not suitable for large-scale purposes, mainly due to low production yields and high cost of preparation/purification procedures.

-*Applications*: Due to their broad substrate specificity, laccases display great biotechnological potential and high market expectative in several fields of industrial applications such as in pulp delignification [112], textile dye bleaching [113; 114], food industries [115], ethanol production [116], bioremediation [102; 117], and organic synthesis [118; 119]. Laccases possess relatively low potential redox (<0.8 V) [120] and, to enhance their activity, it is possible to use small molecules, known as redox mediators. In the presence of these molecules, laccases are able to catalyse the oxidation of substrates which the enzyme is not able to oxidize directly either because of their large dimensions or their high redox potential [121]. Once oxidized by the enzyme and stabilized in more or less stable radicals, mediators diffuse far away from the enzymatic pocket and enhance the redox reaction [122]. The ideal redox mediator would be a small-size compound, able to create stable radicals (in its oxidized form) that do not inactivate the enzyme, and whose reactivity would allow its recycling without degeneration. In addition, taking into consideration their industrial and environmental applications, mediators should be environmental-friendly and

available at low cost. Mediators can be synthetic (e.g. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate, ABTS) and natural (e.g. acetosyringone).

3.1. Fungal laccases: heterologous expression and immobilization

For the aforementioned reasons, laccases represent one 'ecofriendly' enzyme thanks to their industrial versatility; however, laccases secreted from native sources are usually not suitable for large-scale purposes, mainly due to low production yields and high cost of preparation/purification procedures. Heterologous expression may provide higher enzyme yields and may permit to produce laccases with desired properties (such as different substrate specificities, or improved stabilities) for industrial applications [102]. One of the most common organisms used for recombinant expression of laccases is the yeast *Saccharomyces cerevisiae*. Indeed yeasts show fast-growing on simple medium and a high frequency of homologous recombination that facilitates genetic manipulation. Other yeasts, as *Pichia pastoris,* can be used to obtain over-production of these enzymes. In fact, level of expression in *P. pastoris* is 10-100 times higher than that of other expression systems. Moreover, it is characterized to easy genetic manipulation and the ability to perform higher eukaryotic protein modification [123]. These features make *P. pastoris* very useful as a protein expression system [124]. On the other hand, it is possible to increase enzymatic production using as expression host filamentous fungi as *Aspergillus niger*, a particular suitable fungus that shows the capability to secrete large amounts of heterologous proteins into growth medium [125].

However, two major obstacles hamper the use of enzymes in industrial bioprocesses: their sensitivity to various environmental denaturants such as salts, solvents, and proteolytic enzymes and the difficulty of retaining the enzyme in a continuous flow bioreactor. These obstacles make the use of enzymes a costly alternative. To enhance the industrial applicability of enzymes, including the improvement of their stability and their repeated utilization, substantial efforts have been made to immobilize them in the presence or in the absence of a solid support. Well-known strategies to immobilize enzymes are followed reported:

- **Carrier-binding method** is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. The carrier-binding method can be further sub-classified into: ***physical adsorption***-based on the physical adsorption of enzyme protein on the surface of water-insoluble-, ***ionic binding***-relied on the ionic binding of the enzymatic protein to water-insoluble carriers containing ion-exchange residues-, ***covalent binding***-based on the binding of enzymes and water-insoluble carriers by covalent bonds.
- **Cross-linking method** is centred on the formation of covalent bonds between enzyme molecules, by means of bi- or multi-functional reagent, leading to three-dimensional crosslinked aggregates. The most common reagent used for cross-linking is glutaraldehyde.
- **Entrapment method** of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane.

Immobilization technology creates exciting new opportunities for commercial development and profits in a wide range of industrial sectors including, healthcare and medicine, agriculture and forestry, fine and bulk chemicals production, food technology, fuel and energy production, pollution control and resource recovery. Over the past ten years, immobilization of enzymes has been the subject of increased interest, and a number of papers on potential applications of immobilized enzymes

have been published [126; 127]. In bioremediation field, laccases are immobilized to eliminate xenobiotics using continuous stirred membrane reactor [128]. In this frame, benefits in terms of cost, and especially, improving enzyme performances under optimal process reaction conditions (e.g., higher activity and stability at extreme pHs, elevated temperatures or in organic solvents) are obtained.

4. Fungi

Recently, growing interest in white-rot fungi (WRF) is arisen. WRF are able to degrade lignin, a complex natural compound, which forms the hard cover that protects soft wood, by nonspecific extracellular enzymes (e.g. laccases). In addition, they show the capacity to degrade different xenobiotics [96; 129; 130]. The removal performances of xenobiotics by white-rot fungi depend on various factors including the chemical structure of the considered micropollutants, fungal species and their specific enzymes, culture medium, and methods to enhance fungal degradation capacity [131]. In this context, three different WRF were selected: *Pleurotus ostreatus, Phanerochaete chrysosporium* and *Trametes versicolor.* These fungi have showed efficient decolouration capability of synthetic anthraquinone and azo dyes [96; 132] making them very interesting for biotechnological applications.
Marco-Urrea and co-workers [133] reported degradation rate of *T. versicolor* higher than degradation rate of *P. chrysosporium* against the same xenobiotic. On the other hand, considering the performances of *P. chrysosporium* and *P. ostreatus* in decolourization processes, different results were obtained. Indeed, *P. chrysosporium* is active in decolorizing direct wastewater model containing dyes with complex-Trisazo, Polyazo, Stilbene- structures, whilst *P. ostreatus* provides decolourization of the acid wastewater model [96].

5. The thesis

Global aim of this project has been the development of biosystems, based on the use of enzymes and/or fungi, for the degradation of endocrine disruptors. With this aim, the project is divided in different steps:

- Selection of endocrine disruptors belonging to different chemical classes, based on information about the amount of molecules discharged per year and their commercial availability.
- Analyses of the oxidative capabilities of different laccases against xenobiotics -single EDCs and/or their mixture-, also in the presence of both synthetic and natural mediators. Optimization of reaction conditions by enzyme immobilization.
- Analyses of the degradative capabilities of different fungal species against selected xenobiotics; selection of better performing fungi and analyses of their degradative capabilities against mixture of EDCs.
- Screening of aquatic ascomycetous fungi for their ability to grow in the presence of EDCs*.
- Isolation of new fungal species growing on EDCs and their characterization*.

*Work carried out in collaboration with Dr. Dietmar Schlosser at the Helmholtz Centre for Environmental Research, Leipzig (Germany)

In this context, this work has been organised in the following sections:

1. Application of laccases for the degradation of endocrine disrupting chemicals:

Taking into account the role played by laccases in bioremediation processes, POXA1b laccase from *P. ostreatus* and its variant, 1H6C, were recombinantely expressed in the same host and fully characterised. Furthermore, their degradative capabilities together with those of other laccases were analysed against selected EDCs.

2. Application of fungal biosystems for the treatment of endocrine disruptors:

Different kinds of biosystems based on the white-rot fungi *P. ostreatus, P. chrysosporium* and *T. versicolor* have been developed and tested for the biodegradation of EDCs.
Investigation about degradative capabilities of aquatic fungi was also performed, and new fungal species were isolated considering their ability to growth on EDCs.

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Section 1

Application of laccases for the degradation of endocrine disrupting chemicals

Chapter 1

Enzymatic characterization of POXA1b laccase from *P. ostreatus* and its variants

Introduction

Laccases have been intensely studied for their potential uses in industrial processes. They generally work under mild conditions: room temperature and atmospheric pressure. For a feasible industrial use of these enzymes, it is important to obtain both enhanced expression levels and also to produce new engineered laccases with improved properties such as a higher redox potential, optimal activity at neutral or alkaline pH, and thermostability.

Aim of this work has been the overproduction, structural and chemical-physical characterization of two fungal laccases, POXA1b and 1H6C. The former is high redox potential laccase (+650 mV), highly stable in the pH interval of 7 to 10. The latter is a POXA1b variant, obtained through random mutagenesis.

Both laccases have been heterologously expressed in the filamentous fungus *Aspergillus niger.* Productions yields obtained for the two enzyme- 35,000 U/L for rPOXA1b and 60,000 U/L for 1H6C- are among the highest production levels obtained for laccase expression in this and other hosts. Both recombinant enzymes were purified to homogeneity, and theirs biochemical and catalytic properties were investigated. The mutant enzyme shows higher redox potential in comparison to POXA1b.

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Effective mutations in a high redox potential laccase from *Pleurotus ostreatus*

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Abstract Since the first report on a laccase, there has been a notable development in the interest towards this class of enzymes, highlighted from the number of scientific papers and patents about them. At the same time, interest in exploiting laccases—mainly high redox potential—for various functions has been growing exponentially over the last 10 years. Despite decades of work, the molecular determinants of the redox potential are far to be fully understood. For this reason, interest in tuning laccase redox potential to provide more efficient catalysts has been growing since the last years. The work herein described takes advantage of the filamentous fungus *Aspergillus niger* as host for the heterologous production of the high redox potential laccase POXA1b from *Pleurotus ostreatus* and of one of its in vitro selected variants (1H6C). The system herein developed allowed to obtain a production level of 35,000 U/L (583.3 μkat/L) for POXA1b and 60,000 U/L (1,000 μkat/L) for 1H6C, corresponding to 13 and 20 mg/L for POXA1b and 1H6C, respectively. The characterised proteins exhibit very similar characteristics, with some exceptions regarding catalytic behaviour, stability and spectro-electrochemical properties. Remarkably, the 1H6C variant shows a higher redox potential with respect to POXA1b. Furthermore, the spectro-electrochemical results obtained for 1H6C make it tempting to claim that we spectro-electrochemically determined the redox potential of the 1H6C T2 site, which has not been studied in any detail by spectro-electrochemistry yet.

Keywords Protein mutagenesis · Electrochemistry · Electron paramagnetic resonance (EPR) · Recombinant protein expression · Filamentous fungus

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Introduction

Laccase (*p*-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) is an enzyme able to oxidise a broad spectrum of substrates using only oxygen to work and producing water as the only by-product. Interest towards exploitation of laccase as a green tool for several applications is notably increasing, thus making it one of the 'greenest' enzymes of the twenty-first century (Maté et al. 2010).

Laccases couple the four single electron oxidations of the reducing substrate to the four electron reductive cleavage of the dioxygen bond, using four Cu ions distributed among three sites, defined according to their spectroscopic properties (Giardina et al. 2010). Typical metal content of laccases includes one type 1 (T1) copper (Cu1) and one type 2 (T2) and two type 3 (T3) copper ions (Cu2 and Cu3), with Cu2 and Cu3 arranged in a trinuclear cluster (TNC). Laccases are widely distributed in nature, and laccase-like enzymes have been found in insects, bacteria, plants and fungi (Giardina et al. 2010).

Since the first report on a laccase from *Rhus vernicifera* (Yoshida 1883), there has been a notable development in the interest towards laccases, highlighted from the increasing number of scientific papers and patents about this enzyme (Rodgers et al. 2010). At the same time, due to its broad

substrate specificity, interest in exploiting laccases—mainly high redox potential—for various functions has been growing exponentially over the last 10 years, either in the bioremediation field or in biotechnological applications (Lomascolo et al. 2011; Piscitelli et al. 2010). Actually, only fungal laccases are exploited in industrial applications thanks to their high redox potential compared to that of laccases obtained from other sources. For this reason, interest in laccase heterologous expression in different hosts and in tuning its redox potential to provide high yields of more efficient catalysts has been rising since the last decades (Rodgers et al. 2010). As a matter of fact, laccases from fungal sources have been heterologously expressed in different hosts and their properties have been optimised to meet different industrial needs, even if the molecular determinants of the redox potential are to be fully understood yet (Piscitelli et al. 2010; Camarero et al. 2012).

Despite having very similar structures, laccases differently modulate their redox potential, covering a wide range from + 0.430 to +0.800 V (vs. normal hydrogen electrode) (Cambria et al. 2012). The redox potential is a key parameter for substrate specificity since the higher the laccase redox potential is, the wider the range of oxidised substrates. A debate about the origins of these differences was started in the 1990s (Xu et al. 1998, 1999), and several determinants were proposed as crucial ones to redox potential tuning (Li et al. 2004). Among these factors, protein constrains and intra-protein interactions seem to play a major role (Cambria et al. 2012; Garavaglia et al. 2004; Li et al. 2004; Piontek et al. 2002).

Among hosts for heterologous expression, filamentous fungi, such as, for example, *Aspergillus niger* and *Trichoderma reesei*, are natural excellent producers of extracellular enzymes and hence are good candidates for the production of recombinant proteins (Demain and Vaishnav 2009; Iwashita 2002; Wang et al. 2005). Indeed, in these two fungi, production yields of 70 mg/L for a laccase from *Pycnoporus cinnabarinus* (Record et al. 2002) and of 230 mg/L for a laccase from *Melanocarpus albomyces* (Kiiskinen et al. 2004) were achieved. Being recognized as a generally recognized as safe (GRAS) organism, *A. niger* is even more an attractive host for production of homologous and heterologous proteins (Ward 2012).

In this work, the high redox potential laccase POXA1b (+ 0.650 V) (Garzillo et al. 2001; Giardina et al. 1999) from *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (type: Florida) (ATCC no. MYA-2306), a suitable candidate for different industrial application, was heterologously expressed in the filamentous fungus *A. niger*. 1H6C, a POXA1b variant obtained through random mutagenesis (Miele et al. 2010), was also expressed in the same host. This variant shows five amino acidic substitutions (K37Q, K51N, L112F, V148L and P494T), probably involved in conformational changes of the reducing substrate binding site (Miele et al. 2010). This variant, showing an increased decolourization ability towards coloured wastewater model with respect to POXA1b (Piscitelli et al. 2011), represents a good candidate for colourized wastewater bioremediation. The opportunity to obtain high yields of production of these enzymes opens the possibility of its real exploitation.

This article reports the optimisation of the heterologous production of the two laccases along with a characterisation and a comparison between them. We integrate these results with hypotheses that allow us to get a word into an issue debated for a long time, such as laccase redox potential variation.

Materials and methods

Strains and plasmids

poxa1b and *1h6c* cDNAs were used for the construction of plasmids for the expression of laccases in *A. niger*. The *Escherichia coli* strain Top 10 (F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL*(Str^R) *endA1* λ^-) (Life Technologies, Monza, Italy) was used in all DNA manipulations. For the expression in *A. niger*, *poxa1b* and *1h6c* cDNAs were cloned in the pAN52.4 expression vector, containing the sequence for the signal peptide for laccases secretion of the 24 amino acid glucoamylase (GLA) pre-pro-sequence from *A. niger* (Record et al. 2002). The *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) promoter, the 5′ untranslated region of the *gpdA* mRNA and the *A. nidulans trpC* terminator were used to drive the expression of the laccase encoding sequences. The *A. niger* D15#26 strain (Gordon et al. 2000) is a mutant derived from a protease-deficient strain of AB1.13 (Mattern et al. 1992) which acidifies the culture medium far less and is a strain deficient in oritidine-5′-phosphate decarboxylase (*pyrG*). In a co-transformation experiment, *A. niger* D15#26 was transformed with a mixture of plasmid pAB4-1 (van Hartingsveldt et al. 1987), containing the *pyrG* gene as a selection marker, and the expression vectors containing the laccase cDNAs from *P. ostreatus* (Jacq.: Fr.) Kummer (type: Florida) (ATCC no. MYA-2306).

Vector construction

The GenBank accession number of the sequences of the *P. ostreatus* laccase cDNA *poxa1b* (Giardina et al. 1999) reported in this paper is AJ005018. 1H6C cDNA has been obtained after mutagenesis on the *poxa1b* cDNA (Miele et al. 2010). The mature sequences of both cDNAs were amplified (Takara polymerase, Takara, Otsu, Japan) with (POXA1b-1H6C)/Eco (5′T*TGAATTC***GCGCGCTAGCATTGGGC**

C3′) and (POXA1b-1H6C)/Hind primers (5′TCCGG*CAAG CTT***TCATGCTTTCAATGG**3′), using pUC18 (Roche Applied Science, Milan, Italy) containing *poxa1b* and *1h6c* cDNA as templates in order to insert the cDNAs in frame with the sequence for the *A. niger* glucoamylase (GLA) signal peptide. The cloned PCR products were checked by sequencing. The amplified cDNAs, hydrolysed with *Bss*HII and *Hin*dIII restriction enzymes were then inserted in the expression vector digested with the same enzymes.

Fungus transformation, cultivation and laccase production

Fungal co-transformation was basically carried out as described by Record et al. (2002) using each of the laccase expression vectors and pAB4-1 containing the *pyrG* selection marker, in a 10:1 ratio. Transformants were selected for their ability to grow on a minimum medium plate without uridine. POXA1b expression vector (pAN52.4-A1b) and 1H6C expression vector (pAN52.4-1H6C) were successfully transformed into the filamentous fungus (Record et al. 2002). Co-transformants containing laccase cDNA were tested for laccase production by growing on minimum medium plates supplemented with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). *A. niger* was grown for selection on solid minimal medium (in the absence of uridine) containing 70 mM $NaNO_3$, 7 mM KCl, 11 mM KH_2HPO_4, 2 mM $MgSO_4$, 1 % (*w*/*v*) glucose, 0.1 mM $CuSO_4$, 0.2 mM ABTS and trace elements (1,000× stock; 76 mM $ZnSO_4$, 25 mM $MnCl_2$, 18 mM $FeSO_4$, 7.1 mM $CoCl_2$, 6.4 mM $CuSO_4$, 6.2 mM Na_2MoO_4, 174 mM ethylenediaminetetraacetic acid (EDTA)). Recombinants producing laccase were identified by the appearance of a green zone around the colonies after 7–10 days at 30 °C (Bugg et al. 2011); 1×10^6 spores of the best laccase producing transformants were inoculated into liquid medium (300 mL) containing 70 mM $NaNO_3$, 7 mM KCl, 200 mM Na_2HPO_4, 2 mM $MgSO_4{\cdot}7H_2O$ and glucose 5 % (*w*/*v*). Culture aliquots (1 mL) were daily collected and analysed.

Medium composition was optimised in this work by adding casamino acids 2 g/L and yeast extract 5 g/L. Cultures were monitored for 5 days at 28 °C in a shaker incubator (150 rpm) (Julabo, Seelbach, Germany). pH was adjusted to 5.0 daily by adding 1 M citric acid.

Laccase purification

After 5 days of culture, culture media were harvested on the optimal laccase production day, and cells were sedimented by centrifugation at 10,000 rpm at 4 °C for 20 min. Culture broths were filtered through Whatman sheet and then gauze. Samples were concentrated and dialyzed with the Quix Stand Benchtop (GE Healthcare, Milan, Italy) using ultrafiltration membranes with cut-off 5,000 NMWLC and further concentrated on an Amicon PM-10 membrane (Merck Millipore, Billerica, MA, USA).

Then samples were loaded onto DEAE-Sepharose® Fast Flow column (GE Healthcare, Milan, Italy) equilibrated with 50 mM sodium phosphate buffer, pH 6.0. All fractions with laccase activity were concentrated on an Amicon PM-10 membrane and loaded onto SP Sepharose® Fast Flow column (GE Healthcare, Milan, Italy) equilibrated with 50 mM sodium phosphate buffer, pH 6.0 and eluted with step gradient of 0.5 M sodium chloride in the same buffer. Active fractions were pooled, concentrated and dialyzed.

Protein determination and electrophoresis

Protein concentration was determined using the BioRad Protein Assay (Bio-Rad Laboratories, Segrate (MI), Italy), with BSA as standard. The protein homogeneity was checked by electrophoresis on SDS/polyacrylamide gel (10 % polyacrylamide).

Assay of laccase activity

Laccase activity was assayed at 25 °C by monitoring the oxidation of ABTS at 420 nm ($\varepsilon_{420}=36\times10^3$ M^{-1} cm^{-1}) (Childs and Bardsley 1975). The assay mixture contained 2 mM ABTS in 0.1 M sodium citrate buffer (pH 3.0).

Laccase activity towards 2,6-dimethoxyphenol (DMP) was assayed in a mixture containing 1 mM DMP in McIlvaine's citrate phosphate buffer adjusted to pH 5.0. Oxidation of DMP was followed by an absorbance increase at 477 nm ($\varepsilon_{477}=14.8\times10^3$ M^{-1} cm^{-1}) (Martínez et al. 1996).

K_M values were estimated using the software GraphPad Prism (GraphPad Software, La Jolla, CA, USA; http://www.graphpad.com) on a wide range of substrate concentrations (0.05–3 mM). Enzyme activity was expressed in international units (IU).

Effect of temperature

The effect of temperature on laccase activity towards ABTS was evaluated in the temperature range of 20–95 °C in 50 mM Na phosphate buffer adjusted to pH 6.0. The activity was assayed as previously described.

Stability at pH and temperature

Phenol oxidase stability at 65 °C was measured in 50 mM Na phosphate buffer adjusted to pH 6.0. Stability at different pH values was measured using McIlvaine buffer adjusted at pH values 3.0 and 5.0, 50 mM sodium phosphate buffer adjusted at pH 7.0 and 50 mM Tris–HCl buffer adjusted at pH 9.0 at room temperature.

Spectroscopic analysis

Circular dichroism (CD) spectra were recorded on a Jasco spectropolarimeter J-715 (Jasco Corporation, Cremella (LC), Italy) using 0.1 cm quartz cuvettes. Far-UV CD (205–270 nm) spectra were recorded at an enzyme concentration of 0.2 mg/mL in 50 mM potassium phosphate buffer, pH 6.0 at 65 °C.

Fluorescence measurements were performed on a Perkin-Elmer luminescence spectrometer LS 50B (Perkin-Elmer, Waltham, MA, USA). The excitation wavelength was set at 280 nm and the emission spectra were recorded from 300 to 500 nm. 1-Anilino naphthalene-8-sulfonate (ANS) (0.05 mM) binding was studied measuring the fluorescence emission spectra (from 400 to 600 nm) of the probe using a 350-nm excitation wavelength. All experiments were carried out at 65 °C with protein concentrations of 0.2 mg/mL in 50 mM potassium phosphate buffer, pH 6.0. All spectra were corrected by blank subtraction.

Continuous Wave (CW) X-band (9.4 GHz) Electron Paramagnetic Resonance (EPR) measurements were carried out with a Bruker E500 Elexsys Series using the Bruker ER 4122 SHQE cavity (Bruker, Milan, Italy) and an Oxford helium continuous flow cryostat (ESR900) (Oxford Instruments, Mannheim, Germany). EPR solutions had a final concentration of 4.5 mg/mL in 2-(*N*-morpholino)ethanesulfonic acid (MES) 20 mM pH 6.0. The EPR spectra were recorded at 40 K; ν=9.39 GHz, 0.5 mT modulation amplitude and 20 mW microwave power.

The amplitudes of both experimental and simulated spectra were normalized and fitted together to minimize the root mean square deviation between experimental and simulated spectra. The EPR spectra simulations were performed by Easy spin software package using the pepper function as the spectra are in a frozen state (Stoll and Schweiger 2006). Best fitting optimisation was carried out using the procedure combining simplex, Monte Carlo and Levenberg-Marquardt algorithms (Della Lunga and Basosi 1995; Della Lunga et al. 1994, 1998).

Cyclic voltammetry and spectro-electrochemical analyses

Cyclic voltammograms were obtained either with a planar Au electrode or with a screen-printed electrode with low-temperature curing gold ink (DRP-250BT, DropSens, Asturias, Spain). In the first case, an Ag/AgCl (NaCl 3 M) was used as the reference electrode and a Pt wire as a counter electrode. In the second case, the working (4 mm diameter) electrode is made of porous gold, counter electrode is made of platinum and reference electrode is made of silver; in the experiments with the screen printed electrode, the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ couple ($E°$=+0.430 V vs. normal hydrogen electrode (NHE)) has been used as internal reference. All potentials are referred to NHE. UV/Vis spectro-electrochemical measurements were carried out with a Perkin-Elmer Lambda 2 UV/Vis spectrophotometer (Perkin-Elmer, Waltham, MA, USA) and an optically transparent thin layer spectro-electrochemical (OTTLE) cell in quartz glass with an optical path length of 1 mm, equipped with a platinum minigrid working electrode, a Pt auxiliary electrode and an Ag/AgCl (NaCl 3 M) reference electrode. A nitrogen-saturated water solution of the enzymes was used with either MES (20 mM, pH 6.0) or sodium phosphate (50 mM, pH 6.0) used as both buffer and supporting electrolyte. In all the experiments, a BAS 100 A electrochemical analyser (BASi Corporate, West Lafayette, IN, USA) was used as a polarizing unit. Spectro-electrochemical redox titrations have been performed either by monitoring in the time a fixed wavelength during the potential scan or by repeatedly acquiring the whole spectrum during the potential scan. In both cases, the potential scan rate has been 2 mV/s.

Enzymatic hydrolysis

Both proteins were dissolved in denaturing buffer (Tris 300 mM pH 8.0, urea 6 M, EDTA 10 mM), and disulphide bridges were reduced with 1,4-dithiothreitol (DTT) (tenfold molar excess on the Cys residues) at 37 °C for 2 h and then alkylated by adding 2-iodoacetamide (IAM) (fivefold molar excess on thiol residues) at room temperature for 30 min in the dark. Protein sample was desalted by size exclusion chromatography on a Sephadex G-25 M column (GE Healthcare, Milan, Italy). Fractions containing protein were lyophilized. Lyophilized fractions were dissolved in 10 mM ammonium bicarbonate (AMBIC) buffer pH 8.0. Enzymatic digestion was performed using both trypsin (Sigma-Aldrich, Milan, Italy) and V8 (Sigma-Aldrich, Milan, Italy) protease using an enzyme/substrate ratio of 1:50 (*w*/*w*) at 37 °C for 16 h.

Enzymatic deglycosylation

Digested samples were subjected to enzymatic treatment for N-deglycosylation by using PNGaseF (Roche Applied Science, Milan, Italy). Five EU of PNGaseF were added to each sample dissolved in 10 mM AMBIC buffer pH 8.0. Reaction was carried out at 37 °C for 16 h. After deglycosylation, the peptide fraction and the oligosaccharide fraction were separated by RP-HPLC (Agilent Technologies Italia, Cernusco (MI), Italy) on a C18 column (Grace Vydac, Hesperia, CA, USA), via a flow of 0.5 μL/min, with a 0 to 95 % linear gradient in 10 min (A solvent—0.1 % trifluoroacetic acid, 0.07 % acetonitrile in MilliQ water; B solvent—0.1 % trifluoroacetic acid, 0.07 % MilliQ water in acetonitrile).

Mass spectrometric analyses

MALDI–MS experiments were performed on a Voyager-DESTR MALDI/TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) equipped with a nitrogen laser (337 nm). The analyses were performed in positive mode, in the reflector mode. One microliter of peptide mixture was mixed (1/1 *v*/*v*) with a 10-mg/mL solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/50 mM citrate buffer, 70/30 (*v*/*v*). The spectra were acquired using a mass (*m*/*z*) range of 400–5,000 atomic mass unit (amu). For oligosaccharide mixture, 1 μL was mixed (1/1 *v*/*v*) with a 50 mg/mL solution of dihydroxybenzoic acid in acetonitrile/H_2O citrate buffer, 10/90 (*v*/*v*). Spectra were acquired using a mass range (*m*/*z*) of 500–1,000 amu. All acquisitions were generated automatically in the instrument software and based on averaging 5,000 shots per spectrum.

Bisphenol A degradation

Bisphenol A (BPA) (0.1 mM) was incubated in reaction mixture containing 1.5 U/mL of purified laccase in 50 mM sodium citrate buffer (pH 5.0) at 25 °C. The reaction mixture was incubated for 1 h and the remaining amount of BPA was quantified at intervals by reverse-phase HPLC.

High-performance liquid chromatography

BPA was quantitatively analysed using a C18 column (Grace Vydac, Hesperia, CA, USA) on a HPLC instrument (Agilent Technologies Italia, Cernusco (MI), Italy). The fractions were eluted by using a linear gradient of water-acetonitrile at a flow rate of 1 mL/min. The gradient program was 0–3 min (acetonitrile 30 %), 3–9 min (acetonitrile 30–90 %), 9–12 min (acetonitrile 90 %), 12–13 min (acetonitrile 90–30 %) and 13–15 min (acetonitrile 30 %). The eluted sample was monitored by UV absorbance at 227 nm. The retention time of BPA standard was 6.9 min under these conditions. The peak area on the chromatogram was used to calculate the remaining amount of BPA as a percentage of the initial value.

Results

Heterologous production and purification of recombinant laccases from *A. niger*

In co-transformation experiments (Record et al. 2002), *A. niger* D15#26 was transformed with a mixture of plasmid pAB4-1 and one of the two expression vectors containing the *poxa1b* cDNA from *P. ostreatus* or the *1h6c* cDNA. Transformants were selected for their abilities to grow on a minimum medium plate in the absence of uridine. For each construct, approximately 100 uridine prototrophic transformants were obtained per microgram of expression vector. Either POXA1b or 1H6C expression vectors (pAN52.4-A1b and pAN52.4-1H6C) were successfully transformed into the filamentous fungus. Co-transformants containing both laccase-coding cDNAs were tested for laccase activity production by growing on minimum medium plates supplemented with ABTS. Recombinants producing laccases were identified by the appearance of a green zone around the colonies after 7–10 days at 30 °C. Coloured zones on plates were not observed in the case of control transformants lacking the laccase cDNAs. Seventeen and 22 clones were grown in liquid cultures and evaluated for laccase production for POXA1b and 1H6C, respectively. The best producing clones were used to study the time course of laccase production and to characterise the recombinant enzymes.

Laccase production from *A. niger* was analysed in an ad hoc designed medium to individuate the day of maximal production, and a constant increase of secreted activity was observed for both recombinant enzymes with a peak of activity after 5 days of culture. A maximal production of 35,000 and 60,000 U/L for POXA1b and 1H6C, respectively, was obtained.

Purification and characterisation of both recombinant enzymes were carried out from *A. niger* cultures at the day of maximal production. In both cases, a preliminary step of broth scouring was necessary to remove undesirable pigments interfering with laccase activity determination. Laccases were then purified at homogeneity through ionic exchange chromatography. It is worth noting that both enzymes show a very high specific activity towards ABTS, exhibiting values of 2,782 and of 2,865 U/mg for POXA1b and 1H6C, respectively.

Characterisation of recombinant laccases

Kinetics Catalytic parameters of the two recombinant proteins were determined for the non-phenolic ABTS substrate and the phenolic DMP substrate. As far as ABTS is concerned, both POXA1b and 1H6C show an improved affinity towards this substrate with respect to the native enzyme (Giardina et al. 1999), comparable with the affinity of the same protein expressed in other recombinant systems (Table 1) (Festa et al. 2008; Piscitelli et al. 2005). On the other hand, catalytic turnover of both proteins, towards ABTS, is comparable with that of the native protein. As far as DMP is concerned, a decrease of both POXA1b and 1H6C affinity towards this substrate was revealed, as already reported for the protein expressed in other yeast systems (Festa et al. 2008; Giardina et al. 1999; Piscitelli et al. 2005). POXA1b and 1H6C catalytic turnover towards this substrate is highly improved with respect to the native enzyme (Giardina et al. 1999), as already observed for POXA1b expressed in *Kluyveromyces lactis* (Piscitelli et al. 2005) (Table 1).

Table 1 Catalytic parameters of recombinant and native laccases

Laccases	ABTS		DMP	
	K_M (mmol l^{-1})	K_{cat} (min^{-1} 10^4)	K_M (mmol l^{-1})	K_{cat} (min^{-1} 10^4)
1H6C[a] (produced in *A. niger*)	0.07±0.01	8.0±0.1	0.42±0.04	10.0±0.1
POXA1b[a] (produced in *A. niger*)	0.08±0.01	9.0±0.1	0.63±0.06	14.0±0.1
POXA1b[b] (produced in *S. cerevisiae*)	0.09±0.01	ND	0.54±0.02	ND
POXA1b[c] (produced in *K. lactis*)	0.04±0.01	9.0±1.2	0.29±0.04	5.5±0.7
POXA1b[c] (produced in *P. ostreatus*)	0.47±0.06	9.0±1.7	0.26±0.09	1.5±0.1

ND not determined
[a] This work
[b] Festa et al. (2008)
[c] Piscitelli et al. (2005)

Physico-chemical analyses Laccase stability has been analysed as a function of pH and temperature. No significant difference between POXA1b and 1H6C behaviour has been observed both at acid and neutral pH values. The only variation has been noticed at alkaline pH values where 1H6C shows a twofold enhanced stability in comparison with POXA1b. As far as the effect of temperature is concerned, both enzymes show similar characteristics, with the maximum of activity lying within the range 40–55 °C.

Redox potential Spectro-electrochemical redox titration confirmed the redox potential of +0.650 V vs. NHE for recombinant POXA1b, and this value is the same of that previously observed for the *P. ostreatus* POXA1b enzyme (Garzillo et al. 2001) (Fig. 1a). The same redox potential value has been determined by cyclic voltammetry and square wave voltammetry (SWV) by using a bare planar gold electrode (Fig. 1b). The fact that the same potential has been obtained with either an indirect technique, by using mediators (Garzillo et al. 2001) or by using a bare unmodified electrode, comes out in favour of direct electron transfer by the T1 centre to the electrode and supports the retention of the enzyme conformation at the electrode surface (Shleev et al. 2005a).

The spectro-electrochemical redox titration of 1H6C has been performed by following the spectral changes in the wavelength range of 200–1,000 nm by scanning the potential from +1.200 to 0 V (Fig. 2a). As shown in Fig. 2a and its inset, the electrochemical reduction of 1H6C makes the band at

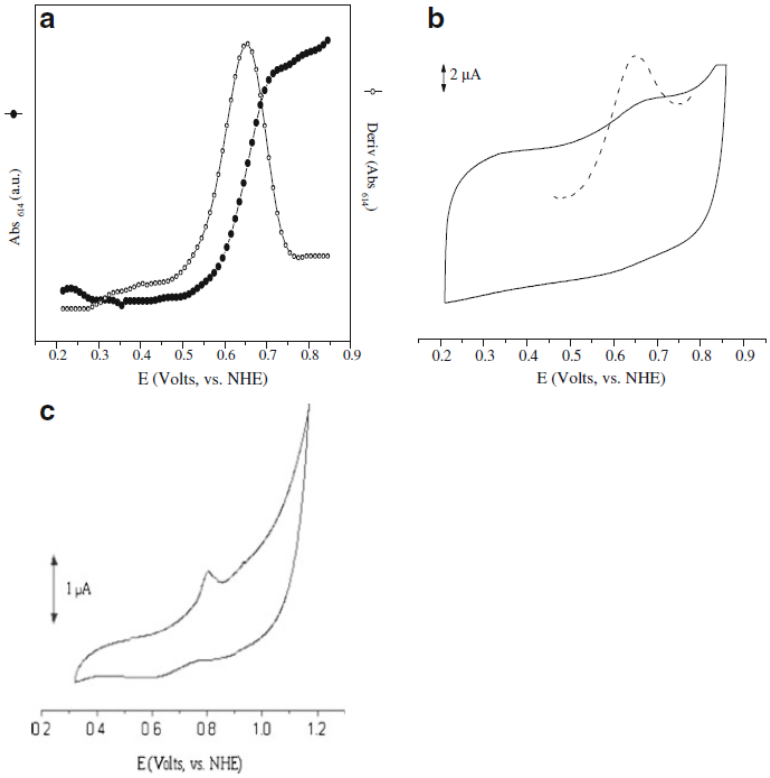

Fig. 1 a Dependence of absorbance (*full circle*) and its derivative (*empty circle*) of POXA1b at 614 nm vs. the applied potential. Potential range +0.850 to +0.200 V vs. NHE; *a.u.* arbitrary units. **b** Cyclic voltammetry of a water solution of 1.5 mg POXA1b (1.5 mg/mL) in 50 mM sodium phosphate buffer, pH 6. Working electrode planar gold, scan rate 50 mV/s. **c** Cyclic voltammetry of a water solution of 2 mg 1H6C (2.5 mg/mL) in 50 mM sodium phosphate buffer, pH 6. Working electrode gold-based screen-printed with low T curing ink DRP-250BT. Scan rate 50 mV/s

616 nm to progressively disappear, as expected. The growing of a band at 343 nm and of another small one at 423 nm are two unexpected changes of the spectra accompanying the electrochemical reduction of 1H6C, as evident from Fig. 2a. The absorbance (616 nm) vs. the applied potential plot allows to establish $E°$=+0.770 V vs. NHE as redox potential of the T1 site (Fig. 2b). This value has been substantially confirmed by cyclic voltammetry by using a commercial electrode screen-printed with low-temperature curing gold ink (Fig. 1c). In these experimental conditions, the anodic peak potential is +0.370 V vs. $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ and + 0.800 V vs. NHE.

Surprisingly, as shown in Fig. 2c, the band at 343 nm continues to grow also by switching the redox potential and reoxidising the 1H6C enzyme. On the other side, reoxidising makes the band at 423 nm disappear while the band at 616 nm grows back, even if in this case the original intensity is not fully recovered, as it is apparent by the comparison with the initial spectrum in Fig. 2c. As for the band at 423 nm, to the best of our knowledge, its origin is presently unknown. Anyway, it clearly exhibits a monotone correlation with the potential, i.e. it progressively grows upon reduction and disappears upon oxidation. For this reason, and in spite of the ill-defined features and the weakness of this band, we made an attempt to monitor its absorbance vs. the applied potential plot, as shown in Fig. 2b, obtaining that the maximal rate of spectral change corresponds to +0.360 V vs. NHE. This value is very close to the redox potential of the T2 site, expected at ~+0.400 V vs. NHE in many multicopper oxidases and considered to be almost invariant in the different enzymes because of the conservation of the structure of the T2 redox centres in terms of ligands and copper–ligand distances (Shleev et al. 2005a, b).

Spectroscopic analyses In order to analyse the possible conformational changes induced by mutations, we have used far-UV CD and fluorescence techniques. In circular dichroism studies, both laccase structures have been analysed after incubation at 65 °C at neutral pH. Both laccases show a stable structure, since no significant alterations have been observed, even after 6 h of incubation. On the other hand, both enzymes lost half of their activity after only just 1 h of incubation at 65 °C (data not shown).

Intrinsic and extrinsic fluorescences of both laccases were also examined with the aim to get insight into protein tertiary structure behaviour after incubation at high temperature (65 °C). POXA1b shows an increased exposure of internal tryptophan residues (Fig. 3a), indicating a progressive loosening of its tertiary structure. A similar behaviour is also evident when analysing 1H6C emission spectra (Fig. 3b); however, higher emission intensity, after 3 h of incubation, was observed with respect to POXA1b emission spectrum, thus indicating a higher tendency of 1H6C to expose

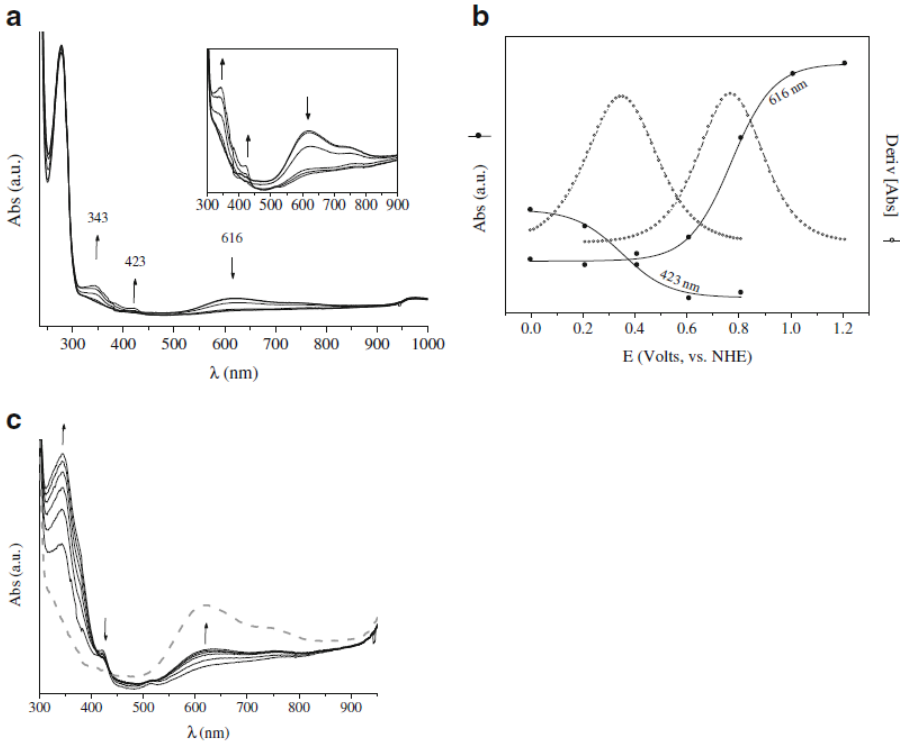

Fig. 2 **a** Absorbance spectra recorded during the reduction of 1H6C in the OTTLE cell. Potential range +1.200 to 0 V vs. NHE. **b** Dependence of absorbance (*full circle*) and its derivative (*empty circle*) of 1H6C at 616 and 423 nm vs. the applied potential. Potential range +1.200 to 0 V vs. NHE. **c** Absorbance spectra recorded during the reoxidation of 1H6C in the OTTLE cell. Potential range 0 to +1.200 V vs. NHE; *a.u.* arbitrary units

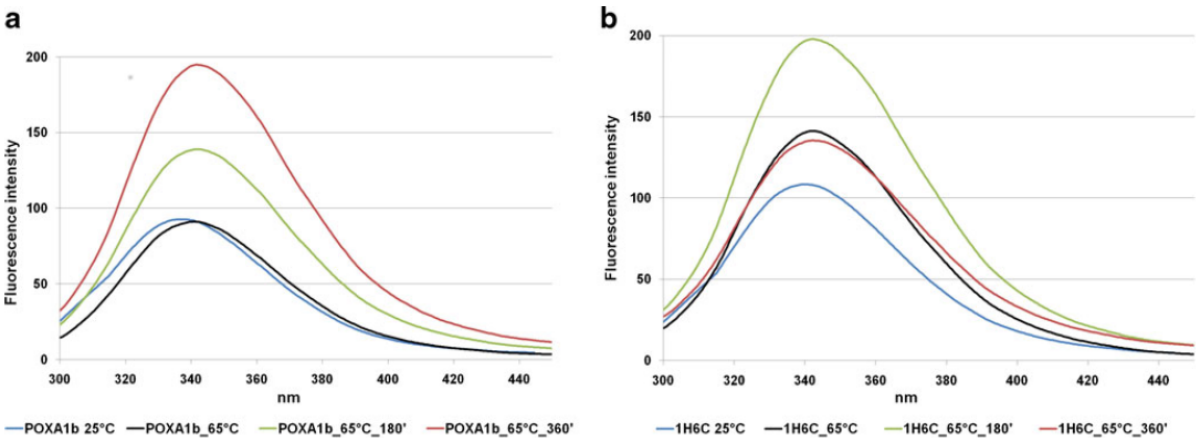

Fig. 3 Dependence of the fluorescence emission maximum wavelength on the time of incubation at 65 °C. **a** POXA1b. **b** 1H6C

tryptophan residues. Furthermore, in 1H6C spectra, it is possible to note an emission decrease after 6 h of incubation (Fig. 3b), possibly due to intrinsic quenching within the protein. Protein extrinsic fluorescence was studied in the presence of ANS, a fluorophore which increases its fluorescence upon non-covalently binding to hydrophobic regions of proteins. By increasing the time of incubation, the fluorescence intensity increases of about seven- and sixfold for POXA1b and 1H6C, respectively (data not shown), thus confirming a tendency to expose hydrophobic residues with time increasing.

Low-temperature EPR spectra of both proteins have been recorded in order to better understand the structure and function of T1 and T2 Cu(II) centres. Figure 4 shows the continuous wave X-band EPR spectra of POXA1b in black and 1H6C in grey, overlapped with the best simulation (dotted line). Both EPR spectra have been simulated using a unique set of magnetic parameters. Therefore, it is possible to infer that the differences between the two experimental spectra are not so evident. For the T1 centre, the g and the hyperfine coupling constant are as follows: $g_{\|}$=2.195, $A_{\|}$=87 cm^{-1}, $g_{\perp}$=2.044; and for T2: $g_{\|}$=2.21, $A_{\|}$=204 cm^{-1}, $g_{\perp}$=2.048.

Mass spectral analyses Recombinant proteins were submitted to an accurate structural characterisation, both at the protein and the carbohydrate level by exploiting MALDI mass spectrometric methodologies. A protein sample was reduced, alkylated and submitted to trypsin digestion; the resulting peptide mixture was deglycosylated by PNGaseF treatment and directly analysed by MALDI/MS. The mass spectral analysis of POXA1b and 1H6C led to the verification of about 77.3 and 78.9 % of the entire protein sequence, respectively. Some of the tryptic peptides could not be detected in the spectra because of the well-known suppression phenomena occurring in MALDI/MS experiments (Knochenmuss and Zenobi 2003). This investigation revealed a series of structural features of the recombinant proteins. On the basis of amino acid sequence, it was possible to predict six N-glycosylation consensus sequences Asn-Xxx-Ser/Thr for the POXA1b

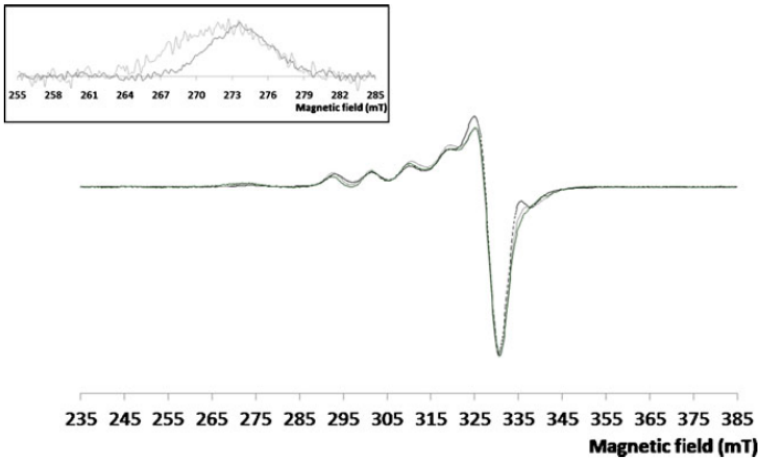

Fig. 4 CW X-band EPR spectra of POXA1b in *black* and 1H6C in *grey* paired with the best simulation (*dotted line*) at 40 K (experimental conditions: ν= 9.39 GHz, 0.5 mT modulation amplitude, 20 mW microwave power). In the *inset*, an enlargement of the T2 first parallel component of the POXA1b and 1H6C experimental spectra

protein, while for 1H6C, the putative N-glycosylation sites were seven because of the mutation P494T. Their occurrence was essentially confirmed by the mass mapping data, with POXA1b showing three sites fully glycosylated and 1H6C two sites, while a partially glycosylated site was mapped for both enzymes. The asparagine residues at positions 342 escaped mass spectral analysis (Table 2).

The intact N-linked oligosaccharides were released from the peptide backbone by PNGaseF treatment of the tryptic digests. The glycan mixture was separated from the peptides by a reverse phase chromatographic step and analysed by MALDI mass spectrometry. The N-linked glycosidic moiety of recombinant proteins constitutes a largely heterogeneous mixture of high mannose type glycans ranging from 6 to 59 U of mannose for POXA1b and from 6 to 50 U of mannose for 1H6C (data not shown).

BPA degradation

POXA1b and 1H6C were also compared in terms of their ability to oxidise the well-known endocrine compound BPA (Sajiki and Yonekubo 2003). The results obtained after incubation and HPLC analysis showed that 1H6C is able to degrade BPA more efficiently than POXA1b (13 vs. 3 % degradation).

Discussion

The characterisation of high redox potential fungal laccases POXA1b and 1H6C required their recombinant expression. Indeed, the filamentous fungus *A. niger* was selected as host to improve the production yield of POXA1b laccase from *P. ostreatus* ($E°$=+0.650 V) (Garzillo et al. 2001; Giardina et al. 1999). 1H6C, a POXA1b variant previously obtained through random mutagenesis (Miele et al. 2010), was also expressed in the same host. This variant shows five amino acidic substitutions (K37Q, K51N, L112F, V148L and P494T), probably involved in conformational changes of the reducing substrate binding site (Miele et al. 2010).

Table 2 Mass spectral analysis carried out on the tryptic peptide mixture

Predicted N-glycosylation site	POXA1b	1H6C
Asn201	Full glycosylated	Full glycosylated
Asn294	Full glycosylated	Full glycosylated
Asn342	ND	ND
Asn434	Partially glycosylated	Partially glycosylated
Asn470	Not glycosylated	Not glycosylated
Asn490	Full glycosylated	Not glycosylated
Asn493	–	Not glycosylated

The position of the consensus sequence and the result concerning the site occupancy are reported

ND not determined

The heterologous system developed in this work allowed a maximal production of 35,000 U/L for POXA1b and of 60,000 U/L for 1H6C, with both enzymes showing a very high specific activity towards ABTS (2,782 U/mg for POXA1b and of 2,865 U/mg for 1H6C), corresponding to a production yield of 13 and 20 mg/L for POXA1b and 1H6C, respectively. Considering that POXA1b production from *P. ostreatus* is around 500 U/L (Garzillo et al. 2001) and that previously optimised recombinant expression systems from *K. lactis* and *Saccharomyces cerevisiae* yielded, respectively, an amount of 4,200 and 200 U/L (Piscitelli et al. 2005), this new developed system in *A. niger* allows to noticeably increase the production of this enzyme up to almost 175-fold. The yield is even doubled when the 1H6C variant is produced. Enzymatic production levels herein obtained for the two laccases are comparable to those obtained for laccases in this and other hosts taking into consideration the high specific activity of these enzymes. As a matter of fact, when laccases from *Cryphonectria parasitica* (Kwon et al. 2009) and from *P. cinnabarinus* (Daly and Hearn 2005) were expressed in *A. niger*, an activity of 6,000 and 8,400 U/L, respectively, was obtained. In addition, production levels reported for the expression of the ascomycete *M. albomyces* laccase in *T. reesei* are among the highest heterologous laccase expression levels reported so far, allowing to obtain 230 mg/L in shaken flask cultures, 290 mg/L in batch fermentations and 920 mg/L in fed batch fermentation (Kiiskinen et al. 2004), corresponding to about 47,000 U/L. An even higher expression yield was achieved when the *Trametes versicolor* laccase IV was expressed reaching a level of 800–1,000 mg/L (Baker and White 2000). Expression yields of fungal laccases in yeasts are generally lower, with only a few reported exceptions. A laccase from *Botrytis aclada* was recently expressed in *Pichia pastoris* with a yield of 517 mg/L (53,300 U/L) (Kittl et al. 2012) using a constitutive promoter. In the same host, after an optimised fermentation strategy, Hong et al. (2002) reported a production of 140,000 U/L of a laccase from *Trametes* sp. An astonishing production of 239,000 U/L was obtained for a *Trametes* spp. laccase (Cui et al. 2007).

Both POXA1b and 1H6C laccases produced by *A. niger* were purified to homogeneity, and their biochemical and catalytic properties were investigated and compared. Both enzymes show similar characteristics, with some significant exceptions regarding catalytic behaviour towards BPA, stability and spectro-electrochemical properties.

Surprisingly, the specific activities and the kinetic parameters of 1HC6 and POXA1b with both ABTS and DMP are quite similar (Table 1), whereas 1H6C shows better catalytic features when both enzymes were expressed in *S. cerevisiae* (Miele et al. 2010). Even if it would appear that expression of the proteins in *A. niger* obscured any catalytic improvements

of 1H6C, we have to consider that the two systems are not fully comparable. As a fact, when produced in *A. niger*, both enzymes are purified at homogeneity, whereas in *S. cerevisiae*, they are just enriched; thus, we cannot rule out that the two purified proteins would have shown a different behaviour.

Mutations have an effect on the stability of the enzyme's three-dimensional structure, since both intrinsic and extrinsic fluorescences indicate that 1H6C is more inclined to expose hydrophobic residues and/or surfaces in the presence of demanding conditions.

As far as spectro-electrochemical analysis is concerned, two unexpected changes of the spectra accompanying the electrochemical reduction of 1H6C have been observed (Fig. 2a): the growing of a band at 343 nm and of another small one at 423 nm. In multicopper oxidases, absorptions at ~330 nm are ascribed to the hydroxo-bridged T3 Cu(II) dimer in the resting oxidised form. The lack of absorption at this wavelength is associated to the so-called alternative resting form, with dioxo- or oxo-bridged T3 Cu(I). A recent paper (Kjaergaard et al. 2012) demonstrates that the alternative resting form can be converted to the resting oxidised form by reduction-oxidation cycling, as indicated by the appearance of the band at 330 nm, initially absent, after this treatment. Once the conversion has been achieved, the resting oxidised form remains and further redox cycling does not show evidence of the alternative resting form anymore. In fact, these details seem to be appropriate to describe the behaviour we experimentally found, even if the reason for the formation of the oxidised T2/T3 site upon lowering the applied potential remains unclear. An educated guess is that at the starting positive potential, the T3 site is oxidised, and then its oxidation state remains unaltered being the T3 Cu ions deeply buried below the surface of the enzyme. As for the band at 423 nm, to the best of our knowledge, its origin is presently unknown. A pulse radiolysis experiment on *R. vernicifera* laccase (Farver et al. 2011) led to the hypothesis that the formation of a transient RS-S*R^{-} radical anion, with an absorption maximum at 410–420 nm, could be the first step in the T1 reduction mechanism. On the other side, the results obtained monitoring the 423-nm absorbance vs. the applied potential (Fig. 2b) reveals that the maximal rate of spectral change corresponds to a value very close to the redox potential of the T2 site measured in many multicopper oxidases, including the high potential laccase from *Trametes hirsuta* (Shleev et al. 2005a). In the case of the high potential *T. versicolor* laccase, the redox potential of T2/T3 has been also inferred to be lower than that of the T1 site (Ivnitski and Atanassov 2007). The role of such low value of the T2 redox potential is still under study (Shleev et al. 2005b). Nevertheless, it seems that thermodynamically unfavourable tunnelling uphill is not an unprecedented phenomenon in biology (Shleev et al. 2005b). These facts make it tempting to claim that we could also have been able to spectro-electrochemically determine the redox potential of the 1H6C T2 site, which, being unrevealed by UV/vis spectroscopy in its oxidised state, has not been studied in any detail by spectro-electrochemistry, so that the spectral features of its reduced state are not described. Realistically, this fascinating hypothesis has to be taken with care and a more detailed study is unavoidable.

The EPR spectra were very similar for both laccases and allowed to measure the g and the hyperfine coupling constant for the T1 centre, and the values found are in agreement with those of other laccases with a comparable redox potential (Brogioni et al. 2008; Pogni et al. 2007; Solomon et al. 1996). However, considering the magnetic parameters of the T2 centre, an uncommon high value of $A_{\parallel}$ was found (Pogni et al. 2007; Solomon et al. 1996) and an even broader first parallel component of T2 is evident for POXA1b with respect to 1H6C. The inset of Fig. 5 shows an enlargement of the T2 first parallel component of the two experimental spectra. Such broadening might be due to the contemporary presence of two T2 contributions even though no further detail can be provided due to low intensity and lack in resolution of the peak. Despite the two laccases exhibiting similar characteristics, the 1H6C variant shows a higher redox potential with respect to POXA1b, with a difference of +0.120 V.

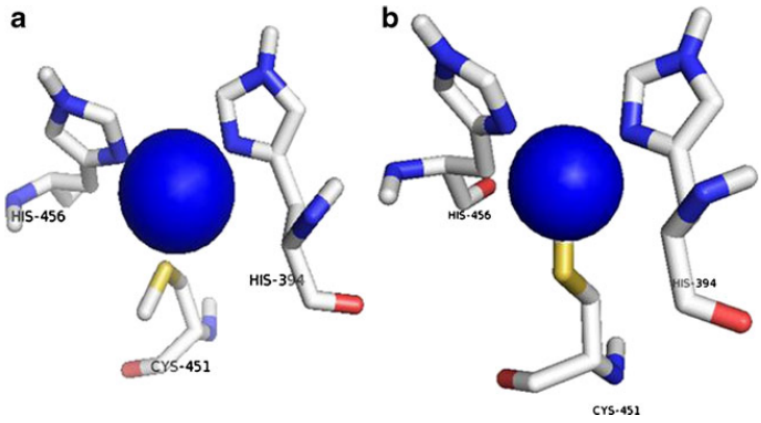

Fig. 5 Close up of T1 close surroundings, elaborated with PyMol (DeLano 2002) from the model. Remarkable amino acid residues are shown as *sticks*; copper is shown as *pale blue sphere*. **a** 1H6C. **b** POXA1b

The mutations, although not directly involved in catalysis, have been proven to influence substrate oxidation, as verified when the two enzymes are compared in terms of their ability to oxidise BPA. If we consider the substitutions present in 1H6C (K37Q, K51N, L112F, V148L and P494T), none of the mutated amino acids is present in the copper coordination sphere. Furthermore, following the debate regarding the determinants proposed as crucial to redox potential tuning, such as protein constrains and intra-protein interactions (Cambria et al. 2012; Garavaglia et al. 2004; Li et al. 2004; Piontek et al. 2002), we would have not been able to foresee the effect of the amino acidic substitution. As a matter of fact, CD spectra of the parent type and 1H6C mutant were recorded in order to compare the secondary structure of the enzymes, but no differences have been found (data not shown). On the other hand, the presence of some subtle perturbations charged to the copper geometry has been theorized on the basis of spectro-electrochemistry results. If we rule out the two substitutions occurring in positions 37 (K37Q) and 51 (K51N) that are generally conserved in laccase sequences and occupied by amidic residues (Festa et al. 2008), the remaining substitutions to consider are L112F, V148L and P494T. As far as L112F is concerned, previous analyses showed a movement of the subdomain around position 112 as a consequence of a conformational rearrangement due to the presence of the bulkier residue of phenylalanine (Festa et al. 2008). The presence of the substitution in position 494, together with the substitution L112F in 3M7C mutant (Festa et al. 2008), lowers the flexibility of that subdomain, while increasing mobility of loops forming the reducing substrate binding site leading to higher accessibility of water molecules to the T1 copper site and possibly leading to an increased activity. Furthermore, position 494 is located in the C-terminal loop that has already been ascertained to affect the function of fungal laccases (Hakulinen et al. 2002). In addition, Zumarraga et al. (2008) recently stated that in ascomycetes laccases, somehow the C-terminal tail exerts a strong influence during processing steps which eventually is affecting on how mature enzyme behaves. In the case of 1H6C, P494T substitution may have affected the observed stability at alkaline pH, in accordance with the results reported by Autore et al. (2009) who demonstrated a role of POXA1b C-terminal tail in affecting enzyme stability properties. Moreover, Gelo-Pujic et al. (1999) have changed the redox potential of the T1 Cu of a laccase from *T. versicolor*, produced in *P. pastoris*, replacing 11 amino acids at the C-terminus with a single Cys residue. Position 148 is located in a closely packed region of the domain 2 adjacent to the reducing substrate binding site (Bertrand et al. 2002). The substitution of V with L, increasing the size of the side chain, could further increase the packing of this region (Miele et al. 2010). Moreover, the close contact of the leucine side chain with the aromatic ring of Y208 could change the conformation of the loop 204–208 forming the bottom of the reducing substrate binding site where the D205 involved in the interaction with the aromatic substrate is located (Bertrand et al. 2002). This change could, in turn, influence the oxidation rate of the reducing substrate but also the interaction between domains 2 and 3. Moreover, it is worth noting that a leucine residue is also present in the same position in the POXC *P. ostreatus* laccase (Palmieri et al. 1993), whose redox potential is +0.760 V (Garzillo et al. 2001), thus very similar to that of 1H6C. Thus, the amino acid substitutions present in 1H6C seem to act in a synergistic way in modifying POXA1b properties.

Substitutions (K37Q, K51N and V148L) were mapped on the 3D model obtained for one of the mutants previously isolated (3M7C) (Festa et al. 2008) and used to highlight changes in T1 close surroundings (Fig. 5). Figure 5 shows a different orientation of the T1 coordinating Cys 451, moving away from copper in 1H6C with respect to POXA1b. This picture is in agreement with the hypothesis first proposed by Piontek et al. (2002), about the distance between the type 1 copper and the coordinating atoms as a key factor for the modulation of the redox potential in laccases.

In conclusion, the unexpected differences between POXA1b and 1H6C laccases coming from diverse techniques (e.g. spectro-electrochemistry and EPR) resounded like an alarm bell persuading us to suppose the presence of subtle perturbations charged to copper geometry in 1H6C variant. Fascinating ideas coming out from these results have to unavoidably become a starting point for further study to progressively understand multicopper oxidase chemistry.

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Chapter 2

Endocrine disruptors degradation by fungal laccases

Introduction

In the last years, high levels of contaminants are found in wastewaters. To remove these xenobiotics from the environment, biodegradation processes can be used. In this frame, oxidative enzymes represent a considerable choice. In particular, among oxidative enzymes, laccases are interesting enzymes, because of their versatility, the possibility to produce them in large-scale, and to modify their properties.

Four different fungal laccases were chosen to test their ability to degrade five different EDCs. Three out of four of the selected enzymes are high redox potential laccases: POXC, POXA1b and 1H6C. The latter two enzymes were heterologously expressed in *Aspergillus niger,* and their characterization has already reported in the first chapter. A commercial laccase was also used.
Laccase oxidative capabilities against selected EDCs were studied in the presence and in the absence of two mediators, ABTS and acetosyringone.

This study also evaluated the effect of oxidative capabilities of better performing enzyme towards the pollutants mixture.
Implementation of its performances against EDCs mixture was achieved through immobilization on glass beads.

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FUNGAL LACCASES DEGRADATION OF ENDOCRINE DISRUPTING COMPOUNDS

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Abstract

Over the past decades, water pollution by trace organic compounds (ng/L) has become one of the key environmental issues in developed countries. This is the case of the emerging contaminants called Endocrine Disrupting Compounds (EDCs). EDCs are a new class of environmental pollutants able to mimic or antagonize the effects of endogenous hormones, and are recently drawing scientific and public attentions. Their widespread presence in the environment solicits the need of their removal from the contaminated sites. One promising approach to face this challenge consists in the use of enzymatic systems able to react with these molecules. Among the possible enzymes, oxidative enzymes are attracting increasing attention because of their versatility, the possibility to produce them in large-scale, and to modify their properties. In this study five different EDCs were treated with four different fungal laccases, also in the presence of both synthetic and natural mediators. Mediators significantly increased the efficiency of the enzymatic treatment, promoting the degradation of substrates recalcitrant to laccase oxidation. The laccase showing the best performances was chosen to further investigate its oxidative capabilities against micropollutant mixtures. Improvement of enzyme performances in Nonylphenol degradation rate was achieved through immobilization on glass beads.

1. Introduction

In the last years assessment and conservation of environmental quality has represented an interesting field of technologic applications. The main problem in industrialized states is represented by a constant and continuous pollution of soil, water-bearing stratum, surface water and air. This is due to the introduction, in the environment, of toxic and dangerous contaminants for many organisms, including humans. In this context Endocrine Disrupting Chemicals (EDCs) play a significant role. EDCs have been found to disturb the endogenous hormone pathway and interrupt the function of hormone receptors via estrogens-mimicking chemicals, resulting in the alteration of physiological functions, such as reproduction and development of different species, including humans [1]. EDCs are found in many products derived from cosmetic industries and working environment [2]. Many natural chemicals (e.g. phytoestrogens, including genistein and coumestrol), found in human and animal food, can also act as endocrine disruptors [2, 3].

Between 2000 and 2006 the European Commission has contracted diverse studies on the identification and evaluation of this class of substances, and a list of substances potentially endocrine disruptor has been drawn up [4]. Efficient and applicable techniques for removing EDCs in wastewater treatment processes remain a challenge of high environmental and public health significance [5]. One promising approach consists in the use of enzymatic systems able to degrade EDCs into non-toxic or easy to remove products [6]. The promise of phenol oxidases (laccases and tyrosinases) and peroxidases for the elimination of EDCs from aqueous solutions has been established over the last few years and is attracting an increasing attention [7, 8]. Nonetheless, the application of enzymes in continuous systems such as wastewater treatment plants remains a challenge as it is limited by their non reusability, the instability of their structures and their sensitivity to harsh process conditions. Many of these undesirable limitations may be overcome by the use of immobilized enzyme. In the immobilized form, enzymes are more robust and more resistant to environmental changes allowing easy recovery and multiple reuses [8].

As a fact, examples referring to treatment of EDCs molecules [9-11], as well as of contaminated synthetic water and municipal wastewater [12] with different fungal

peroxidases, laccases and tyrosinases are present in the recent literature. In all reported cases, estrogenic activities were completely removed. Recent efforts have been focused on the immobilization of bio-catalysts in order to tackle this major limitation and to facilitate their possible reuse [8].
Laccases (p-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) are blue multicopper oxidases, catalysing the oxidation of a broad range of xenobiotics concomitantly with the reduction of molecular oxygen to water. This renders them very attractive compared to other enzymatic systems because no additional/expensive co-substrate or cofactor is required apart from oxygen. These enzymes usually contain four copper ions distributed in three active sites, which are involved in the electron transfer from the substrate (T1 active site) towards oxygen (T2/T3 active sites) [13].
In this project, among various chemical classes, the EDCs bisphenol A (BPA), nonylphenol (NP), methylparaben (MTPRB), buthylparaben (BTPRB) and dimethylphthalate (DMPTL) (Figure 1) have been selected, based on information about their toxicity, the amount discharged per year and their commercial availability. BPA is a high production volume chemical used as an intermediate in the fabrication of polycarbonate plastic and epoxy resins [14]. Due to its daily use, high concentrations of BPA are observed in wastewater and in wastewater sludge (0.004–1.36 mg kg^{-1}). NP is a mixture of para-, ortho-, and meta-isomers, the most prevalent of them is para-NP. It is a viscous, colourless liquid and it is subjected to ethoxylation to give alkylphenol ethoxylates [15]. This compound is very toxic and recalcitrant, thus it shows a high potential to bio-concentrate [16]. Parabens are esters of para hydroxybenzoic acid, widely used as preservatives in food, pharmaceutical and cosmetic industries to prevent bacterial growth [17, 18]. Phthalates are a group of persistent, high production volume chemicals, used for a variety of products, including personal care products (e.g. perfumes, lotions, and cosmetics), varnish, medical devices, pharmaceuticals, solvents, additives, insect repellents [19].
Four different fungal laccases were used in this study to set-up EDCs enzymatic treatment, also in the presence of both synthetic and natural mediators. Three out of four selected enzymes were high redox potential laccases from *Pleurotus ostreatus:* POXC [20, 21], POXA1b [21, 22] heterologously expressed in the filamentous fungus *Aspergillus niger* [23], 1H6C, a POXA1b variant obtained through random mutagenesis [24] and produced in *A. niger* [23]. Finally, a commercial laccase, the Novoprime Base 268 (Novozymes), was also used for enzymatic treatment. Moreover, considering that in the natural environment pollutant mixtures are common, this study also evaluated the effect of the best performing enzyme, both free and immobilized, towards the presence of pollutants mixtures.

2. Materials and Methods

2.1 Organism and culture conditions

The *P. ostreatus* (Jacq.:Fr.) Kummer (type:Florida) (ATCC no. MYA-2306) fungus was maintained through periodic transfer at 4°C on Potato Dextrose Yeast extract (PDY) medium (potato dextrose 24 g/L; yeast extract 5 g/L). Growth were carried out at 28°C in the dark by preinoculating 300 mL of PDY in 500 mL shaken flasks with 6 agar plugs of mycelium grown on solid state on Petri dishes (11 mm diameter). 50 mL of a 5-day-old culture were transferred in 1 L flasks containing 450 mL of PDY broth. Cultures were incubated in the dark at 28°C under agitation (150 rpm).
A. niger D15#26 strain [25] was grown in liquid medium (300 mL) containing 70 mM $NaNO_3$, 7 mM KCl, 200 mM Na_2HPO_4, 2 mM $MgSO_4$ $7H_2O$, glucose 5% (w/v), 2 g/L

casamino acids and 5 g/L yeast extract. pH was adjusted to 5.0 daily by adding 1 M citric acid [23].

2.2 Enzymes

Laccase POXC [20] was purified from *P. ostreatus* with slight modifications in the purification protocol. After 10 days of culture, the medium was collected and filtered through gauze. 1 mM of the serine protease inhibitor, phenylmethanesulfonylfluoride (PMSF), was added to the surnatant. Secreted proteins were precipitated from the filtered medium by addition of $(NH_4)_2SO_4$ up to 80% saturation and loaded on Phenyl Sepharose High Performance 35/100 (GE Healthcare, Milan- Italy). POXC was eluted with a linear gradient of decreasing $(NH_4)_2SO_4$ concentration from 1 M to 0 M. Fractions corresponding to POXC were pooled, equilibrated in buffer 50 mM sodium phosphate (NaP) pH 6.5 and loaded onto a DEAE Sepharose Fast Flow column (GE Healthcare, Milan- Italy) with a linear gradient 0 M to 0.5 M NaCl, and fractions corresponding to POXC were pooled and desalted.

POXA1b and 1H6C were heterologously expressed and purified from *A. niger*, as previously described [23].

Laccase Novoprime Base 268 (Novozymes) was dissolved in 50 mM NaP pH 6.5.

2.3 Assay of enzymatic activity

Laccase activity was assayed at 25°C by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm ($\varepsilon_{420}=36\times10^3$ M^{-1} cm^{-1}). The assay mixture contained 2 mM ABTS in 100 mM sodium citrate buffer, pH 3.0.

Immobilized enzyme activity was assayed incubating 10 mg of glass beads in 1 mL of 2 mM ABTS in 0.1 M sodium citrate buffer (pH 3.0). The activity was determined by measuring the absorbance at 420 nm every 30'' following the reaction for 2 min. Enzymatic units were expressed as U/g.

2.4 Laccase immobilization on glass beads

Glass beads type S (0.4- 0.6 mm diameter) were supplied by Silibeads (Sigmund Lindner GmbH, Germany). Beads were pre-treated with 1.2 M HNO_3 at 60°C for 4 hours and then, extensively washed with water and dried at 60°C. Carrier derivatization was performed as follows: 5 g of dry pre-treated beads were mixed with 10% APTES (□-aminopropyltriethoxysilane, Sigma-Aldrich) in 50 mL distilled water and incubated at 80°C for 2 h under constant mixing. The suspension was then washed thoroughly with 50 mM NaP buffer pH 6.5 and treated with 2.5% glutaraldehyde for 1 h at room temperature. The activated beads were extensively washed with the over cited buffer and finally incubated for 1h with a solution of laccase mixture in 50 mM NaP buffer, pH 6.5 at room temperature. Residual active glutaraldehyde was inactivated by 1 h incubation with 100 mM glycine at room temperature. Immobilization yield (Y) was defined as the ratio between laccase activity assayed on the solid biocatalyst and total activity available in the liquid solution at the beginning of the immobilization processes. A yield of 83% was obtained following this procedure.

2.5 EDCs enzymatic degradation

1 mM stock solution of each EDC (Sigma-Aldrich, Milan- Italy) was prepared in hot water. To improve the solubility of NP and DMPTL in hot water, methanol (0.4% v/v) and Tween 80 (0.1% w/v) were added, respectively. 100 µM of each EDC was incubated for 1 h at 25°C in a reaction mixture containing 1.5 U/mL of purified laccase in 50 mM sodium citrate buffer, pH 5.0, total reaction volume was set to 4 mL. Amounts of EDC were quantified every 30 minutes (t_0, $t_{30'}$, $t_{60'}$) by reverse-phase HPLC. Enzymatic reaction was stopped by adding 50 µL of hydrochloric acid (HCl) to 500 µL of reaction mixture and centrifuging at 15,100 g for 15 min at room

temperature. 100 µL of the surnatant were analysed by HPLC. Degradation of EDCs mixture was performed in the same condition, using a final concentration of 25 µM of each EDC. Thus, the final concentration of EDCs mixture was of 100 µM. Control reactions were performed in the same conditions without enzyme addition. Mediators used were ABTS, dissolved in sodium citrate buffer 50 mM, pH 5.0, and acetosyringone (AS), dissolved in hot sodium citrate buffer, 50 mM, pH 5.0. Concentrations used for both mediators were 20 µM and 200 µM.
Degradation of EDCs mixture by means of immobilized enzyme was performed in the same condition, using an amount of beads corresponding to 6 U total in the presence of 20 □M AS.

2.6 High-Performance Liquid Chromatography

All EDCs were quantitatively analysed using a C18 column (Grace Vydac, Hesperia, California- USA) on a HPLC instrument (Agilent Technologies Italia). The fractions were eluted by using a linear gradient of water-acetonitrile (A solvent 0.1% trifluoroacetic acid in Milli-Q (MQ) water; B solvent 0.07% trifluoroacetic acid, 5% MQ water in acetonitrile) at a flow rate of 1 mL/min. The gradient program for BPA analysis was 0–3 min (acetonitrile 30%), 3–9 min (acetonitrile 30–90%), 9–12 min (acetonitrile 90%), 12–13 min (acetonitrile 90- 30%), and 13–15 min (acetonitrile 30%). The eluted sample was monitored by UV absorbance at 227 nm. The retention time for BPA was 6.9 min under these conditions. As regards as NP, the applied gradient was 0–3 min (acetonitrile 20%), 3–9 min (acetonitrile 20–90%), 9–12 min (acetonitrile 90%), 12–13 min (acetonitrile 90- 20%), and 13–15 min (acetonitrile 20%). The detection wavelength was 277 nm. The retention time for NP was 14.5 min under these conditions. The gradient program for parabens analyses was 0–7 min (acetonitrile 30–70%), 7–8 min (acetonitrile 70–90%), 8–11 min (acetonitrile 90%), 11–12 min (acetonitrile 90- 30%), and 12–14 min (acetonitrile 30%). The detection wavelength was 254 nm. Under these conditions the retention times for MTPRB and BTPRB were 5.8 min and 8.8 min, respectively. As regards as DMPTL, the applied gradient was the same used for parabens, while the detection wavelength was 274 nm. The retention time for DMPTL was 6.8 min under these conditions.
As far as the EDCs mixtures is concerned, each molecule was analysed with its optimised program.
The peak area on the chromatogram was used to calculate the remaining amount of EDC as a percentage of the initial value.

3. Results and Discussion

3.1 Endocrine disruptors degradation by enzymes

Enzymatic degradation of EDC bisphenol A (BPA), nonylphenol (NP), methylparaben (MTPRB), buthylparaben (BTPRB), and dimethylphthalate (DMPTL) was tested in solution at pH 5.0 in the presence of the different selected laccases. Among the EDC molecules, only BPA was degraded by enzymes in the absence of any mediator within the time of incubation analysed (Figure 2). After 1 hour of incubation Novoprime Base 268 was able to degrade 60% of BPA, whereas POXC degradation rate was slower than that obtained by Novoprime 268, reaching 30% of BPA degradation after both 30 minutes and 1 h incubation. Both POXA1b and 1H6C were less efficient, with the latter being more able to degrade BPA, probably thanks to its higher redox potential [23]. The rate of BPA degradation was comparable with that obtained for other laccases in similar condition. A carefully comparison of results present in the recent scientific literature reveals that different strategies have been used to obtain BPA removal, along with different time of reaction and concentration

of both enzyme and substrate. Gassara and co-workers [26] reported a rate of BPA degradation of 13% after 2 hours incubation in the presence of 0.05 U/mL of a laccase from *Phanerochaete chrysosporium*. A purified laccase from *Grifola frondosa* was able to degrade 15% BPA (0.65 mM) in 1 hour [27], whereas a purified laccase from *Phlebia tremellosa* [28] removed around 65% of BPA estrogenic activity after a 3 h-incubation with 50 U of enzymatic activity. Interesting results were obtained using a purified laccase by *Trametes villosa*, able to totally degrade 2.2 mM BPA in 3 h-incubation [29].

3.2 EDCs degradation by enzymes in the presence of mediators

With the aim to enhance laccase efficiencies towards selected EDCs, two different mediators, a synthetic and a natural one, were added to the reaction mix. The selected mediators were ABTS, the first acknowledged laccase mediator [30], and the natural mediator AS, an eco-friendly, easily and economically available mediator [31].

As it is shown in Figure 3A, the presence of both mediators enhances laccase performances towards BPA but for Novoprime 268, and ABTS mediator is more effective than AS with all the tested laccases. As a fact, in the presence of ABTS, POXC was able to almost fully degrade BPA (95%) in 1 hour reaction. It is also possible to note that in the presence of both mediators POXA1b and 1H6C showed the same efficiency. Unexpectedly, the presence of mediators did not influence or even decreased Novoprime base 268 efficiency. A similar effect has also been observed for a *Coriolopsis polyzona* laccase towards NP using 1-hydroxybenzotriazole (HBT) as substrate [10].

Also when considering nonylphenol, the presence of both mediators enhances laccase performances, with ABTS being more effective than AS with all tested laccases (Figure 3B). In this case, POXC and Novoprime base 268 showed almost the same degradation rate both in the presence of ABTS and AS. On the other hand, POXA1b and 1H6C showed an opposite behaviour. As a fact, in the presence of ABTS, 1H6C was more effective than POXA1b, whereas in the presence of AS, POXA1b proved to be more efficient than its variant. This result seems indicate that no simple rule regarding redox potential or affinity can be easily drawn, being the whole reaction mechanism quite complex. The obtained results seem promising if carefully compared with other systems. Indeed, a laccase from the white rot fungus *C. polyzona* was able to eliminate 50% BPA and 66% NP in the presence of 10 □M ABTS as mediator [10]. When the synthetic mediator HBT (200 □M) was used to improve laccase degradation, an enhanced degradation of almost 1.3 fold for both substrates was observed, reaching a degradation of 95% and 80% for BPA and NP, respectively [32].

When the mediator concentration was increased up to 200 □M, AS revealed to be the best mediator, since all enzymes were able to also degrade methylparaben and butylparaben after 1 h-incubation (Table 1). Also in this case, POXC showed the best performances, being able to degrade in 30 minutes 50% and 60 % of methyl and butylparaben, respectively (degradation did not improve after 1 h-incubation). Among parabens, butylparaben was more susceptible to laccase degradation in the presence of mediators than methylparaben. In the scientific literature are present only few reports regarding paraben degradation by laccases. Mizuno and coworkers [33] demonstrated that both iso- butylparaben and n- butylparaben were almost completely removed (95%) after 2 h of treatment and completely disappeared after 4 h of treatment with 0.5 U/mL of laccase activity in the presence of 2 mM HBT. The

only substrate recalcitrant to laccase oxidation in all the tested conditions was dimethylphthalate.

3.3 Degradation of EDCs mixture by free and immobilized POXC

POXC, the best performing enzyme, was chosen for further degradation analyses against a mixture of the selected EDCs in a total final concentration of 0.1 mM. The analyses were conducted in the presence of four out of five substrates. As a fact, DMPTL was not used, considering its recalcitrance to laccase degradation under all the tested conditions. It is worth to note that in the absence of any mediator POXC is able to degrade almost 40% BPA and 80% NP after 1 h-incubation, whereas methyl and buthylparaben were not degraded (Figure 4). As far as BPA is concerned, a slower degradation rate was observed when BPA concentration was lowered if compared with the degradation observed with high BPA concentration. When mediator was added to the reaction, the efficiency was greatly enhanced, and fully disappearance of BPA was observed in the presence of AS. On the other hand, POXC is able to efficiently degrade NP at low concentration also in the absence of mediators, and no increase is observed when mediators are added to the reaction mix. Thus, it may be hypothesized that the enzyme shows a higher affinity towards NP than towards BPA. Parabens at low concentration were not oxidised in presence of both mediators.

When immobilized POXC was used towards EDCs mix in the presence of AS, NP degradation improved with respect to the free enzyme, reaching the same extent of degradation (80%) within only 15 min, and no further increase was observed. On the other hand, a slightly lower BPA removal was observed (80%) using the immobilized enzyme respect to the free one. Parabens were not degraded, following the same trend already observed for the soluble counterpart. No adsorption of EDC molecules on activated carrier was observed. Tests were carried out using the silanized and derivatized carrier (without enzyme) against the mix of EDCs and no adsorption on the carrier was observed. Laccase immobilized on glass beads maintained significant activity during storage at 4°C in 50 mM phosphate buffer pH 6.5. After one month of storage, the retained laccase activity was 100%.

In order to assess reusability of the immobilized laccase against mixture of EDCs, six successive cycles of batch degradation were performed. After six cycles, there was a 20% drop in laccase activity (Figure 5). As far as EDC removal is concerned, a gradual loss of BPA degradation during six cycles was observed. On the other hand, NP degradation was decreased up to 40% after the first cycle, but no further drop was observed during the following 5 cycles.

Conclusions

The growing attention accorded to the removal of EDCs from environmental matrices makes oxidative enzymes an attractive candidate in the bioremediation arsenal. Four different laccases were chosen for their interesting characteristics and tested towards EDC molecules. The obtained results have shown that all laccases are able to oxidize different EDCs. In particular, BPA is the only substrate oxidized under all conditions tested. Furthermore, to improve laccase capabilities, mediators were added to reaction mixtures. Among the chosen laccases, POXC was the enzyme with the highest bioremediation capacity under all conditions analysed. Its performances were increased in the presence of both mediators. Interesting results were obtained in the presence of the natural mediator acetosyringone. When used at high concentration, this natural mediator enhanced the bioremediation capacity of POXC determining a rate degradation of 50% of both parabens in 30 minutes. Thus,

results herein obtained confirm laccase capabilities [33] to degrade this kind of substrates, very poorly investigated till now. Furthermore, oxidative capabilities of POXC were also studied in the presence of EDCs mixtures. Removal rates were different in micropollutant mixtures if compared with removal rates obtained treating individually the different molecules with alternating results towards BPA and NP, respectively. These results highlight the influence in the enzymatic degradation efficiency of the ratio between xenobiotic concentration and enzyme affinity. Thus, a challenge still open to face EDCs degradation is the discovery/tailoring enzymes capable of degrading the target compounds with an affinity constant of the same order of magnitude respect to the actual concentrations of the EDCs in the environment. As a fact, since EDC concentration in real wastewater is very low (ng/L), enzyme displaying a very high efficiency (high turnover together with high affinity) towards this molecule are excellent candidates to efficiently achieve their removal. Improvement of enzyme performances in NP removal was achieved through immobilization on glass beads.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article

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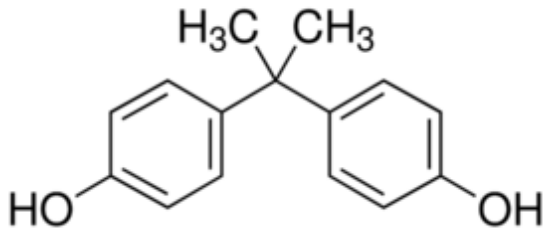

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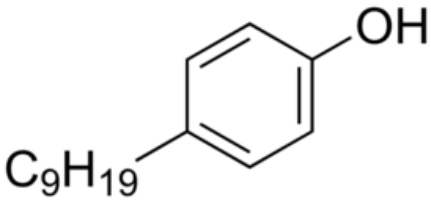

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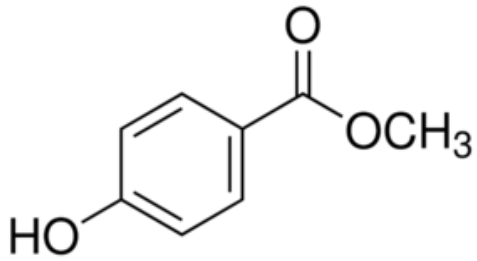

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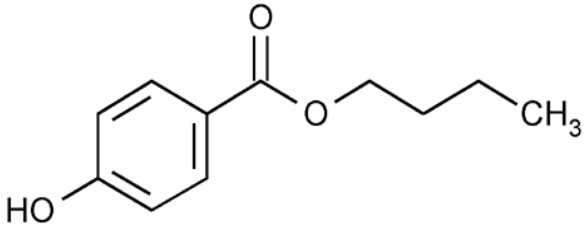
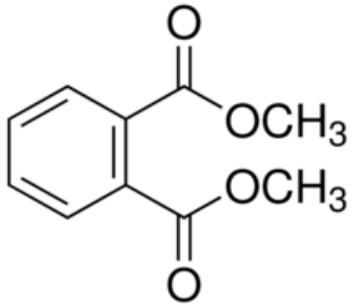


Buthylparaben (BTPRB)



Dimethylphthalate (DMPTL)

FIGURE 1 Chemical structure of endocrine disrupting substances used in this study.

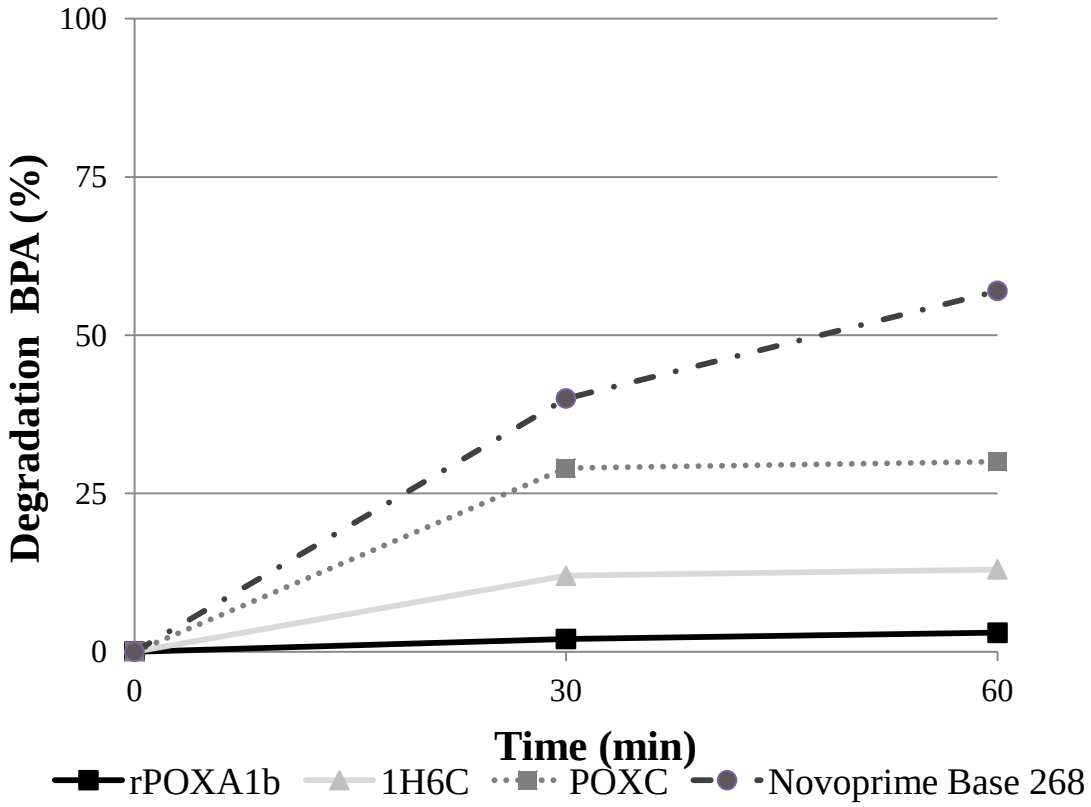


FIGURE 2 Percentage degradation (%) of BPA by fungal laccases. Reaction conditions: 100 □M BPA, pH 5.0 (50 mM sodium citrate buffer), 25°C and 1.5 U/mL laccase, with a reaction time of 1 h. All results are averages from two replicate experiments and the standard deviation is less than 10%

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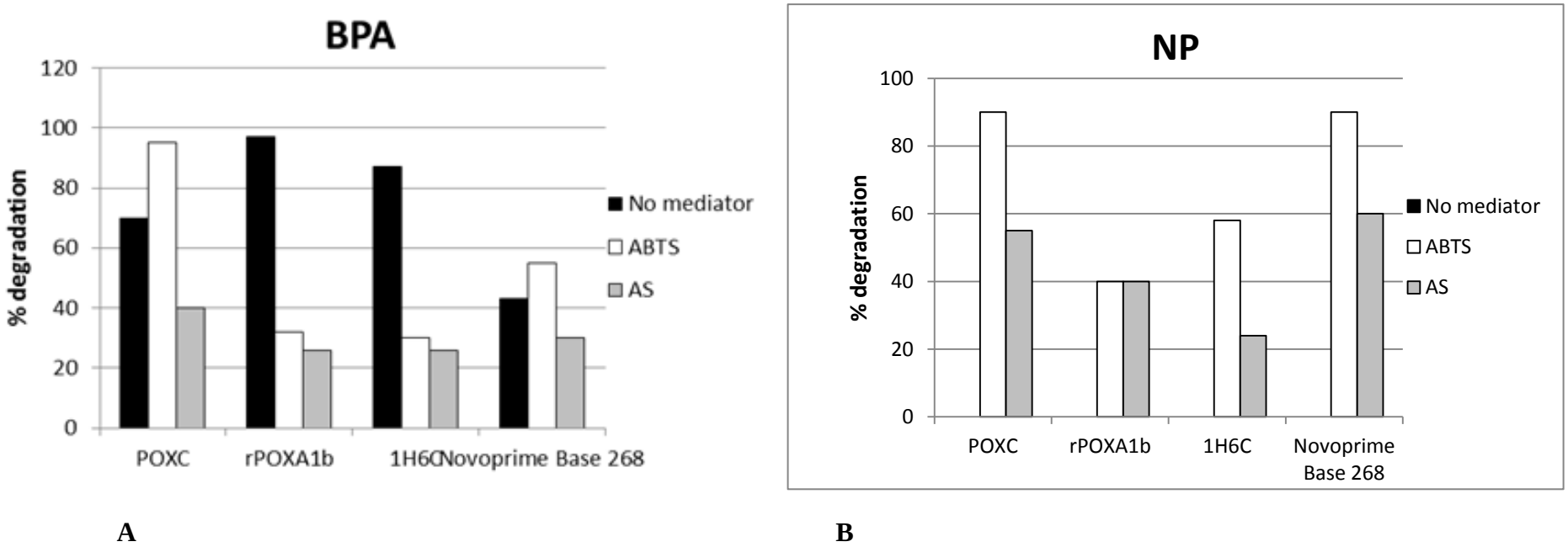


A **B**

FIGURE 3 Effect of absence of mediator (■), 20 □M of ABTS (□), or AS (■) on the removal of EDCs after a 1-h treatment at pH 5.0 and at a temperature of 25°C with 1.5 U/mL of laccases. **A.** BPA; **B.** NP. All results are averages from two replicate experiments and the standard deviation is less than 10%.

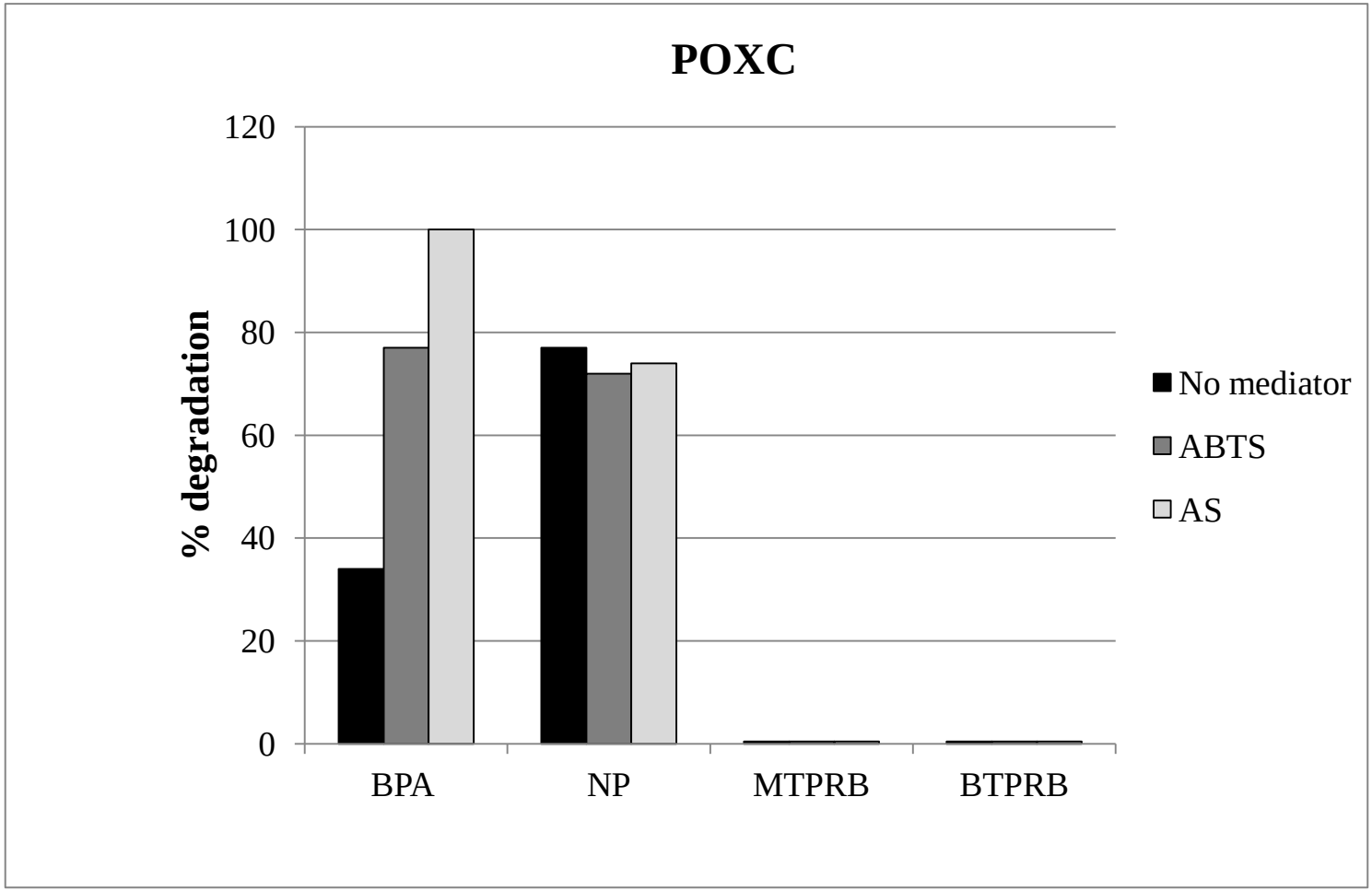


FIGURE 4 Effect of absence of mediator (■), 20 □M of ABTS (□), or AS (■)on the removal of EDCs mixtures by POXC. Reaction conditions: 25 □M of each molecule, pH 5.0 (50 mM sodium citrate buffer), 25°C and 1.5 U/mL laccase, with a reaction time of 1 h. All results are averages from two replicate experiments and the standard deviation is less than 10%.

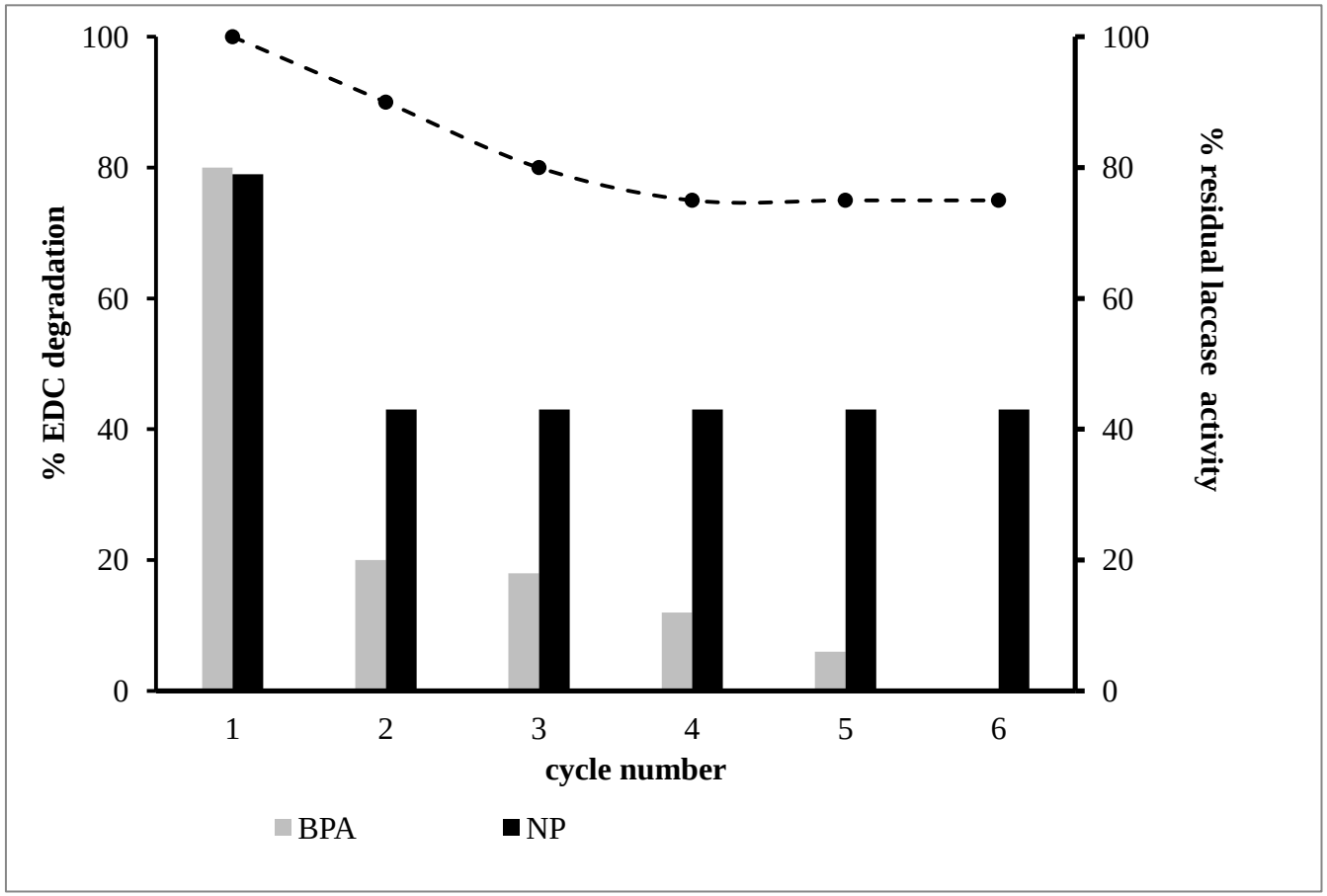


FIGURE 5 Percentage degradation (%) of BPA (■) and NP (■) by immobilized POXC. Reaction conditions: 6 U_{TOT} versus 25 □M each EDC, pH 5.0 (50 mM sodium citrate buffer), 25°C and, in the presence of 20 □M AS with a reaction time of 1 h. Residual laccase activity is reported as filled black circle (●). All results are averages from two replicate experiments and the standard deviation is less than 10%.

Table 1. Degradation of MTPRB and BTPRB in the presence of 200 □M of ABTS, or AS after a 1-h treatment at pH 5.0, 25°C with 1.5 U/mL of laccases. All results are averages from two replicate experiments and the standard deviation is less than 10%.

Enzymes	MTPRB		BTPRB	
	(% degradation)		**(% degradation)**	
	ABTS	**AS**	**ABTS**	**AS**
POXC	--	50	15	60
rPOXA1b	--	35	--	40
1H6C	5	7	7	8
Novoprime Base 268	--	40	--	50

CONCLUSIONS OF SECTION 1

The work described in Section 1 has been aimed at evaluating the performances of laccases for wastewater treatment contaminated by endocrine disrupting chemicals. Four different fungal laccases were chosen in this study to set-up EDCs enzymatic treatment.

Four different fungal laccases were chosen to test their ability to degrade five different EDCs. Three out of four of the selected enzymes are high redox potential laccases: POXC, POXA1b and 1H6C. The latter two enzymes were heterologously expressed in *Aspergillus niger,* and their characterization has already reported in the first chapter. A commercial laccase was also used.
Analyses about oxidative capabilities of selected laccases were studied in the presence of different EDCs. Moreover, laccase activity was enhanced by presence of both synthetic and natural mediators. The best performaces were obtained by POXC, which showed higher degradation rate in almost all tested condition and its performances were increased by the addition of both mediators. Interesting results were obtained in the presence of the natural mediator acetosyringone. Indeed, it was able to degrade 50% of both parabens after 30 min of incubation. Moreover, considering that in the natural environment pollutant mixtures are common, analyses of degradative capabilities of POXC was also evaluated towards xenobiotics mixtures. Removal rates were different in micropollutant mixtures if compared with removal rates obtained treating individually the different molecules. Indeed, only an implementation of enzyme performances in nonylphenol degradation rate was achieved, without the use of any mediator. In the end, studies about oxidative capabilities of POXC were carried out through immobilization on glass beads. Also in this case, the best performance was monitored against NP. These results underlie the influence in the enzymatic degradation efficiency of the ratio between xenobiotic concentration and enzyme affinity. Thus, a challenge still open to face EDCs degradation is the discovery/tailoring enzymes capable of degrading the target compounds with an affinity constant of the same order of magnitude respect to the actual concentrations of the EDCs in the environment.

Section 2

Application of fungal biosystems for the treatment of endocrine disruptors

INTRODUCTION

In the last decades, a large number of materials and compounds have been produced without adequate knowledge on their interaction with environment and human health. The result is a continuous pollution by a wide array of hazardous chemicals with different structures and toxicity levels showing an effect on the reproductive systems of various animals, including humans [1; 2]. Among these substances, there are particular chemicals -the endocrine disrupting chemicals (EDCs)- known and/or suspected to alter the normal hormone regulations and damage the health of intact organisms or their progenies or subpopulations [3; 4]. Due to their widespread presence in the environment and toxic activity even at low concentrations, EDCs have received increased attention in water quality management and health care. Moreover, these compounds are characterized by high bioactivity, ubiquitous nature, toxicity and persistence. Among various EDCs, bisphenol A (BPA), nonylphenol (NP), two different parabens, methyl- and buthyl-paraben, and dimethylphthalate (DMPTL) have been investigated; this selection was based on information about the amount of molecules discharged per year and their commercial availability. BPA is widely used for the production of epoxy and phenol resins, polycarbonates, polyester, and lacquer coatings on food containers, its production is 650,000 tonnes per year [5], determining an high concentration of BPA in wastewater and in wastewater sludge (0.004-1.36 mg/kg) [6]. Nonylphenol and phthalate esters, which are largely produced for use as surfactants and/or plasticizers in a variety of plastics industries, are also known to have estrogenic activities [7; 8]. Parabens are esters of p-hydroxybenzoic acid, they are widely used as preservatives in food, pharmaceutical and cosmetic industries in order to prevent bacterial growth [9; 10].

For aforementioned peculiarities, it is extremely important to found a valid decontamination treatment. In the last years, physical and chemical processing were used, but they are expensive and may generate a large volume of sludge [11; 12]. On the other hands, biological wastewater treatments are an attractive option as they could be cost-effective and environmentally friendly [13-15]. A promising alternative is the application of ligninolytic fungi or isolated fungal enzymes for the biodegradation of these compounds [16; 17]. Among the mushrooms, most white-rot fungi (WRF) have the capability to degrade EDCs such as nonylphenol, bisphenol A and phthalates [18-21]. These white-rot fungi stand a wide range of pH and considerably high concentrations of aromatic substrates, allowing an extensive utilization of their pollutant degradation capabilities [22]. Indeed these mushrooms are already used for bioremediation of coloured industrial wastewaters [13; 23; 24].

Another approach to solve this problem can be the use of filamentous ascomycetes, ascomycetous yeasts, or mitosporic fungi, which have gained considerable attention in the bioremediation process [25-29]. Indeed, in this context, due to the high variety of physicochemical conditions, the aquatic environment is a promising source for the isolation of interesting fungi. These organisms may be more suitable than other organisms for the treatment of micropollutants in wastewater, thanks to their living conditions and their possible adjustment mechanisms in different aquatic habitats. Furthermore, they are able to grow at high contents of inorganic ions found in both aquatic environments and industrial effluents [30-32]

In this section, different approaches were carried out, testing their different degradative capabilities.

Chapter 3

Degradation of Endocrine Disrupting Chemicals throught white-rot fungi

RESULTS AND DISCUSSION

3.1 Endocrine disruptors degradation by fungi

Analyses of degradative capabilities of different fungi against various EDCs were performed. All fungi were able to grow in presence of 100 µM of EDCs. In Fig. 1 results obtained at the 3rd growth day are showed, they are representative of the tendency of degradative capabilities of mushrooms in the presence of single EDCs; even if *T. versicolor* gives the best results after only 2 days. It is worth to note that *T. versicolor* is the better performing fungus. Indeed, it was able to totally degrade BPA in 2 days, *P. chrysosporium* and *P. ostreatus* were able to degrade 50% BPA in 3 days, and their performances remained constant during the growth. Cajthaml and co-workers (2009) [17] reported the ability of *P. ostreatus* and *T. versicolor* to degrade 44 µM of BPA in 3 and 7 days, respectively; on the contrary *P. chrysosporium* degraded BPA until 30%. Variances showed may be due to different concentrations analysed, indeed higher BPA quantity could inhibit degradative capabilities of *P. ostreatus*. As for *T. versicolor*, differences on the degradative capabilities may be due to the different culture conditions used.

In the presence of NP, *T. versicolor* was the only fungus able to totally degrade this substrate in 8 days, *P. chrysosporium* and *P. ostreatus* degraded 80% NP in 2 days, but this value remained stable during their growth. The data are in accordance with literature in which *T. versicolor* was the most efficient NP degrader among other tested ligninolytic strains [17; 33].

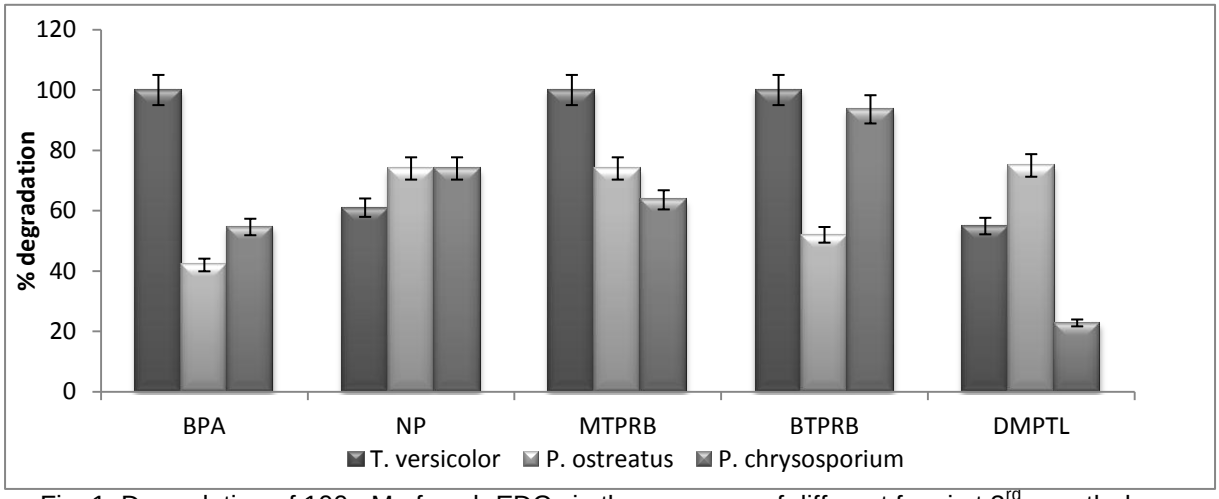
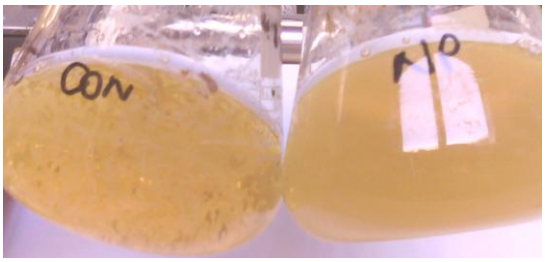


Fig. 1. Degradation of 100 µM of each EDCs in the presence of different fungi at 3rd growth day.

Furthermore, analyzing cultures in the presence of nonylphenol, all mushrooms showed a morphologic change. As a fact, cultures exhibited smaller size mycelium with respect to the control and they displayed cellular lysis, maybe due to the presence of nonylphenol degradation compounds (Fig. 2). As for parabens, *T. versicolor* showed high rate degradation, indeed both substrates were degraded totally in 2 days. The performances of *P. chrysosporium* towards these EDCs were also interesting, indeed parabens disappearance was after 4 days. Only *P. ostreatus* was not able to degrade these compounds completely. This is the first work in which parabens have been studied and good results have been obtained. In the case of DMPTL, *P. ostreatus* gave the best performances showing 80% of degradation after 8 days. These data are in accordance with results of Hwang and co-workers (2008) [34] demonstrating that *P. ostreatus* showed better characteristics in comparison of *T. versicolor* performances.

No EDCs absorption was monitored in control growth in all tested conditions. Laccase activity was monitored in all conditions and no significant increase was observed.



2A

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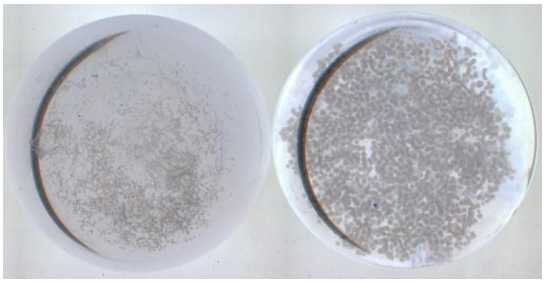

2C

Fig 2. Morphologic changes in nonylphenol culture broths. **2A**. *T. versicolor* in PDY vs *T. versicolor* in 0.1 mM NP; **2B**. *P. ostreatus* in 0.1 mM NP vs *P. ostreatus* in PDY; **2C.** *P. chrysosporium* in 0.1 mM NP vs *P. chrysosporium* in PDY.

3.2 EDCs degradation by T. versicolor in minimal culture broth

Since *T. versicolor* gave the better results in all conditions studied, it was chosen for further investigations. The next step was the analysis of fungus degradative capabilities against an EDCs mixture (20 µM final concentration of each substrate) in PDY. In this frame, *T. versicolor* totally degraded MTPRB and DMPTL in 2 days, the other substrates in 4 days. Comparing these results with those obtained in the presence of single compound, it was possible to observe that, at low concentration, this fungus totally degrades DMPTL. On the contrary, the degradation rate of BPA and BTPRB in the mixture was lower than that exhibited in the presence of single compound.

To verify the performances of *T. versicolor* in real world condition, formulation of minimal culture broth was performed. This goal was obtained by reduction of both carbon and nitrogen source, using ten times diluted PDY and only BPA as growth medium. BPA was chosen as reference xenobiotic because of its high concentration in the environment. Degradation rate of 100 µM BPA was analysed in both different growth conditions showing, in both cases, totally disappearance of this substance after 1 day of growth (data not showed). Studies on the reuse of same biomass for BPA degradation were also carried out. Fifteen cycles of degradation were performed by daily addition of 100 µM BPA to the same culture. Interesting results were found, indeed totally disappearance of this micropollutant was found displaying that degradative performances of *T. versicolor* are constant during the growth (fig. 3).

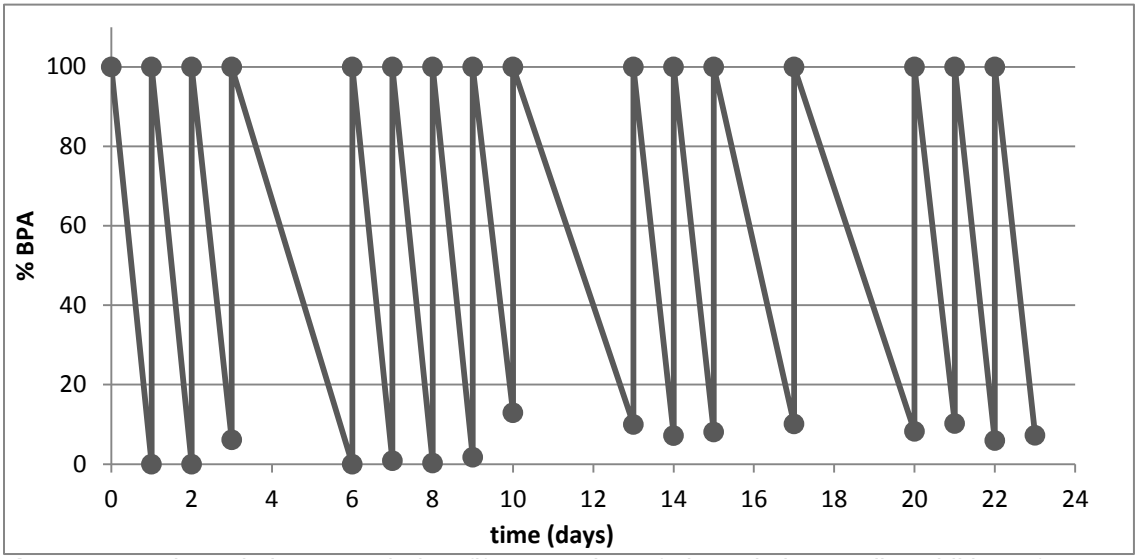

Fig. 3. BPA degradation rate during fifteen cycles of degradation. Daily addition of BPA was performed. Data represent means from triplicate cultures.

The next step was carried out analysing degradative capabilities of *T. versicolor* against an EDCs mixture and using only water and EDCs mixture as nutrients. Surprisingly, all xenobiotics disappeared in only 1 day. Fifteen cycles of degradation were performed by daily addition of EDCs mixture to the same biomass. It is worth to note that BPA and BTPRB were daily degraded during all cycles. On the other hands, MTPRB and NP disappeared day by day in the first two cycles, then only partial degradation was monitored for the next five cycles. Anyway, performances were restored showing continue and total degradation of these substrates. As regards as DMPTL, it was totally degraded only in the first day of culture, but its slow degradation was monitored determining its accumulation after daily addition (Table 1).

EDCs	EDCs concentration (µM)					
	1 cycle	2 cycle	4 cycle	6 cycle	8 cycle	15 cycle
BPA	0	0	0	0	0	0
NP	0	0	23	25	0	0
MTPRB	0	0	31	73	60	0
BTPRB	0	0	0	0	0	0
DMPTL	0	33	57	107	116	163

Tab. 1. EDCs concentration (µM) in mixture before daily addition. EDCs degradation was monitored every day. BPA and BTPRB were daily degraded; other substrates showed a partial degradation determining theirs accumulation. Data represent means from triplicate cultures.

MATERIALS AND METHODS

Organism and culture conditions- *T. versicolor* (NBRC4937) and *P. ostreatus* (Jacq.: Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) were maintained through periodic transfer at 4°C on Potato Dextrose Yeast extract (potato dextrose 24 g/L; yeast extract 5 g/L) (PDY); *P. chrysosporium* Burdsall M1 (DSM 13583) was maintained through periodic transfer at 4°C on 1/10 PDY. In order to study EDCs degradation, liquid fermentations using PDY medium were conducted. Incubations were carried out at 28°C in the dark by preinoculating 300 mL of PDY in 500 mL shaken flasks with 6 agar plugs of mycelium grown on solid state on Petri dishes (11 mm diameter), both for *T. versicolor* and *P. ostreatus*. The preinocula for liquid cultures of *P. chrysosporium* were prepared with 6 agar plugs of mycelium grown on solid state on Petri dishes (14 mm diameter) at the same way used for the other fungi. After 5 days of growth, preinoculum was homogenized and diluted 1:10 in 250 mL flasks containing 150 mL of PDY broth (1/10 PDY broth for *P. chrysosporium*) supplemented with 100 µM EDC and 150 µM copper sulphate. The cultures were incubated in the dark at 28°C on a rotary shaker (150 rpm) and monitored for 8 days.
Degradation of EDCs mixture was performed in the same condition, using a final concentration of 20 µM of each EDC. Thus, the final concentration of EDCs mixture was of 100 µM. In this case, the inocula were performed filtering fungal mycelium through cellulose nitrate filters (0.45 mm pore size; Sartorius) and resuspended it in the same broth of inoculum. Mycelial suspension obtained was homogenized and inoculated in broth and EDCs mixture.
Autoclaved, tyndallized and inactivated inoculi were used as controls and they were performed in the same way of active cultures; inactivated cultures were prepared by adding sodium azide (500 mg/L). Laccase activities were monitored every day.

Assay of enzymatic activity- Laccase activity was assayed at 25°C by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm ($\varepsilon_{420}=36\times10^3$ M^{-1} cm^{-1}). The assay mixture contained 2 mM ABTS in 100 mM sodium citrate buffer, pH 3.0.

EDCs degradation- 1 mM stock of each EDCs was prepared in hot water. To improve the solubility of NP and DMPTL in hot water, both methanol (0.4% v/v) and Tween 80 (0.1% w/v) were added.
100 µM of each EDC was added to culture broth at the time of inoculation. Residual EDCs in the fungal cultures were analyzed every day by reverse-phase HPLC. Samples cell-free were mixed with an equal volume of methanol and vigorous mixing, kept at -20°C for 15 minutes and centrifuged at 15,100 g at 4°C for 15 min. Then, 100 µL was injected into the HPLC column and analysed. Degradation of EDCs mixture was performed in same condition of previous experiments.

High-Performance Liquid Chromatography- All EDCs were quantitatively analysed using a C18 column (Grace Vydac, Hesperia, California- USA) on a HPLC instrument (Agilent Technologies Italia). The fractions were eluted by using a linear gradient of water-methanol (A solvent 0.1% trifluoroacetic acid in Milli-Q (MQ) water; B solvent 0.07% trifluoroacetic acid, 5% MQ water in methanol) at a flow rate of 1 mL/min. The gradient program for BPA analysis was 0-3 min (methanol 30%), 3-9 min (methanol 30-90%), 9-12 min (methanol 90%), 12-13 min (methanol 90-30%), and 13-15 min (methanol 30%). The eluted sample was monitored by UV absorbance at 227 nm. The retention time for BPA was 6.9 min under these conditions. As regards as NP, the applied gradient was 0-3 min (methanol 20%), 3-9 min (methanol 20-90%), 9-12 min (methanol 90%), 12-13 min (methanol 90-20%), and 13-15 min (methanol 20%). The detection wavelength was 277 nm. The retention time for NP was 14.5 min

under these conditions. The gradient program for parabens analyses was 0-7 min (methanol 30-70%), 7-8 min (methanol 70-90%), 8-11 min (methanol 90%), 11-12 min (methanol 90-30%), and 12-14 min (methanol 30%). The detection wavelength was 254 nm. Under these conditions, the retention times for MTPRB and BTPRB were 5.8 min and 8.8 min, respectively. As regards as DMPTL, the applied gradient was the same used for parabens, while the detection wavelength was 274 nm. The retention time for DMPTL was 6.8 min under these conditions. As far as the EDCs mixtures is concerned, each molecule was analysed with its optimised program. The peak area on the chromatogram was used to calculate the remaining amount of EDC as a percentage of the initial value.

Chapter 4

Aquatic fungi for Endocrine Disruptors Chemicals Degradation.

RESULT AND DISCUSSION

4.1 Endocrine disruptors degradation by aquatic fungi

Six different strains representing various species (Tab 1) were compared in term of their ability to degrade selected xenobiotics.

Fungal strains	Genera
Phoma sp. UHH 5-1-03	Aquatic mitosporic fungus
Clavariopsis aquatica WD(A)-00-01	Aquatic hypomycetes
Trichosporon porosum JU-K-2	Basidiomycetes
Acephala sp. JU-A-2	Ascomycetes
Stachybotrys chlorohalonata A-2008-2	Ascomycetes
Cladosporium herbarum AP2-2009-7	Ascomycetes

Table 1. Fungal strains used within the present study

A comparison was performed in 6-well plates, analysing the degradation rate of each fungus towards each EDC and EDCs mixture. Cultures were monitored during six days, and, in both cases, only parabens were totally degraded by three of the selected mushrooms (Tab. 2). It is worth to note that *C. herbarum* AP2-2009-7 was able to fully degrade these two micropollutants after only 1 day. Moreover, *C. herbarum* and *Phoma* sp also showed degradative capabilities against EDCs mixture, exhibiting total degradation of parabens after 6 days. On the other hand, no fungus was able to degrade BPA, NP and DMPTL, in all of the conditions tested.

			EDCs mix				
	MTPRB	BTPRB	BPA	NP	MTPRB	BTPRB	DMPTL
Phoma sp. UHH5	6	6	n.d.	n.d.	6	6	n.d.
C. aquatica WD(A)-00-01	6	6	n.d.	n.d.	n.d.	n.d.	n.d.
T. porosum JU-K-2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acephala sp. JU-A-2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. chlorohalonata A-2008-2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C. herbarum AP2-2009-7	1	1	n.d.	n.d.	6	6	n.d.

Table 2. Total degradation of EDC expressed in days. n.d.=not degraded. Data represent means from triplicate cultures.

Considering these results, *C. herbarum* AP2-2009-7 and *Phoma* sp. UHH 5-1-03 were chosen for further scale up. Indeed, the degradation capabilities of these fungi were investigated in shaken flasks, both in single and in mixed cultures.

C. herbarum AP2-2009-7 showed a total degradation of MTPRB after only 2 days. The fungus also exhibited interesting results in the presence of dimethylphthalte, being able to totally degrade it after 7 days. As for the other xenobiotics, included BTPRB, the fungus was not able to degrade them.

In order to demonstrate the possibility of reusing this biosystem for EDCs degradation, fungal biomass was recycled after the first growth in EDCs. Obtained results were summarized in table 3.

		C. herbarum AP2-2009-7	
		1st cycle	2nd cycle
MTPRB		2	2
DMPTL		7	3
EDCs mixture	BPA	>15	>15
	NP	15	11
	MTPRB	2	2
	BTPRB	2	2
	DMPTL	>15	>15

Table 3. Total degradation of selected EDC by *C. herbarum* AP2-2009-7 expressed in days. Data represent means from triplicate cultures.

During second cycle, *C. herbarum* AP2-2009-7 was able to completely degrade MTPRB and DMPTL after 2 and 3 days, respectively, showing in the case of DMPTL, an enhanced of degradation rate.

Analyses of degradative capabilities of *C. herbarum* AP2-2009-7 against EDCs mixture were also carried out and all results were summarized in table 3. Also in this case, recycling biomass in the presence of EDCs mixture was performed (table 3). During second cycle, an enhanced of NP degradation was monitored.

No EDCs absorption on fungal biomass was monitored in control growth in all tested conditions.

Furthermore, laccase activity was monitored and no significant increase was monitored in the presence of EDCs with respect to the values obtained in the absence of the micropollutants. The inability of some fungi to degrade xenobiotics could be related to the absence of laccase activity. Indeed, Junghanns and co-workers (2005) [28] analysed the NP degradation rate by *C. aquatica* WD(A)-00-01 and *Phoma* sp. and a slow degradation of this molecule was monitored for both fungi. When laccase activity was induced, an enhancement of NP degradation rate was observed in *Phoma* [35].

As for *Phoma* sp. UHH 5-1-03, its performance in the presence of methylparaben was fascinating. Indeed, at the third day of growth, analysing the amount of xenobiotic in the culture broth, 67% degradation was monitored. However, monitoring the amount of MTPRB in control growth, also bioadsorption of xenobiotic on fungal biomass was observed. For this reason, the real percentage of degradation was calculated as the difference between the amount of MTPRB present *ab initio* and

both the amount bound on biomass surface and that remaining in the culture broth. At the 3rd day of growth, 33% MTPRB was present in culture broth, while 24% was found bound on fungal biomass and 43% was really degraded (Table 4).

As regards as BTPRB degradation by *Phoma* sp. UHH 5-1-03, only the bioadsorption process was monitored, without degradation (Table 4).

EDCs	EDCs remaining in culture broth (%)				BIOADSORPTION (%)				BIODEGRADATION (%)			
	2nd	3rd	4th	7th	2nd	3rd	4th	7th	2nd	3rd	4th	7th
MTPRB	75	33	0	0	0	24	10	0	25	43	90	0
BTPRB	95	62	30	0	5	32	70	100	0	0	0	0

Table 4. Biodegradation and bioadsorption processes of *Phoma* sp. after 2, 3, 4 and 7 days of growth. Measured data represent means from triplicate cultures.

Contrarily to behaviour of cultures in multiwell, the fungus also exhibited interesting results in the presence of dimethylphthalte, as already observed for *C. herbarum* AP2-1009-7. Indeed, total DMPTL degradation was accomplished in 7 days. No fungal absorption occurred in this case. No degradation of the other compounds was observed. Reuse of fungal biomass, as previously described, was performed both for MTPRB and DMPTL and all results were summarized in table 5. In this case, no bioadsorption was monitored.

Analysing *Phoma* sp. performance against EDCs mix, this fungus was able to completely degrade all EDCs in two days. Recycle of fungal mycelium was performed and results were showed in table 5. Methyl- and buthyl- paraben absorption was monitored in control growth in the presence of EDCs mixture, both in the first and in the second growth. For the other xenobiotics, no EDCs absorption was monitored in both control growth.

		Phoma sp. UHH 5-1-03	
		1st cycle	2nd cycle
MTPRB		7	3
DMPTL		7	3
EDCs mixture	BPA	2	>15
	NP	2	>15
	MTPRB	2	2
	BTPRB	2	2
	DMPTL	3	>15

Table 5. Total degradation of selected EDC by *Phoma* sp. UHH 5-1-03 expressed in days. Data represent means from triplicate cultures.

Considering particular performances showed by *Phoma* sp. in the presence of parabens, further investigation about its degradative and adsorption capabilities were performed.

4.2 Biodegradation and bioadsorption ability of Phoma sp. UHH 5-1-03

In order to study the different degradation processes of parabens from *Phoma* sp., a different growth was carried out. Preinoculum and inoculum of *Phoma* sp. was performed as in materials and methods section and the amount of inoculums was around 14 g/L, in all tested condition.

Analysing the degradation rate of parabens after four hours, fascinating results were obtained. Indeed, their total biodegradation was monitored. Furthermore, washing active biomass with methanol and analysing the obtained sample, no bioadsorption was monitored. On the other hand, total bioadsorption of both parabens on control cultures was observed after four hours. These obtained results were opposite and did not underline the differences between the bioadsorption and biodegradation process. For this reason, new cultures were carried out and inoculum phase and only 25% of preculture biomass was inoculated. Degradation rates of these xenobiotics were monitored in 6 hours. As previously observed, in active cultures, no bioadsorption process was monitored for both parabens, being biodegradation the only process. On the other hand, bioadsorption profile was again observed in control growths, determining a total disappearance of both parabens in six hours.

Thus it seems that in the active cultures, even if bioadsorption occurs, it is followed by a kind of xenobiotic uptake, since no xenobiotic is detected after methanol extraction form cells.

In conclusion, *Phoma* sp. UHH 5-1-03 can be used in both processes, showing high rate of decontamination.

MATERIALS AND METHODS

Organism and culture conditions- All fungi selected in this study were maintained through periodic transfer at 4°C on 2% (w/v) malt extract. In order to compare EDCs degradation, different liquid fermentations were carried out. The first analysis was performed in 6-well plates in 10 mL Stanier mineral medium (1966) [36] supplemented with 250 µM of each EDC or 250 µM EDCs mixture, at the time of inoculum. Incubations were carried out at 28°C in the dark with agitation (120 rpm) inoculating 0.5 mL of a mycelial suspension. This suspension was prepared by homogenizing of 30 agar plugs (7 mm diameter) derived from the margins of well-grown agar plate cultures (3-4 weeks old) of each fungus with 30 mL of Stanier medium using an Ultraturrax. The cultures were monitored for 6 days. The second step was conducted in 100 mL Erlenmeyer flasks containing 30 mL Stanier medium supplemented with 250 µM EDC and 250 µM EDCs mixture for selected fungi. The inoculum suspension was prepared as previously described. In both steps, control cultures were prepared by adding sodium azide (0.5 g/L) to wells or flasks inoculated by each fungus in the presence of both single compound and EDCs mixture.

Fungal biomass recycling was tested after full degradation of EDC molecule using 5% of fungal biomass to start a new degradation cycle in the condition already described.

Liquid precultures of *Phoma* sp. UHH 5-1-03 were conducted in Erlenmeyer flasks (100 mL) containing 30 mL of 2% (w/v) malt extract medium (pH 5.6-5.8) inoculated with 0.5 mL of a mycelial suspension, obtained as previously described. Flasks were incubated at 14°C and 120 rpm in the dark for nine days. After nine days, fungal mycelia were removed from culture media by sterile filtration through filter papers, and washed 3 times with 30 mL of Stanier medium. Then, the whole fungal biomass, or a 25% of fungal biomass, is transferred into new sterile 100 mL flasks containing 30 mL mineral medium supplemented with 250 µM of each EDC or 250 µM EDCs mixture, at the time of inoculum. Finally, flasks were incubated at 28°C and 120 rpm. As regards as control, sodium azide was added at a final concentration of 0.5 g/L to inactivate the fungal cultures after 8 days of incubation of preinoculum. The laccase activity was daily monitored.

Assay of enzymatic activity- Laccase activity was assayed at 25°C by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm (ε_{420}=36×10^3 M^{-1} cm^{-1}). The assay mixture contained 2 mM ABTS in McIlvaine buffer, pH 4.0.

EDCs degradation- 50 mM stock of each EDC was prepared dissolving xenobiotic in methanol containing 10% Tween 80 (w/v). As regards as analyses in the presence of EDCs mixture, a 25 mM stock solution was prepared as previously described. Residual EDCs in the fungal cultures were daily analyzed by reverse-phase UPLC. Samples cell-free were vigorously mixed with an equal volume of methanol, kept at -20°C for 15 minutes and centrifuged at 15,100 g at 4°C for 15 min.

To analyse the EDC amount on biomass surface, 0.5 mL of fungal mycelium was washed with an equal volume of methanol, vigorously mixed, kept at -20°C for 15 minutes and centrifuged at 15,100 g at 4°C for 15 min. After centrifugation, supernatants (900 µL) were transferred into 1.5 mL UPLC vials. Samples (3.3 µL) were injected into the UPLC column and analysed.

Ultra-Performance Liquid Chromatography- Samples were subjected to an Acqutity^{TM} UPLC system comprising a Binary Solvent Manager (BSM), a Sample Manager (SM), and a PDA eλ photo diode array detector, and equipped with an Acquity^{TM} UPLC BEH C18 column (1.7 µm particle size; 2.1 x 50 mm; Waters,

Eschborn, Germany) operated at a column temperature of 40°C. The following solvents serve as mobile phases: solvent A - 10% methanol in deionised water, acidified to pH 3.0 with concentrated phosphoric acid; solvent B - 100% methanol, acidified to pH 3.0 with concentrated phosphoric acid. The following elution profile was applied: isocratic elution at 20% B for 0.14 min; linear increase to 100% B for 2.8 min; isocratic elution at 100% B for 3.2 min; linear decrease to 20% B for 3.25 min; isocratic elution at 20% B for 3.5 min (0.5 mL/min flow rate). A wavelength range from 220 to 400 nm was recorded. Calibration of the method was carried out with external standards. In this condition, the retention times for EDCs were 1.076 min MTPRB, 1.278 min DMPTL, 1.783 min BPA, 1.992 min BTPRB and 2.756 min NP. The peak area on the chromatogram was used to calculate the remaining amount of EDC.

Determination of fungal dry weights- Fungal cultures were harvested after 9 days; mycelia were removed from fungal cultures by filtration through filter papers, washed with 50 mL distilled water, dried at 80°C for 24 h, and weighed.

Chapter 5

Isolation of a new mushroom for Endocrine Disruptors Chemicals Degradation.

RESULT AND DISCUSSION

5.1 Analyses of degradative capabilities of new mushroom

Fungal growth on agar plates was monitored for 10 days. One fungal strain was growth on EDCs agar plates placed at 28°C showing radial expansion. Four sequential transfers were carried out on agar plates supplemented with 250 µM EDCs and a slowdown in growth rate was monitored. Liquid cultures were also performed analysing the degradation rate of this fungus against each xenobiotic and towards EDCs mixture.

Cultures were monitored for 15 days, and nonylphenol and methylparaben partially disappeared, while the other xenobiotics were recalcitrant to biodegradation (Fig. 1).

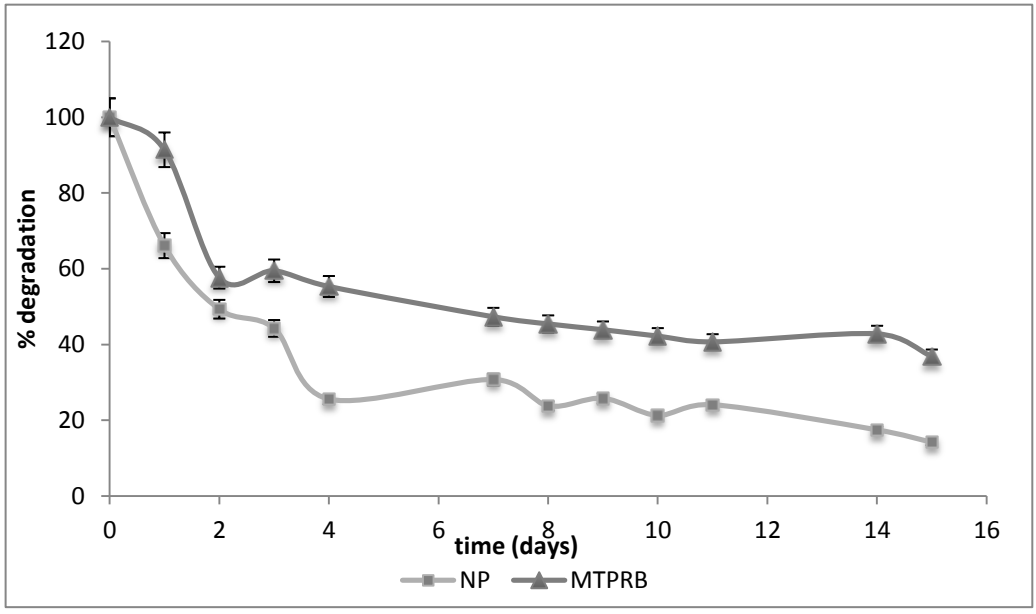

Fig. 1. Degradation rate of nonyphenol and methylparaben in the presence of new fungus. Data represent means from triplicate cultures.

When the fungus was grown in the presence of EDCs mixture, after two days of growth, all xenobiotics showed 50% disappearance, and a complete degradation was monitored for NP after 10 days (Fig. 2). Laccase activity was monitored in all conditions, and no detectable values were showed in the presence of EDCs.

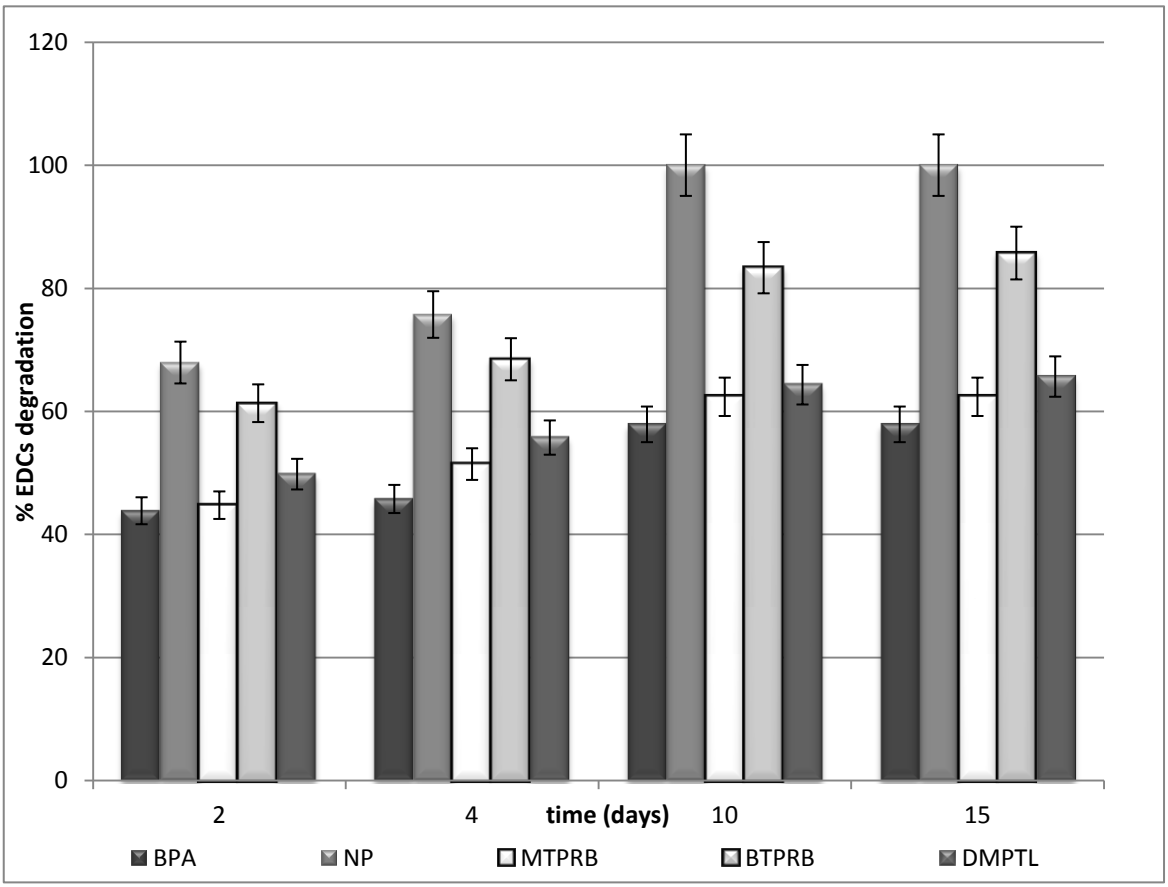

Fig. 2. Degradation percentage of EDCs mixture. Data represent means from triplicate cultures.

5.2 Identification of the newly isolated strain and enzymatic characterization

Both the ITS1 and ITS2 regions were successfully amplified from DNA using specific universal primers ITS1–ITS2 and ITS3–ITS4. Results showed a 99% identity with ITS of *Trichoderma virens*. *Trichoderma* species are free-living fungi that are common in soil and root ecosystems. They have been widely studied for their capacity to produce antibiotics, parasitize other fungi, and compete with deleterious plant microorganisms [37]. In particular, *T. virens* is a plant beneficial fungus, enhances biomass production and promotes lateral root growth [38]. It also shows beneficial features to the plant conferring resistance to necrotrophic fungi [39]. This fungus is also studied for its biotransformation capabilities and tolerance to growth in the presence of aromatic amines [40]. Considering these interesting peculiarities, in particular its application in bioremediation field, an analysis of its genome was performed, and genes related to oxidative and hydrolytic enzymes were found.

Analyses were carried out exploring the capabilities of this mushroom to produce laccase enzymes. With this aim, different culture broths and various laccase inducers were used to valuate this peculiarity, but no laccase activity was detected (data not showed). On the other hand, the production of hydrolytic enzymes was also studied. In this frame, culture medium was performed using minimal and synthetic broth in the presence of palm oil as inducer. Interesting results were obtained, reaching a production of 7 U/mL of lipase activity after 2 days of growth in the presence of minimal broth and palm oil (data not showed), a production similar to that of other fungi, as *Penicillium* sp., *Trichoderma* sp., *Aspergillus* sp., *Mucor* sp. after 3 days of growth [41]. To improve lipase production, different oils were used as carbon sources. These oils were selected considering their availability and their fatty acid composition. All selected carbon sources were chosen for high percent concentration

of oleic and linoleic acid (C_{18}), a well know lipase inducer. Mustard Seed Oil, sunflower Seed Oil, palm oil and agro-industrial wastes (rapeseed cake and olive mill wastewater (OMW)), were used.

		FATTY ACID COMPOSITION (PERCENTAGE)										
Name of the Oil	MP-Melting point (°C)	Caproic (Hexoic) C10	Caprylic (Octoic) C8	Capric (Decoic) C10	Lauric (Dodecanoic) C12	Myristic (Tetradecanoic) C14	Palmitic (Hexadecanoic) C16	Stearic (n-Octadecanoic) C18	Oleic (C18:1)	Linolenic (C18:3)	Linoleic (C18:2)	Arachidic C20 (Eicosanoic)
Coconut Oil	20-24	0-0.8	5.0-9.0	6.0-10.0	44.0-52.0	13.0-19.0	8.0-11.0	1.0-3.0	5.0-8.0	----	0-1.0	0-0.5
Corn (Maize) Oil	14-21	----	04.00	07.00	----	0.2-1.0	8.0-12.0	2.0-5.0	19.0-49.0	----	34.0-62.0	----
Hemp Seed Oil	15-17	----	----	----	----	----	5.0-7.0	1.0-3.0	11.0-13.0	24.0-26.0	54.0-56.0	----
Linseed Oil (Flax Seed Oil)	19-21	----	----	----	----	----	4.0-7.0	2.0-5.0	12.0-34.0	35.0-60.0	17.0-24.0	0.3-1.0
Mustard Seed Oil (Rap Seed	11.5-15	----	----	----	----	----	01.05	00.04	22	06.08	14.02	----
Palm Kernal Oil	24-26	tr	3.0-5.0	3.0-7.0	40.0-52.0	14.0-18.0	7.0-9.0	1.0-3.0	11.0-19.0	----	0.5-2.0	tr
Palm Oil	42-45	----	----	----	----	0.5-2.0	32.0-45.0	2.0-7.0	38.0-52.0	----	5.0-11.0	----
Rice Bran Oil	24-28	----	----	----	----	0.4-1.0	12.0-18.0	1.0-3.0	40.0-50.0	0.5-1.0	29.0-42.0	----
Soya Bean Oil	22-27	----	----	----	----	tr.0.5	7.0-11.0	2.0-6.0	22.0-34.0	5.0-11.0	43.0-56.0	----
Sunflower Seed Oil	16-20	----	----	----	----	----	3.0-6.0	1.0-3.0	14.0-35.0	----	44.0-75.0	0.6-4.0
Tea Seed Oil	21-22	----	----	----	----	----	----	----	----	----	7.0-14.0	----

Table 1. Fatty acid profiles for selected oils (in grey).

As reported in table 2, lipase production was around 15 U/mL after one day of growth in almost all tested condition, only in the presence of palm oil 3 U/mL were produced. The interest for lipase activity is due to the reported application of this enzyme for phthalate biodegradation. Indeed, Dulazi and co-worker (2010) [42] reported interesting degradation rates using an immobilized lipase for the degradation of mixtures of different phthalates. It is possible that an enhancement of degradative capabilities of this fungus towards EDCs could be obtained inducing production this enzymatic activity.

Carbon source	U/mL
Mustard Seed Oil (Rap Seed Oil)	15
Palm Oil	3
Sunflower Seed Oil	13
Repeseed cake	13
OMW	9

Table 2. Lipase activity in the presence of different carbon source. Measured data represent means from duplicate cultures.

MATERIALS AND METHODS

Source and maintenance of fungal strains- Fungal strain was isolate by ground sample from Cospudener See. Sediment samples (10 g) were shaken together with 7.5 g glass beads in 100 mL of sterile 0.2% (w/v) sodium pyrophosphate solution for 30 min at room temperature and 130 rpm. After sedimentation of the solid particles, aliquots (150 µL) from serial dilutions of the supernatants were poured onto 250 µM EDCs agar plates, in the presence of both Tetracyclin (250 mg/L) and Streptomycin (0.4 g/L). Moreover, agar medium was supplemented with 2.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ x 7 H_2O, 0.1 g/L $CaCl_2$, 1.0 g/L $(NH_4)_2SO_4$, 10 mL mineral salt solution (3.0 g/L $MgSO_4$ x 7 H_2O; 1.5 g/L Nitrilotriacetat; 1.0 g/L NaCl; 0.5 g/L $MnSO_4$ x H_2O; 181.2 mg/L $CoSO_4$ x $7H_2O$; 178.0 mg/L $CaCl_2$ x 2 H_2O; 100.0 mg/L $FeSO_4$ x 7 H_2O; 100.0 mg/L $ZnSO_4$; 18.4 mg/L $AlK(SO_4)_2$ x 12 H_2O; 12.0 mg/L $NaMoO_4$ x 2 H_2O; 10.0 mg/L $CuSO_4$ x 5 H_2O; 10.0 mg/L H_3BO_3), 1.5 mL Vitamin solution (10.0 mg/L pyridoxine x HCl; 5.0 mg/L 4-aminobenzoic acid; 5.0 mg/L D-(+)-calcium pantothenate; 5.0 mg/L lipoic acid; 5.0 mg/L nicotinic acid; 5.0 mg/L riboflavin; 5.0 mg/L thiamine x H_2O; 2.0 mg/L biotin; 2.0 mg/L folic acid), 15 g/L agar, pH 5-6. Fungal strain able to growth on this plate was transferred on 1% malt extract and was routinely used to maintain mushroom at 14°C in the dark.

DNA preparation- Fungus was subcultured in Potato Dextrose Yeast extract (potato dextrose 24 g/L; yeast extract 5 g/L) (PDY), incubation was carried out at 28°C, 120 rpm, for 48 to 72 h in the dark by inoculating 50 mL of PDY in 250 mL shaken flasks with 1 agar plug of mycelium grown on solid state on Petri dishes (11 mm diameter). 10 mL of culture were mixed by vortex for 5 min. The mycelium was collected by centrifugation for 10 min at 9500 rpm, 0.5 mL mycelium were resuspended in 1 mL of water, mixed by vortex and centrifuged for 10 min at 9500 rpm. 0.4 mL lysis solution (2% triton X-100; 1% sodium dodecyl sulphate; 100 mM sodium chloride; 100 mM Tris, pH 8; 1 mM EDTA), 0.5 mL phenol-chloroform and 0.5 mL glass beads were added to the mycelium and mixed by vortex for 10 min. Then 0.2 mL TE (10 mM Tris pH 8, 1 mM EDTA) were added and centrifugation for 5 min at 13000 rpm was performed. Obtained surnatant was transferred to a new tube and addition 1 mL 100% ethanol to precipitate the DNA was carried out. After centrifugation for 2 min at 13000 rpm, the obtained pellet was resuspended in 0.4 mL TE and treated with 30 µg RNasi A, at 37°C for 30 min. 10 µL 4 M ammonium acetate plus 1 mL 100% ethanol were added to the sample, and, after inversion, the tube was placed on ice for 10 min. Pellet was collected by centrifugation for 2 min at 13000 rpm, air dried and the obtained pellet was resuspended in 50 µL TE.

ITS amplification- The fungus-specific universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCATCGATGAAGAACGCAGC-3') were used to amplify the ITS1 region, while universal primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-GCATATCAATAAGCGGAGGA-3') were used to amplify the ITS2 region. PCR was performed in a total reaction volume of 50 µL consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates (0.2 mM each), 1.2 U of *GoTaq* DNA polymerase, 0.4 µM (each) of the ITS1 region primers (ITS1/ITS2) or the ITS2 region primers (ITS3/ITS4), 2 µL (1 to 5 ng) of DNA template, and 50 µL of a mineral oil overlay. PCR was carried out using the following conditions: initial denaturation at 95°C for 10 min; 35 cycles of denaturation (95°C for 1 min), annealing (60°C for 1 min), and extension (72°C for 1 min); and a final extension step at 72°C for 10 min.

All amplicons were purified using the QIA quick® Nucleotide Removal kit (Qiagen) following manufacturer instructions.

Identification of fungus by ITS sequencing- Fungal specie was identified by searching databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/). The ITS1 or ITS2 sequence was compared using nucleotide-nucleotide BLAST (blastn) with default settings. Species identification was determined from the higher expect value of the BLAST output.

Growth condition for laccase and lipase activity- Fungus was subcultured in PDY, incubation was carried out at 28°C, 120 rpm, in the dark by inoculating 100 mL of PDY in 500 mL shaken flasks with 2 agar plugs of mycelium grown on solid state on Petri dishes (7 mm diameter). After 3 days, preinoculum was filtered, washed three times with water and resuspended in the same broth of inoculum. Obtained mycelial suspension was homogenized and inoculated in a ratio of 1:10 in 250 mL flasks containing 150 mL of PDY broth. To enhance laccase activity, different inducers were used in potato dextrose broth (PDA) (24 g/L): 0.15 mM copper sulphate ($CuSO_4$), 0.05 mM $CuSO_4$ plus 1 mM vanillic acid, 0.05 mM $CuSO_4$ plus 2 mM ferulic acid.

To enhance lipase activity, during the first phase, different culture broths were used in the presence of the same inducer (0.2% tween 80, 2% palm oil): 1/10 PDA; Gal broth (1 g/L peptone, 20 g/L yeast extract, 2 g/L glucose, 5 g/L NaH_2PO_4, 1 g/L KH_2PO_4); African salt (5 g/L $(NH_4)_2SO_4$, 6 g/L NaH_2PO_4, 3 g/L $MgSO_4$, 3g/L $CaCl_2$). During second step, different oils were added to African salt and 0.2% tween 80: 2% rapeseed cake; 2% olive mill wastewater; 2% Mustard seed oil; 2% sunflower seed oil

Growth condition for EDCs degradation- Cultures were performed in 100 mL Erlenmeyer flasks containing 30 mL medium according to Stanier *et al.* (1966) [36] supplemented with 250 µM EDC and 250 µM EDCs mixture. Incubations were carried out at 28°C in the dark with agitation (120 rpm) by inoculating with 0.5 mL of a mycelial suspension. This suspension was prepared by homogenizing of 30 agar plugs (7 mm diameter) derived from the margins of well-grown agar plate cultures with 30 mL of Stanier medium using an Ultraturrax. The laccase activity was monitored in all flasks every day.

Control cultures were prepared by adding sodium azide (0.5 g/L).

Assay of enzymatic activity- Laccase activity was assayed at 25°C by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm ($\varepsilon_{420}=36\times10^3\ M^{-1}\ cm^{-1}$). The assay mixture contained 2 mM ABTS in McIlvaine buffer, pH 4.0.

Lipase activity was assayed by a spectrophotometric method using p-nitrophenyl decanoate as a substrate. The p-nitrophenyl decanoate was dissolved in isopropanol at a concentration of 10 mM. The assay mixture contained 0.2 mM p-nitrophenyl decanoate in 50 mM Tris-HCl buffer, pH 8.0. The activity was assayed by detecting the product, p-nitrophenol, spectrophotometrically at 405 nm. The molar extinction coefficient of pNP was 3.39 $x10^3 M^{-1}\ cm^{-1}$

EDCs degradation- 50 mM stock of each EDC was prepared dissolving xenobiotic in methanol containing 10% Tween 80 (w/v). 250 µM of each EDC was added to culture broth at the time of inoculation. As regards as analyses in the presence of EDCs mixture, a 25 mM stock solution was prepared in the same way previously described. Residual EDCs in the fungal cultures were analyzed every day by reverse-phase UPLC. Samples cell-free were mixed with an equal volume of methanol and vigorously mixed, kept at -20°C for 15 minutes and centrifuged at 15,100 g at 4°C for 15 min. To analyse the EDC amount on biomass surface, 0.5 mL of fungal mycelium was washed with equal volume of methanol, vigorously mixed, kept at -20°C for 15 minutes and centrifuged at 15,100 g at 4°C for 15 min. After centrifugation,

supernatants (900 µL) were transferred into 1.5 mL UHPLC vials. Samples (3.3 µL) were injected into the UPLC column and analysed. Degradation of EDCs mixture was performed in same condition of previous experiments.

Ultra-Performance Liquid Chromatography- Samples were subjected to an Acqutity^{TM} UPLC system comprising a Binary Solvent Manager (BSM), a Sample Manager (SM), and a PDA eλ photo diode array detector, and equipped with an Acquity^{TM} UPLC BEH C18 column (1.7 µm particle size; 2.1 x 50 mm; Waters, Eschborn, Germany) operated at a column temperature of 40°C. The following solvents serve as mobile phases: solvent A - 10% methanol in deionised, acidified to pH 3.0 with concentrated phosphoric acid; solvent B - 100% methanol, acidified to pH 3.0 with concentrated phosphoric acid. The following elution profile was applied: isocratic elution at 20% B for 0.14 min; linear increase to 100% B for 2.8 min; isocratic elution at 100% B for 3.2 min; linear decrease to 20% B for 3.25 min; isocratic elution at 20% B for 3.5 min (0.5 mL/min flow rate). A wavelength range from 220 to 400 nm was recorded. Calibration of the method was carried out with external standards.

The retention times for EDCs were 1.076 min MTPRB, 1.278 min DMPTL, 1.783 min BPA, 1.992 min BTPRB, 2.756 min NP. The peak area on the chromatogram was used to calculate the remaining amount of EDC.

CONCLUSIONS OF SECTION 2

The work described in Section 2 has been aimed at evaluating the performances of different biosystems for wastewater treatment contaminated by endocrine disrupting chemicals. In this context, three different approaches were pursued, using ligninolytic fungi, aquatic fungi and exploiting microflora present in the environment.

As regard as ligninolytic mushrooms, interesting results were obtained, because all selected fungi were able to degrade EDCs, and the best results were showed by *T. versicolor*. In only two days it was able to totally biodegrade three of the selected EDCs. For this reason, it was used for biodegradation process in the presence of EDCs mixture and, also in this case, good degradation rates were monitored.

The performance of this fungus was also studied in the presence of BPA as the only carbon source. Surprisingly, after 1 day this xenobiotic was totally disappeared. This result led us to investigate the possibility of recycling the same biomass for multiple cycles of BPA degradation. During fifteen cycles of degradation, the performances of *T. versicolor* were constant, showing totally disappearance of this micropollutant.

Same analysis was carried out against EDCs mixture as nutrients. In this case, all xenobiotics were degradaded, only DMPTL resulted recalcitrant to biodegradation and its accumulation in colture broth was monitored.

As for aquatic fungi, *Phoma* sp. UHH 5-1-03 and *C. herbarum* AP2-2009-7 showed interesting degradative capabilities against parabens and phthalates. Indeed, they degraded these compounds in seven days. Moreover, *Phoma* sp. UHH 5-1-03 also showed bioadsorption capabilities joined to degradative performances in the presence of parabens. Experiments to understand the different rates of these processes were carried out.

Furthermore, a fungal strain able to growth on EDCs mixture was selected from ground sample. Degradative capabilities of this fungus against EDCs were studied in liquid cultures, in the presence of both single and mixture compounds. Results show that this fungus is able to totally degrade NP and MTPRB. On the other hand, in the presence of the mix only NP was completely degraded, other xenobiotics were partially removed. Molecular characterization allowed the identification of this fungus as *Trichoderma virens*. Analyses were carried out exploring the capabilities of this mushroom to produce laccase enzymes, however no production of laccase activity was detected, in all conditions tested so far. On the other hand, the production of hydrolytic enzymes was studied. The hyghest levels of production were obtained in the presence of Mustard Seed Oil and African broth, reaching up to 15 U/mL of lipase activity after one day of growth.

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Conclusions

CONCLUSIONS

In this project, different biodegradation processes were explored for decontamination of endocrine disrupting chemicals. In particular, two kinds of processes have been specifically analysed: oxidation-based processes and biodegradation-based processes. The former alternative has been explored studying enzymatic oxidative capabilities; the latter was based on degradative capabilities of different fungal strains.

As for degradation based on enzymatic oxidative capabilities, among four different fungal laccases chosen for EDCs oxidation, the best performances were showed by POXC in all tested tested condition. Its oxidative capability was also studied towards an EDCs mixture in both free and immobilized forms.

As for biodegradation by fungi, three different approaches were followed. The first one was related to the use of different white-rot fungi for EDCs degradation. Among selected mushrooms, *Trametes versicolor* was chosen because of its high degradation rate against each xenobiotic. Moreover, degradative capabilities of this fungus were analysed in the presence of EDCs mixture. In order to mimic real condition, the capabilities of *T. versicolor* were analysed in the presence of water and an EDC (BPA) as carbon source. In this way, reuse of same biomass for BPA degradation was carried out, in which the performances of *T. versicolor* were constant and totally disappearance of this micropollutant was monitored. The same analysis was carried out in the presence of only EDCs mixture, and in this case good degradation rates were showed.

The second approach consisted in the use of aquatic ecosystems for biodegradation of water contaminants. In this way, two different fungi were chosen: *Phoma* sp. UHH 5-1-03 and *Cladiosporium herbarum* AP2-2009-7. Their degradation capabilities were analysed in the presence of both single compound and EDCs mixture. Both fungi showed interesting degradative capabilities towards parabens and dimethylphthalate.

Furthermore, a fungal strain able to growth on EDCs mixture was selected by ground sample. Degradative capabilities of this fungus against selected EDCs were studied in liquid cultures, in the presence of both single and mixture compounds, and the best degradation rates were monitored against NP and MTPRB in single culture. Identification of this fungus was carried out and it was identified as *Trichoderma virens.*

This work analysed different biosystems for EDCs degradation and all tested biosystems exhibited different performances. The conclusion is that a single strain/enzyme may be not sufficient to solve the highly variable nature of wastewater effluents, since there are too many factors that may affect the biodegradation rate of EDCs. Thus, further work in this direction should be the analyses of both fungal consortia and enzymatic mixtures performances from different sources in order to exploit their potential synergistic effect. These results indicate that each process alone might not represent a universally applicable solution to wastewater treatment. Combination of different techniques is generally required to devise a technically and economically feasible option, in which the respective advantages of each method would be exploited.

Publications and communications

PUBLICATIONS:

- Piscitelli A., Del Vecchio C., Faraco V., Giardina P., **Macellaro G**., Miele A., Pezzella C., Sannia G. Fungal laccasses: versatile tools for lignocellulose transformation. C R Biol. 2011 Nov;334(11):789-94.
- **Macellaro G**., Baratto M.C., Piscitelli A., Pezzella C., Fabrizi de Biani F., Palmese A., Piumi F., Record E., Basosi R., Sannia G. Effective mutations in a high redox potential laccase from *Pleurotus ostreatus*. Appl Microbiol Biotechnol. 2014 Jan 26.

COMMUNICATIONS:

- "Moving towards improved biocatalysts for new bioremediation processes" G. Sannia, A. Piscitelli, C. Pezzella, **G. Macellaro**, V. Faraco, and P. Giardina. 4th Korea-Italy S&T Forum, Naples (Italy), 3- 4 June 2010.
- "Moving towards improved biocatalysts?" C. Pezzella, V. Faraco, P. Giardina, **G. Macellaro**, A. Piscitelli, and G. Sannia. Oxizymes in Leipzig (Germany), 14-16 June 2010.
- "*Pleurotus ostreatus*: invaluable source of laccases for industrial production?" G. Sannia, C. Del Vecchio, V. Lettera, **G. Macellaro**, C. Pezzella, A. Piscitelli, and P. Giardina. 7th International Conference on Polymer and Textile Biotechnology, Milan (Italy), 2-4 March 2011.
- "High Redox-Potential Oxidative Enzymes for Industrial Applications". C. Pezzella, A. Piscitelli, **G. Macellaro**, V. G. Giacobelli, P. Cicatiello, G. Sannia. Braga (Spain). 2014.
- "Effective Mutations in a High-Redox Potential Laccase from *Pleurotus ostreatus"*. C. Pezzella, A. Piscitelli, **G. Macellaro**, V. G. Giacobelli, P. Cicatiello, G. Sannia. Oxizymes in Vienna (Austria). 2014.

EXPERIENCES IN FOREIGN LABORATORIES

October 2013 – January 2014: The Helmholtz Centre for Environmental Research, Leipzig (Germany) under the supervision of Dr. Dietmar Schlosser.

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Appendix

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Review/Revue

Fungal laccases: Versatile tools for lignocellulose transformation

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ABSTRACT

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

1. Introduction

Lignocellulose is a complex of carbohydrate polymers (cellulose and hemicellolose) tightly bound to lignin, and is a major constituent of a wide variety of materials including waste materials from agriculture, forestry, wood-based industries, and municipal solid waste [1]. These materials are produced in abundance, and represent a good option for conversion to useful, high value products. Lignocellulose conversion requires a pre-treatment step to degrade or loosen the recalcitrant and heterogeneous lignin fraction. This multi-faceted challenge is being addressed by an ever-increasing suite of ligninolytic enzymes isolated from various sources. Among these, fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are known to play an important role in lignin degradation/modification processes. These enzymes can be successfully applied to paper manufacturing, enhancement of fibre properties, production of improved forages and pre-treatment of lignocellulosic biomasses for fuel production.

Biotechnology can contribute to the development of "green tools" for the transformation of lignocellulosic feedstocks by providing tailor-made biocatalysts based on the oxidative enzymes responsible for lignin attack in nature [1]. With this purpose, laccases are currently being improved using (rational and random-based) protein engineering [2].

Laccase is one of the oldest enzymes reported and it is arousing great interest in the scientific community because of its very basic requirements (it just needs air to work and its only released by-product is water) and huge catalytic capabilities, making it one of the "greenest" enzymes of the 21st century [3]. This enzyme is produced by various fungi, plants, and certain bacteria or insects [4]. Laccase is able to catalyze direct oxidation of *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, and aryl diamines as well as some inorganic ions. It couples the four single-electron oxidations of the reducing substrate to the four electron reductive cleavage of the

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dioxygen bond, using four Cu atoms distributed against three sites, defined according to their spectroscopic properties. Typical metal content of laccase includes one type-1 (T1) copper, and one type-2 (T2) and two type-3 (T3) copper ions, with T2 and T3 arranged in a trinuclear cluster (TNC) [4].

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

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

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

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

2. Experimental

2.1. Strains, media, and plasmids

The *S. cerevisiae* strain used for heterologous expression was W303-1A (MAT ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100). The plasmid used for *S. cerevisiae* expression was pSAL4 (copper-inducible *CUP1* promoter). *S. cerevisiae* was grown in selective medium (6.7 g L^{-1} yeast nitrogen base w/o amino acids and ammonium sulfate; 5 g L^{-1} casaminoacids; 30 mg L^{-1} adenine; 40 mg L^{-1} tryptophane; 50 mM succinate buffer pH 5.3; 20 g L^{-1} glucose).

2.2. Random mutagenesis

Random mutations were introduced with low, medium, and high frequency of mutation, into the POXA1b encoding cDNAs using GeneMorphTM PCR Mutagenesis Kit (Stratagene, La Jolla, CA). EP-PCR was performed with primers POXA1bfw (ATAAAAGCTTGAATTCATGGCGGTTGCATTCG) and POXA1brev (TAAGGATCCAAGCTT TTATAATCATGCTTC).

2.3. Construction of mutant library

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

2.4. Library screening

Single clones grown on plate were picked and transferred into 96-well plates containing 30 μL of selective medium per well. Plates were incubated at 28 °C, 250 rpm for 24 h. After 24 h, 130 μL of selective medium was added to each well and the plates were incubated at 28 °C, 250 rpm for 24 h. Thirty microliters of each culture was transferred to a new 96-well plate to measure the OD_{600} value. The plates were then centrifuged for 10 min at 1500 g, 4 °C, and a suitable volume of supernatant was transferred to a new 96-well plate to perform laccase assay. Phenol oxidase activity was assayed at 25 °C using 2 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε 36,000 M^{-1} cm^{-1}), using Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA). Enzyme activity was expressed in international units (U). Cultures in shaken flasks were also performed. Pre-cultures (10 mL) were grown on selective medium at 28 °C on a rotary shaker (150 rpm). A volume of suspension sufficient to reach a final OD_{600} value of 0.5 was then used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of selective medium and cells were then grown on a rotary shaker. Optical density and laccase activity determination were daily assayed.

2.5. Screening of library for stability

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

2.6. Molecular modeling

The structure of POXA1b was obtained by homology modeling from the crystal structure of *Trametes versicolor*

(1GYC pdb entry), with which it shares 60% sequence identity. The last 16 residues of POXA1b were modeled using the coordinates of the corresponding residues at the C-terminus of the crystal structure of the *Melanocarpus albomyces* laccase (1GWO pdb entry). 3D model and *in silico* mutants were generated using the SWISS-MODEL web server by means of the project mode option that allow to select the template and control the gap placement in the alignment. Refinement of the models has been performed by molecular dynamics simulations. Simulations on the wild-type POXA1b and on the *in silico* generated mutants 1M9B and 3M7 C were performed with the GROMACS package as already described [10].

2.7. Stability assays

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

2.8. r4 cDNA construction

To obtain the laccase *r4* cDNA the 3′ terminal portion (from the nucleotide 453) of the cDNA coding for 3M7 C was ligated to pSAL4 vector containing the 5′ terminal portion (the first 5′ terminal 452 nucleotides) of 1M10B encoding cDNA, after KpnI digestion of pSAL4 vectors containing the two cDNAs.

2.9. DNA sequencing

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

2.10. Decolourization experiments

Batch decolourization experiments have been performed incubating (1 mL final volume of reaction) crude preparation of laccase containing different enzyme amounts (0.1 U, 1 U and 3 U) in three wastewater models: Acid (0.1 g L^{-1} Acid blue 62, 0.1 g L^{-1} Acid Yellow 49, 0.1 g L^{-1} Acid Red 266, 2 g L^{-1} Na_2SO_4, pH 5), Direct (1 g L^{-1} Direct blue 71, 1 g L^{-1} Direct Yellow 106, 1 g L^{-1} Direct Red 80, 5 g L^{-1} NaCl, pH 9) and Reactive (1.25 g L^{-1} Reactive blue 222, 1.25 g L^{-1} Reactive Yellow 145, 1.25 g L^{-1} Reactive Red 195, 1–25 g L^{-1} Reactive Black 5, 70 g L^{-1} Na_2SO_4, pH 10) [11].

Performances of selected laccases in model wastewater decolourization were evaluated by recording light absorption spectra between 280 and 800 nm at different times (10 min, 20 min, 1 h, 2 h, 3 h and 24 h), and comparing them with the corresponding spectra of controls (the waste incubated with the supernatant of yeast cultures transformed with the empty expression vector). Decolourization was calculated as the extent of decrease of spectrum area recorded between 380 and 740 nm with respect to a control sample. All spectra were recorded after 1:100 dilution of the sample in milliQ water. All experiments were carried out in duplicates, and the mean values were taken. The standard deviation for the experiments was less than ± 5%.

3. Results and discussion

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

3.1. First generation

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

3.2. Second generation

1m9b cDNA was used as template for a second round of EP-PCR at low and medium frequency of mutation [10]. A second generation library of 1200 clones was obtained and screened using the same *criterium* described before. Three mutants, 1L2B, 1M10B (L112F, K37Q, K51N), and 3M7C

(L112F, P494T), were selected showing an activity increase of up to three fold with respect to POXA1b.

Concerning 1M10B mutations, it finds out how directed evolution can get the same result of natural evolution, preserving the properties of mutated amino acids. As a fact, positions 37 and 51 are generally occupied by amidic residues. The mutant 3M7C displays a high activity and an up to two fold increased stability at acidic and neutral pH, as well as at high temperature. 1M10B variant is more stable at alkaline pH (about two fold), whereas its stability is almost equivalent to that of POXA1b in the other tested conditions. The mutation P494T is located in the C-terminal loop that has already been ascertained to affect the function of fungal laccases [4]. MD simulations of this mutant and comparison with the wild-type POXA1b revealed a lower flexibility of the subdomain around position 112 probably responsible of its higher stability. On the other hand, an increased mobility of loops forming the reducing substrate binding site, has been observed in 3M7C leading to higher accessibility of water molecules to the T1 copper site, and to an increased activity of the enzyme.

First and second generation libraries (2300 clones) were then screened for variants with improved activity at pH 5 using either the non-phenolic substrate ABTS, and the phenolic one 2,6-dimethoxyphenol (DMP).

When DMP was used as substrate, two new variants (2L4A and 3L7H) endowed with higher enzyme activity (about three fold increase) than the wild-type laccase were selected [12]. Both mutants doubled the stability of the wild-type enzyme at pH 5. Q272H mutation found in 2L4A may stabilize the protein structure at pH 5 allowing additional interactions – electrostatic and hydrogen bonds – between the positively charged imidazolic ring of His272 and the side chain of Asp287.

After screening with ABTS, one clone, 1L9A, was selected, showing an increase of about three fold with respect to wild-type. Besides the parental mutation (L112F), 1L9A also presents the mutation R284H, located in the loop Gly282-Thr289. This loop may play an important role in protein stability [12]. As a fact, concerning its properties, the mutant increases stability at pH 5 (1.5 fold), while loosing the high characteristic stability of POXA1b at alkaline pH.

3.3. Rational design

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

3.4. Third generation

The increased stability of R4 makes it an appropriate scaffold to carry out directed evolution [15]. In fact, more stable enzymes should also be more susceptible to evolution since they have higher ability to tolerate functionally beneficial but destabilizing mutations. Therefore, directed evolution of R4 was chosen as strategy to improve its performances [13]. A library of 1000 clones with low, medium and high average of mutation frequency was obtained through EP-PCR. When this new collection was screened by assaying activity towards ABTS at pH 3, two mutants, 4M10G and 1H6C, with higher activity (about four-fold increase) than that of POXA1b wild-type were selected. Both mutants display higher stability than POXA1b at pH 5 (almost four-fold). 1H6C also retains R4 stability features at pH 10 and at pH 7. Sequence analyses of the selected mutants led to the identification of the mutations V126I for the 4M10G variant and V148L for the 1H6C mutant, besides those of the parental R4 enzyme. Position 148 is located in a closely packed region of the domain 2 [16] adjacent to the reducing substrate binding site. The substitution of V with the larger L causes a close contact of the leucine side-chain with the aromatic ring of Y208, thus changing the conformation of the loop 204–208 forming the bottom of the reducing substrate binding site [16]. This change could, in turn, influence the oxidation rate of the reducing substrate but also the interaction between the domains 2 and 3 [13].

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

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

3.5. Application of improved laccases

Laccase improvement, along with elucidation of their structure-activity-stability relationships, allows to design and develop more suitable systems for specific industrial applications either in the bioremediation field or for lignocellulose conversion, increasing their real exploitation. An ideal catalyst should be stable and active in extreme conditions of pH, normally found in the industrial wastewaters, as well as able to operate on a wide range of substrates. Here we describe, as an example, the application of mutated laccases to the decolourization of industrial wastewaters. In these experiments, models of acid, direct and reactive dye wastewaters from textile industry – defined on the basis of discharged amounts, economic relevance and representativeness of chemical

structures of the contained dyes [11] – were used. POXA1b variants represent good candidates thanks to their properties best suited for wastewater conditions. Seven out twelve of selected POXA1b variants show an increased decolourization ability with respect to wild-type toward the acid model. As a fact, a two fold increase in decolourization percentage respect to that of POXA1b has been obtained. In particular the mutant 3M7C shows a higher decolourization capacity (up to 40% decolourization after 3 hours of incubation). No significant decolourization has been obtained towards the others analyzed wastewaters, such as reactive and direct models. All the trials have been performed in a period of time compatible with the stability of the protein, being POXA1b and its derived mutants very stable in a wide range of pH [10,12,13].

4. Conclusion

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

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

Though recombinant production yield of *P. ostreatus* native and mutated laccases in the yeast *S. cerevisiae* achieves modest levels (0.2–1 mg l^{-1}), the developed system represents an invaluable tool for rapid screening and selection of new improved laccase variants. Then, the development of new heterologous expression systems in optimised hosts, i.e. filamentous fungi, will provide higher

Table 1
Properties of selected POXA1b variants.

Mutants	Substitution	Activity	Stability				
			pH 3	pH 5	pH 7	pH 10	60 °C
1M9B	L112F	1.5×	–	–	–	–	–
1L2B	L112F, N248Y, N261K, V350I	2.5×	=	+	–	=	=
1M10B	K37Q, K51N, L112F	2.5×	=	+	=	++	=
3M7C	L112F, P494T	3×	+	++	++	=	+
2L4A	L112F, Q272H	2.7×	–	++	=	=	=
3L7H	L112F, S285N, N328S	2.7×	–	++	–	–	=
1L9A	L112F, R284H	3×	–	+	=	–	=
R4	K37Q, K51N, L112F, P494T	2.5×	–	=	+++	++	+
1H6C	K37Q, K51N, L112F, P494T, V148L	4.5×	–	+++	+++	++	+
4M10G	K37Q, K51N, L112F, P494T, V126I	4.5×	–	++	++	+	=
1L10A	K37Q, V48I, K51N,A391T	4.5×	–	+++	–	+	=
3L2A	K37Q, K51N, L112F, S285N	3×[a]	++	=	+++	++	=

[a] This value represents an increase of stability at pH 3 with respect to the wild-type enzyme.

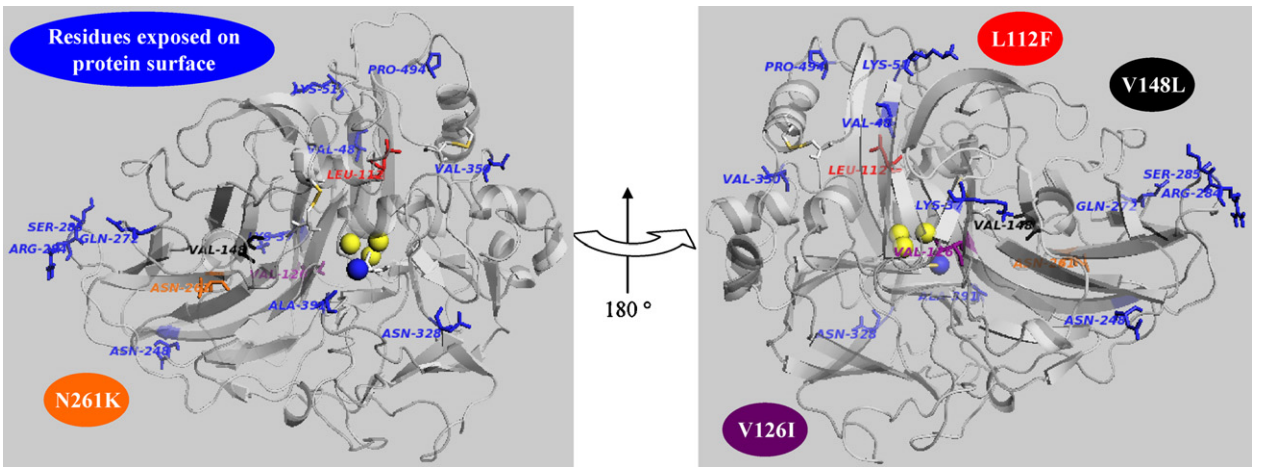

Fig. 1. Ribbon representation of POXA1b model. The mutated residues are in licorice rendering. Trinuclear coppers and T1 copper are highlighted in van der Waals representation (yellow and blue, respectively).

enzyme yields ensuring the exploitation of the selected "tailored" laccase mutant(s) for biotechnological applications on large-scale.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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