DISCOVERING AUTOREGULATOR SYSTEMS IN INDUSTRIALLY USEFUL BASIDIOMYCETES: A NOVEL APPROACH TO IMPROVE FUNGAL BIOPROCESSES BY ENDOGENOUS EXTRACELLULAR SIGNALS

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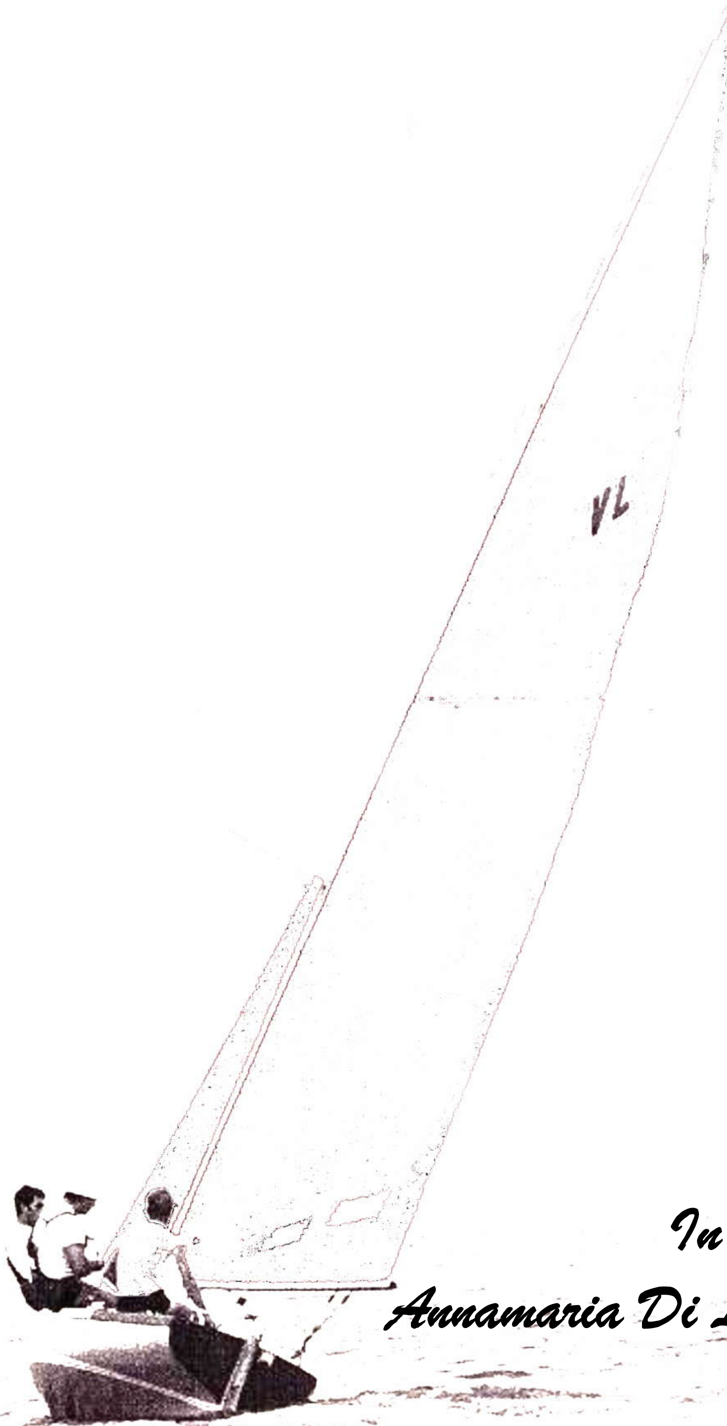
Somewhere, something incredible is waiting to be known.

Carl Sagan



In memory of my aunts

Annamaria Di Lauro, Olga Lettera

and my friend

Sophie Vanhulle

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SUMMARY

Autoregulators are endogenous extracellular metabolites signalling information on environmental conditions or on the status of cells within the mycelium, thus ensuring a coordinated colony function. This mechanism results in the regulation of transitions between alternative morphogenetic and/or functional programmes of the colony as a living unit.

The white-rot fungus *Pleurotus ostreatus* belongs to a subclass of white-rot fungi producing many ligninolitic enzymes such as laccases, MnPs and AAO. Isoenzymes with interesting structural and functional properties are expressed by this fungus and their production is differentially regulated by culture conditions. However, nothing is known about properties of the fungus secondary metabolites and correlation between them and fungal morpho-physiological switch. Regulation of fungal development stages and expression of ligninolytic system through autoregulatory signals may allow controlling fungal phenotype and improving fungal application.

Characterization of the endogenous exo-metabolome of *P. ostreatus* growing in submerged culture model is reported. 11 GC/MS-detected volatile compounds related to shikimic acid pathway (aryl compounds) had been differentially produced during submerged growth. Endogenous exo-metabolites extracted from spent media positively affected the general mechanisms of laccase expression in liquid culture.

Other excreted eight-carbon aliphatic compounds had been previously detected during *P. ostreatus* mushroom fructification and reported in literature.
Effects of some of these commercially available aryl compounds and eight carbon volatile molecules were evaluated in *P. ostreatus* cultures. Fungal pellet morphology, sporal germination, radial growth in agar medium and laccase production profiles in submerged cultures were monitored in presence and absence of each potential autoregulator.

Morphological and physiological analysis showed at least 3 autoregulators involved in different processes. Benzaldehyde indicates a dramatic decrease of nutrient source (starvation) and induces increase of main hyphal length in order to enhance the probability of finding new nutrient sources. 2-hydroxy-4-nitrobenzoic acid is secreted during a long starvation stress. This chemical inhibits mycelium growth and induces, up to 10 times, laccase production in liquid culture. These two physiological reactions can be considered as defence mechanisms in natural behaviours. Finally, 1-octen-3one inhibited basidiospore germination. Analougus mechanism regulating similar sexual processes are reported for other fungal species.

Moreover, the production of new *P. ostreatus* dikaryotic strains with improved efficiencies in laccase expression was performed through inter-crossing different variants. The effect of the 2-hydroxy 4-nitrobenzoic acid autoregulator on laccase production was also tested on the best performing strains. The signal molecule increased laccase production levels confirming that its signalling role is not strain-dependent.
As a result this work allowed the knowledge of autoregulatory mechanisms in *P. ostreatus* in order to control physiological phenomena for industrial purposes such as protein production capability. Because a very efficient heterlogous expression system

in *P. ostreatus* is still not available, this classical natural method, as well as classical breeding, represent valid and eco-compatible methods for bioproductions improvement in comparison with random mutagenesis.

As parallel section a new laccase from *P. ostreatus* phenoloxidase multigene family was isolated from the mature fruiting body and enzymatically characterized for the first time.

RIASSUNTO

I funghi filamentosi hanno vaste potenzialità in molti ambiti di interesse economico, non solo come formidabili produttori di metaboliti secondari, quali antibiotici, chemioterapici o ingredienti alimentari, ma anche come organismi d'elezione nella secrezione di enzimi utilizzabili nell'industria della carta e nel biorisanamento del suolo, delle acque, dei reflui industriali. Purtroppo le applicazioni disponibili per questi organismi sono limitate da numerose problematiche collegate ai relativi processi fermentativi: gli alti costi di produzione correlati a tali microrganismi, o ai loro sistemi ligninolitici, rendono proibitivi i reali campi di applicazione.
É in questo quadro che si inserisce l'opportunità di utilizzare approcci molecolari che consentano di ampliare le conoscenze di specifiche vie metaboliche e delle fasi di adattamento ai segnali extracellulari, nonché di identificare eventuali molecole prodotte dall'organismo stesso con proprietà regolatorie, capaci di veicolare il metabolismo verso determinati prodotti d'interesse e di stabilizzare un certo fenotipo.
I miceli fungini possono essere considerati infatti come complessi ifali capaci di coordinare lo sviluppo cellulare in relazione al microambiente che incontrano, attraverso un sofisticato e multidinamico sistema chemiosensibile.
Recentemente, è divenuto sempre più chiaro che funghi, appartenenti a generi e specie diversi, utilizzino meccanismi di segnalazione extracellulare per regolare alcuni processi come la produzione di antibiotici, la patogenesi e la morfologia.
In funghi filamentosi come in altri organismi il segnale è mediato da molecole accumulate nel mezzo extracellulare sia mediante diffusione passiva attraverso la membrana che per trasporto attivo. Tuttavia, mentre molte funzioni fisiologiche sono state correlate a meccanismi di questo tipo, e numerosi studi sono stati condotti per la caratterizzazione e purificazione di metabolici fungini, le interconnessioni tra questi due aspetti rimangono in buona parte sconosciute.
Il presente progetto di tesi pone come oggetto di studio il fungo basidiomicete *white-rot Pleurotus ostreatus*, le cui proprietà medicali e terapeutiche, nonché le potenziali applicazioni del relativo sistema ligninolitico in campo ambientale e biotecnologico sono ormai largamente conosciute. In particolare tale ricerca si colloca in un quadro più ampio di progettualità volta alla scoperta dei determinanti molecolari che regolano gli stadi dello sviluppo miceliare al fine di migliorare i bioprocessi industriali. Ciò è possibile soltanto raggiungendo una più intima conoscenza del ruolo biologico dei segnali extracellulari negli adattamenti morfologici, fisiologici e metabolici durante le diverse condizioni di crescita fungina.
Il progetto si articola pertanto attraverso il conseguimento di diversi obiettivi di seguito riportati:
1) Caratterizzazione del profilo esa-metabolomico di *P. ostreatus* in condizioni di colture sommerse modello
2) Studio degli effetti di metaboliti endogeni sui vari stadi della crescita fungina nonché sulla produzione di enzimi di interesse commerciale
3) Incremento dei processi di produzione enzimatica mediante miglioramento genetico dei ceppi fungini accoppiato all'uso di autoregolatori extracellulari.

RISULTATI

1) Caratterizzazione del profilo esa-metabolomico di *P. ostreatus* in condizioni di colture sommerse modello

Questa sezione concerne lo sviluppo di un modello fermentativo di crescita basale in coltura sommersa. La formulazione relativamente poco complessa del terreno colturale (a composizione definita, fatta eccezione la presenza di estratto di lievito) è

stata realizzata al fine di semplificare le analisi dell'eso-metaboloma fungino, non solo per ridimensionare il numero di analiti rintracciabili nelle condizioni di riferimento, ma soprattutto per focalizzare la ricerca esclusivamente su metaboliti endogeni prodotti *ex novo*. Ciò ha consentito di escludere l'eventuale presenza di composti prodotti per bioconversione di substrati esogeni ad opera di enzimi extracellulari fungini. Inoltre le condizioni fermentative selezionate hanno permesso di esplorare e monitorale la crescita ifale nella sua totalità, dalla fase esponenziale a quella di stress da affamamento di carbonio, in un arco relativamente breve di tempo.
Sono stati monitorati i seguenti parametri: biomassa secca (g/l), consumo di glucosio (g/l), attività laccasica (U/ml) e proteine secrete totali (g/l). Dai dati rilevati è stato possibile osservare come la produzione massima di enzimi ad attività laccasica sia associata fondamentalmente a due momenti della crescita fungina: il primo collegato al repentino aumento di biomassa miceliare tra il terzo ed il quarto giorno, ed il secondo ascrivibile all'attivazione del metabolismo secondario dopo arresto della crescita del fungo per effetto del depletamento della fonte di carbonio (settimo giorno). Non è da escludere che la produzione di laccasi sia collegata non solo a regolazioni di tipo intracellulare, ma anche a fattori esterni che evolvono proprio in questa seconda fase della crescita e su cui ancora non ci sono dati certi. In natura le laccasi assolvono molteplici compiti non del tutto chiariti e la loro presenza potrebbe essere imputabile a diversi segnali metabolici rispondenti a differenti esigenze del microrganismo. Basandosi sulle considerazioni sopra riportate un accumulo di segnali extracellulari correlati alla fase di affamamento può essere plausibilmente ipotizzato.
Di seguito si è proceduto all'identificazione dell'eso-metaboloma secreto nel terreno di crescita mediante analisi di spettrometria di massa accoppiata alla gas cromatografia: le analisi sono state condotte su campioni prelevati durante tutte le fasi fermentative (3, 5, 7, 10, 13 e 16 giorni).
Su 21 composti rintracciati più della metà risultano strutturalmente correlati alla classe dei **derivati dell'acido benzoico**, spesso differenti da quest'ultimo solo per lo stato di ossidazione, o per la presenza di diversi gruppi funzionali legati all'anello benzenico. La produzione e la secrezione di tali molecole è riconducibile alla via metabolica dei composti arilici già ipotizzata per altri basidiomiceti come *Bjerkandera adusta*.
La produzione, soprattutto nella fase di stress da depletamento di carbonio, di molecole benzil-derivate, strutturalmente correlate ad acido ferulico ed alcool veratrilico, induttori di enzimi ossidativi dei sistemi ligninolitici fungini, ha posto le basi per la ricerca di autoinduttori di laccasi prodotte da *P. ostreatus*. Al fine di verificare l'effettiva potenzialità delle componenti eso-metaboliche sulla regolazione dei sistemi ossidativi extracellulari, sono stati allestiti esperimenti di condizionamento dei terreni colturali basali in concomitanza dell'aggiunta dell'inoculo. Le soluzioni condizionanti sono state ricavate da brodi culturali esausti mediante estrazione in solvente organico: tali mezzi di crescita esausta sono stati prelevati durante l'inizio (7° giorno), nella fase intermedia (10° giorno) e in quella finale (16° giorno) dello stress da carenza di carbonio. Le analisi sono state svolte monitorando le variazioni di biomassa, della produzione di proteine totali secrete e di attività laccasica presente nel mezzo extracellulare: durante il primo massimo di produzione una variazione di 2 e 3 volte superiore alla condizione basale è stata registrata per le fermentazioni condizionate con gli estratti dei mezzi esausti del 10° e 16° giorno (rispettivamente). Nessuna sensibile alterazione è stata invece registrata per ciò che concerne gli altri parametri, indicando che la variazione del profilo di produzione è specifico per

l'attività fenolo-ossidasica. L'assenza di effetti durante la fermentazione condizionata con la soluzione del mezzo esausto vecchio 7 giorni sottolinea, inoltre, una diversa presenza nel tempo di autoinduttori durante la fase di stress.
I risultati sopra riportati hanno fortemente motivato l'indagine sulle proprietà regolatorie dei composti arilici rintracciati proprio in questo arco temporale.

2) Studio degli effetti di metaboliti endogeni sui vari stadi della crescita fungina nonché sulla produzione di enzimi di interesse commerciale

Alla luce dei dati disponibili in letteratura sui metaboliti estratti dal corpo fruttifero e dai pellet miceliari di funghi basidiomiceti e considerando inoltre le analisi svolte sui composti secreti da *P. ostreatus* in colture sommerse, due considerazione possono essere formulate:
esistono classi di molecole strutturalmente correlate appartenenti a vie metaboliche distinte, preponderanti in un certo stadio della crescita fungina;
alcune di queste molecole esplicano un ruolo attivo nella regolazione della morfologia e/o fisiologia del fungo stesso.
Se da una parte *P. ostreatus* produce prevalentemente derivati dell'acido benzoico durante la crescita in terreno liquido, dall'altra è possibile rintracciare dagli estratti del carpoforo prettamente sostanze a catena lineare di 8 carboni derivate dall'acido linoleico.
Alcune ricerche hanno ipotizzato una stretta associazione tra secrezione di proteine in funghi filamentosi e processo di crescita delle estremità ifali, il che sottolinea l'importanza della conoscenza della morfologia del fungo durante la produzione di enzimi extracellulari commercialmente interessanti.
La capacità di controllare parametri della crescita ifale quali la ramificazione, la lunghezza ifale o la velocità di crescita radiale, assumono una nuova importanza se visti sotto l'ottica della massimizzazione della capacità produttiva e secretoria di proteine, sia endogene che ricombinanti. L'identificazione di nuove molecole che possano quindi andare ad influire su tali parametri possono acquisire un interesse biotecnologico fino ad oggi inaspettato.
In questa ottica sono state quindi selezionati, secondo disponibilità commerciale, alcuni metaboliti tra quelli precedentemente identificati per le due rispettive vie metaboliche. Le proprietà dei putativi segnalatori endogeni di *P. ostreatus* sono state verificate sulla crescita del micelio e sulla capacità di influire: sulla morfologia ifale; sulla velocità di crescita radiale; sulla capacità di regolare la germinazione sporale e sulla produzione di laccasi in colture sommerse.
Sono di seguito riportati gli effetti che le singole molecole, testate alla concentrazione finale di 1mM, hanno avuto sui parametri in esame:

	Morfologia (scala microscopica)	Germinazione sporale	Crescita radiale (scala macroscopica)	Attività laccasica (colture sommerse)
Benzaldeide	**Incremento della lunghezza ifale**	Nessun effetto	**Incremento della crescita periferica**	**Induzione in fase esponenziale (2 volte)**
Alcool benzilico	Nessun effetto	Nessun effetto	Nessun effetto	Nessun effetto
Acido benzoico	**Inibizione della crescita**	Inibizione della germinazione	Inibizione della crescita	Diminuzione
Acido 2-idrossi-4-nitrobenzoico	**Forte inibizione della crescita**	**Inibizione della germinazione**	**Forte inibizione della crescita**	**Induzione in fase stazionaria (10 volte)**
4-metossibenzaldeide	Nessun effetto	Nessun effetto	Nessun effetto	Nessun effetto
1-otten-3olo	**Aumento della ramificazione**	Nessun effetto	Nessun effetto	Nessun effetto
1-otten-3one	Diminuzione della ramificazione	**Inibizione della germinazione**	**Rallentamento della crescita periferica**	Nessun effetto
3-ottanolo	Nessun effetto	Nessun effetto	Nessun effetto	Nessun effetto
3-ottanone	Aumento della ramificazione	Nessun effetto	Nessun effetto	Nessun effetto

Basandosi sui dati riportati, per alcune delle molecole sopra elencate è ipotizzabile un loro ruolo fisiologico nella crescita fungina.
La **benzaldeide**, molecola ritrovata in concomitanza con la drastica diminuzione della fonte di carbonio nel brodo di coltura, influisce positivamente sulla velocità di crescita radiale incrementando la lunghezza delle ife primarie senza però aumentarne la ramificazione: questa apparente crescita (di fatto il peso secco del micelio non subisce variazioni sensibili) è spesso riscontrata in situazione di affamamento dove la colonia miceliare estende le proprie ife allontanandosi, alla ricerca di nuove fonti di nutrienti; inoltre vi è un parallelo incremento nella produzione di laccasi extracellulari paragonabile ai livelli indotti dal condizionamento col mezzo esausto, aumento che potrebbe favorire l'attacco di materiale ligno/cellulosico.
Estremamente interessante risultano inoltre gli effetti indotti da nitro-derivati di metaboliti benzilici, identificati per la prima volta come composti ex novo prodotti in *P. ostreatus*: questi composti sono rilevati in soluzione nel brodo durante il processo fermentativo a partire dal settimo giorno di crescita. L'**acido 2-idrossi-4-nitrobenzoico** è probabilmente coinvolto nel tamponamento degli effetti tossici associati allo stress ossidativo/nitrativo (nitrazione delle proteine), stress in cui potrebbe incorrere il fungo durante la fase di lisi cellulare; inoltre questo acido ha un effetto inibitorio sullo sviluppo del microorganismo, precludendo la germinazione delle spore e la crescita ifale in condizioni critiche. Il dato che risulta biotecnologicamente più rilevante è però correlato all'azione induttrice che tale metabolita esplica in colture sommerse. Sia in terreno basale che in terreno ricco (formulato per massimizzare la produzione di enzimi ossidativi), in presenza dell'acido 2-idrossi,4-nitrobenzoico si è registrato infatti un aumento del picco di produzione di laccasi che varia fino ad un ordine di grandezza rispetto al controllo senza variazione effettiva dell'incremento massimo della biomassa.
Al fine di ottenere un quadro completo degli effetti indotti da questa molecola sul secretoma sono stati condotti esperimenti di proteomica differenziale analizzando campioni prelevati in diversi giorni dalle due fermentazioni, allestite in presenza e assenza dell'acido 2-idrossi,4-nitrobenzoico. Le analisi hanno riportato nelle condizioni di induzione, soprattutto per i campioni prelevati al 9° giorno di crescita, una maggiore espressione di numerose proteine, tra cui enzimi ad attività laccasica e

aril-alcool ossidasica. Le lacune dovute ad un'annotazione preliminare e automatizzata del genoma non permettono però una chiara interpretazione del ruolo biologico nella fisiologia fungina di tutte le proteine secrete differenzialmente espresse; tuttavia, i dati relativi ad enzimi ossidativi confermano e ampliano il numero di isoforme sovraprodotte nel mezzo extracellulare appartenenti al sistema ligninolitico. Inoltre l'effetto induttivo si verifica anche sull'espressione di enzimi idrolitici dall'alto valore commerciale come lipasi e proteasi. Nell'ambito dello sviluppo di biosistemi enzimatici, l'uso di composti nitro-benzilici, "naturalmente" prodotti dal fungo, apre quindi la porta all'implementazione di processi industriali maggiormente ecocompatibili, volti alla produzione sia di ossidasi che di idrolasi, classi di enzimi particolarmente interessanti dal punto di vista industriale.
Rivolgendo l'attenzione invece a molecole isolate esclusivamente dal carpoforo, esperimenti effettuati mediante condizionando del mezzo basale con derivati dell'acido linoleico hanno mostrato proprietà regolatorie soprattutto per ciò che concerne il processo germinativo sporale. Dimostrazione delle proprietà autoinibitorie di molecole segnale in relazione allo sviluppo sessuale dei funghi sono note in più di 60 ceppi. Queste molecole sono spesso prodotte durante la fase sporulativa, depositate sullo strato esterno della parete della spora o imprigionate tra le lamelle del carpoforo contenenti i basidi. La loro funzione è chiaramente quella di impedire il prematuro sviluppo miceliare dei nuovi individui nelle zone più limitrofe. Per molti funghi saprofiti il segnale è guidato dalla presenza di molecole costituite da derivati di catene lineari di 8 o 9 carboni, spesso monoinsaturi.
L'1-otten3-olo, sostanza odorosa dal tipico odore fungino, è prodotta in molti funghi superiori come *Agaricus bisporus* e *P. ostreatus*; soprattutto a livello delle lamelle. La sua azione di inibitore della germinazione è stata riportata per il fungo patogeno delle piante da cereale *Pennicillium paneum*; tuttavia non sono noti in letteratura gli effetti di questo composto e di quelli metabolicamente correlati in funghi superiori come *P. ostreatus.* Le analisi effettuate nel presente progetto hanno evidenziato però assenza di regolazione del processo germinativo per ciò che concerne l'1-otten3-olo mentre l'analogo chetonico, **1-otten-3-one**, ha esibito proprietà inibenti a concentrazioni dell'ordine mM. Altri esempi di metaboliti endogeni dello sviluppo sessuale come lo zearalenone sono stati osservati nel fungo *Fusarium graminearum*: anche qui la forma chetonica esplica il suo ruolo sulla fisiologia del fungo mentre quella alcolica (zearalenolo) risulta del tutto inattiva. Considerando i dati proposti in un quadro più generale appare quindi chiaro che i meccanismi di regolazione sono spesso specie specifici e che alcuni elementi strutturali della molecola possono invece essere condivisi da più specie: una stessa molecola prodotta da funghi diversi può avere effetti differenti sui ceppi produttori e composti endogeni non correlati metabolicamente possono invece esplicare ruoli simili in ceppi distinti.
Analisi del pattern isoenzimatico prodotto dalle fermentazione miceliare in colture sommerse in presenza di metaboliti estratti dal carpoforo hanno fornito inoltre nuovi spunti di ricerca. Nelle condizioni sopra descritte risulta infatti variabilmente espressa, anche se in maniera non riproducibile e a concentrazioni al limite della sensibilità delle tecniche zimografiche, una nuova isoforma laccasica. Dall'impossibilità di poter svolgere valide analisi in queste condizioni l'attenzione è stata rivolta allo stadio di sviluppo in cui queste molecole sono naturalmente prodotte in funghi, ovvero durante il processo di fruttificazione.
La sequenza completa del genoma di *P. ostreatus* non esclude che la famiglia genica delle laccasi di questo fungo comprenda ancora altri membri non ancora caratterizzati. Da qui l'interesse verso la ricerca di nuove laccasi da *P. ostreatus*,

finalizzato non solo ad approfondire la caratterizzazione di questa complessa famiglia enzimatica, ma anche ad ampliare la gamma di catalizzatori enzimatici biotecnologicamente interessanti.
Carpofori di *P. ostreatus* sono stati omogeneizzati in tampone fosfato e l'estratto proteico in soluzione è stato purificato, mediante due diverse cromatografie a scambio cationico. Le analisi attraverso elettroforesi su gel di poliacrilammide in condizioni native colorato per attività laccasica hanno rivelato la presenza di questa isoforma ad oggi ancora ignota. Le successive analisi di cromatografia liquida accoppiata a spettrometria di massa tandem hanno identificato la proteina ad attività fenolo-ossidasica come **LACC12**, nome attribuito per analisi *in silico* al relativo gene (progetto *P. ostreatus* genome: http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_1.home.html). La presenza di LACC12 come nuova isoforma arricchisce la famiglia degli isoenzimi ad attività fenolo-ossidasica, differenti per stabilità e specificità di substrato, ampliando le potenzialità che esse possono svolgere in campo biotecnologico. Di seguito è riportata la tabella indicante la Km LACC12 per alcuni substrati modello in relazione a quella delle isoforme già caratterizzate:

	POXA1b	**POXA1w**	**POXA3a**	**POXA3b**	**POXC**	**LACC12**
Km ABTS* (mM)	4.7×10^{-1}	9.0×10^{-2}	7×10^{-2}	7.4×10^{-2}	3.9×10^{-2}	$\mathbf{1.5 \times 10^{-1}}$
Km DMP (mM)**	3.8×10^{-1}	2.1	14	8.8	7.6×10^{-3}	$\mathbf{2.7 \times 10^{-1}}$
Km Syringaldazine (mM)	2.2×10^{-1}	1.3×10^{-1}	3.6×10^{-2}	7.9×10^{-2}	2.0×10^{-2}	$\mathbf{5.5 \times 10^{-2}}$

* acido 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonico)
** N,N-dimetil-p-fenilendiammina

Come è possibile evincere dai dati, l'eterogeneità di specificità per i diversi substrati in comparazione con gli altri enzimi, rendono LACC12 estremamente interessante per studi volti alle reali applicazioni nei bioprocessi industriali.
Questa scoperta pone inoltre nuovi interrogativi sul ruolo specifico che questa laccasi possa svolgere durante il passaggio morfofisiologico che porta alla formazione della struttura riproduttiva del basidiomicete oggetto di studio.

3) Incremento dei processi di produzione enzimatica mediante miglioramento genetico dei ceppi fungini accoppiato all'uso di autoregolatori extra cellulari
Data l'assenza di un efficiente metodo di trasformazione genetica per funghi *white-rot* e impossibilitati dall'utilizzo di metodi di mutagenesi *random,* mediante i quali si producono ceppi non più GRAS, per ottenere una variabilità e un miglioramento delle specie "naturali" di *P. ostreatus* sono stati svolti esperimenti classici di cross-ibridazione. Le basidiospore prodotte per meiosi possono essere isolate e indotte alla germinazione: i corrispondenti monocarionti, caratterizzati per le loro capacità produttive, vengono poi incrociati per determinare i gruppi di compatibilità associati al *maiting*. Le coppie di ceppi compatibili danno vita ad un nuovo dicarionte le cui

proprietà derivano da una combinazione di caratteri multigenetici e/o multiallelici dei ceppi parentali.
In questa sezione di ricerca una collezione di 28 funghi monocarionti derivanti da due ceppi commerciali di P. ostreatus (ATCC 2306 e D1208) è stata prodotta e impiegata per l'ottenimento di nuovi organismi dicarionti, le cui produzioni di attività laccasica in colture sommerse si diversificano notevolmente dai ceppi parentali. In particolare il ceppo **5Ax3D** raggiunge livelli di espressione che superano le **140.000 U/l**, valore che rende tale microrganismo impiegabile in reali bioprocessi su scala industriale.
Al fine di studiare l'effetto del metabolita endogeno 2-idrossi-4-nitrobenzoico, su altri ceppi differenti dal microrganismo modello, sono stati selezionati 4 dicarionti della collezione, 5Ax3D, 5Ax11D, 6Ax11D e 5Ax15A, ed allestite per ciascuno crescite in colture sommerse, in presenza ed in assenza del derivato nitrobenzilico. I dati mostrano anche qui un effetto induttivo del metabolita sul profilo di produzione laccasica, fino a 7 volte superiore rispetto al controllo, per tutti i ceppi ad esclusione di 5AxD3, che risulta invece insensibile alla presenza della molecola. L'assenza di responsività ad induttori endogeni di attività fenolo-ossidasica e l'alta produzione di laccasi relative a questo ceppo fanno presupporre che esso sia deregolato. Tuttavia per gli altri organismi fungini si raggiungono livelli di produzione che sfiorano le 100.000 U/l nelle sole condizioni di induzione.
Il miglioramento dei microrganismi mediante approcci di genetica classica e l'induzione di produzione di laccasi mediante autoregolatori fungini endogeni si sono rivelati quindi sistemi sinergici "naturali", economici ed ecocompatibili per il miglioramento di bioprocessi industriali.

General introduction

0.1 - Primary and secondary metabolites: an ancient issue

In current language, metabolism is defined as the set of chemical reactions that occur in living organisms to maintain life through complex bioprocesses from suitable nutriments [1]. These processes allow organisms to grow and reproduce, maintain their structures, and respond to their environments. All metabolic reactions involve energy transformation and are usually divided into two categories: catabolism breaking organic molecules into smaller compounds with concomitants energy production and anabolism, that require energy to construct cells components such as proteins and nucleic acids or to synthesize energy-storage molecules [1].

The chemical reactions of metabolism are organized into metabolic pathways, in which one chemical is transformed through a series of steps into another chemical, by a sequence of enzymes.

Primary metabolism refers to the anabolic and catabolic processes required for respiration, nutrient assimilation, and growth/development; i.e. those processes required for cell maintenance and proliferation. The main products of this metabolism can be, for instance, polysaccharides, as well as amino acids and proteins.

Under particular conditions, some of these primary metabolites can be transformed into very specific molecules by enzymes involved in side bioprocesses. This is the secondary metabolism, and these products are the secondary metabolites. The term secondary metabolism was coined to describe a wide range of reactions whose products are not directly involved in “normal” growth. In this respect secondary metabolism differs from intermediary metabolism.

In the last century, the function of secondary metabolism was subject to a lively debate; the various theories were compared and contrasted many times [2]. Ancient science considered them as simple waste or breakdown products, food reserves, or evolutionary relics. Another theory was supported by Bu'Lock's maintenance hypothesis: it considers that secondary metabolism keeps cellular metabolism in working order when growth is not possible [3, 4]. From the perspective of this thesis work, the most interesting hypothesis is that secondary metabolites "might function in control of the ordered sequence of events that occurs during differentiation" [5, 6].

Anatomic differentiation and the beginning of secondary metabolism are phase dependent and often occur simultaneously after active growth has ended [7]. In the field of ecological biochemistry the theory that secondary metabolites might serve as an ecological advantage reopened the issue. Studies on plant-plant and plant-other organism interactions showed that secondary metabolites demonstrate a variety of functions in defense, reproduction, and dissemination [8, 9]. According to these studies, secondary metabolism can only be understood in relationship to the interaction of organisms living, competing, and coevolving with each other.

With the maturing of biochemical pathways knowledge we now accept secondary metabolism as a process that must in some manner benefit the organisms in which it occurs [10]. It refers to compounds present in specialized cell’s that are not necessary for the general cells survival but are thought to be required for the survival in the environment: the difference between primary and secondary metabolisms is strictly related to environmental conditions.

Finally, secondary metabolism is recognized to be an activity providing many different intrinsic or extrinsic functions [11]. Intrinsic functions positively affect the growth physiology or reproduction of the producing organism; extrinsic ones impinge on the activities of other organisms and have various roles in regulating the interaction between a producer and its environment.

It is evident from the complex and specialized nature of the biosynthetic pathways by which secondary metabolites are formed that there are wide opportunities for their loss by mutation, and that costs are incurred in maintaining this sophisticated biosynthetic capability: the process must be positively selected in order to persist. Along with selection for persistence of the biochemical pathway there has also been selection for integration of the secondary metabolic activity into the organism's physiology. A substantial volume of research has established the general pattern of physiological control, and at the same time revealed the similarities between control of secondary metabolism and other processes responsive to environmental change [12, 13].
The next decade is certain to witness the clarification of control mechanisms that govern the onset and regulate the intensity of many secondary metabolic processes, thereby giving us potential tools for increasing fermentation yields of antibiotics and other useful products [14]. As metabolic 'add-ons', the pathways we classify as secondary can most plausibly be viewed as having evolved by progressive modification of an available precursor in the direction of metabolic flow, rather than by building up the pathway in reverse through sequential acquisition of new precursors. The former process constitutes inventive evolution by random accumulation of novel metabolites, some of which fortuitously confer a selective advantage on producer organism.

0.2 - Secondary metabolites: regulation, signalling and relationship between microorganisms

Microorganisms have the ability to emit and to receive various short- and long-range signals through excretion of secondary metabolites that can mutually influence cellular behaviour [15]. Such signals can coordinate the behaviour of either individual microorganisms or multicellular communities. Long-range signals are often related to different aspects of the microorganisms' social life. As environmental conditions often change rapidly, bacteria need to respond quickly in order to survive. These responses include adaptation to nutrients availability, defence against other microorganisms which may compete for the same nutrients and avoiding toxic compounds potentially dangerous for the bacteria. It is very important for pathogenic bacteria or yeast during infection of a host (e.g. humans, other animals or plants) to co-ordinate their virulence in order to escape the immune response of the host to be able to establish a successful infection [16]. The most studied example of long-range signalling is *quorum sensing*, which has been described in various bacteria [17, 18, 19].
Quorum sensing was studied for the first time in liquid cultures of the marine *Vibrio fischeri* bioluminescent bacteria and it was observed that the cultures produced light only when large numbers of bacteria were present [20]. The word describes the phenomenon through which the accumulation of signalling molecules enables a single cell to sense the presence of other bacteria and coordinate the expression of specific genes in line with cell density (that is the *quorum*). In the natural environment, there are many different bacteria living together which use various classes of signalling molecules. As they use different languages they cannot necessarily talk to all the other bacteria. Today, several *quorum sensing* systems are intensively studied in various organisms such as marine bacteria and several pathogenic bacteria [21, 22].
Quorum-sensing signals are usually specific for particular microorganisms; for example, *N*-acyl-homoserine lactones in Gram-negative bacteria and post-

translationally modified peptides in Gram-positive bacteria. However, Chen and colleagues have described the existence of a universal signalling molecule, furanosyl-borate diester (AI-2), which is used for interspecies bacterial communication [23]. Quorum sensing regulates diverse physiological processes, including bioluminescence, swarming, antibiotic synthesis, production of virulence determinants in pathogenic bacteria and biofilm formation [23].
Cell density-dependent regulatory networks in microorganisms generally control processes that involve cell-cell interactions, such as group motility [24] and the formation of multicellular structures [25, 26]. In a wide array of environmental and medically relevant bacteria, the development, maintenance, and dispersion of multicellular, surface-associated biofilms are in part controlled by quorum-sensing regulatory pathways [26]. The uptake of extracellular DNA is often regulated in accordance with cell density presumably to enhance the chances of taking up DNA from closely related strains. Both pathogens and symbionts that live in association with plant or animal hosts often use *quorum sensing* to regulate factors involved in microbe-host interactions [27, 28].
As indicated, long-range signalling is usually based on the production of a chemical compound that is spread through liquid or air. Most of the described signalling mechanisms allow individual microorganisms or populations to respond to environmental changes that are associated with nutrient depletion. Such conditions are common in nature but, even in laboratory, microorganisms need to retain properties that allow them to survive under non-optimal growth conditions. It is not yet known whether (and how) these signalling systems differ between natural strains and their laboratory counterparts. The importance of extracellular signalling molecules has also been highlighted by findings in “viable-but-non-culturable” bacteria. Studies on the density and variability of microorganisms that exist in different environments indicate that they represent one-half of all cellular carbon, but that 99% of them are unknown and cannot be grown under standard laboratory conditions [29]; in other words, only a small proportion (1%) of microorganisms that occur in nature can be grown in a laboratory. At least in some cases, these bacteria need specific factors that are produced by other cells for their growth. For example, after long-term (several months) starvation, *Micrococcus luteus* bacteria convert to dormant cells that are able to survive for long periods of time but have lost the ability to grow on agar plates. This ability is restored by adding the specific Rpf resuscitation factor, which is the peptide that is produced by a growing *M. luteus* culture. Rpf homologues have been identified in other bacteria, including those of *Mycobacterium* spp. [30]. Therefore, in nature, a large proportion of bacteria probably survive long periods of starvation and other stresses as dormant cells, and only continue to grow after receiving a signal from growing individuals that indicates a possible favourable change in the environment.

0.3 - Fungal metabolism and natural products

Filamentous fungi, through most of their life cycle (Fig. 0.1) consist of hyphae, cylindrical cells that growth at one end. The start point of typical fungal life cycle start can be considered the germination of a spore when it finds suitable environment conditions [31].
The germ tube emerges as slender hypha that grows and branches and branches turn in branch, to form a modular system known as mycelium. Under natural environmental conditions, or in culture, growing fungi take from their surroundings those nutrients that they can use easily as energy sources to produce materials such

as proteins, lipids and nucleic acids, for continued growth and biomass production (primary metabolism). This allows them to proliferate and colonize new regions to obtain more nutrients. Primary metabolites are formed during the active growth of the fungus and these will accumulate in the surroundings if growth becomes restricted or when supplies of key nutrients are depleted. The majority of fungi produce very similar primary metabolites. These contribute to the biosynthesis of the building blocks of fungal mycelium. Some primary metabolites have commercial importance and large scale cultures are grown industrially with the specific purpose of obtaining large quantities of these fungal products including organic acids such as citric acid (used in food and soft drink manufacturing) [32], ethanol (used in alcoholic drinks production) [33], enzymes (e.g. pectinases, glucose isomerase and lipases used in food processing) [34] and amino acids and vitamins (food supplements) [36].
As nutrient depletion or other conditions in particular environments occur, growth of the fungus slows down and parts of the mycelium switch to using different biochemical pathways [37]. This alteration in metabolism prevents the fungus from poisoning itself and maintains the biochemical machinery of the cells. Primary metabolites and intermediate compounds which have accumulated in the fungus are converted to different products (secondary metabolites) which are not normally made during active growth and are not essential for vegetative proliferation. Some of these secondary metabolites, but not all of them, are complex molecules and are produced when the fungus is not actively growing [38]. These compounds may be synthesized in parallel with differentiation and sporulation of the fungus and are often indicated in literature as natural products.
The fungal kingdom consists of many species with unique and unusual biochemical pathways. Products include useful economically important pharmaceuticals, such as antibiotics (e.g. penicillin, cyclosporine) [39] and statins [40], potent poisons extremely harmful to man and animals, including aflatoxins and trichothecenes mycotoxins [41] and some janus–faced metabolites that are both toxic and pharmaceutically useful, such as ergot alkaloids [42].
Secondary metabolites are more species-specific and products are often unique to a particular species [43]. For this reason it is likely that although a very large number of fungal secondary metabolites have now been identified many more will be described in future as the activities of more fungal species are investigated. The rate of sugar consumption in primary metabolism may reflect the production of a primary metabolite but this is not true for secondary metabolite production. Most secondary products are formed at the end of active growth and derive from primary products which were synthesized earlier.

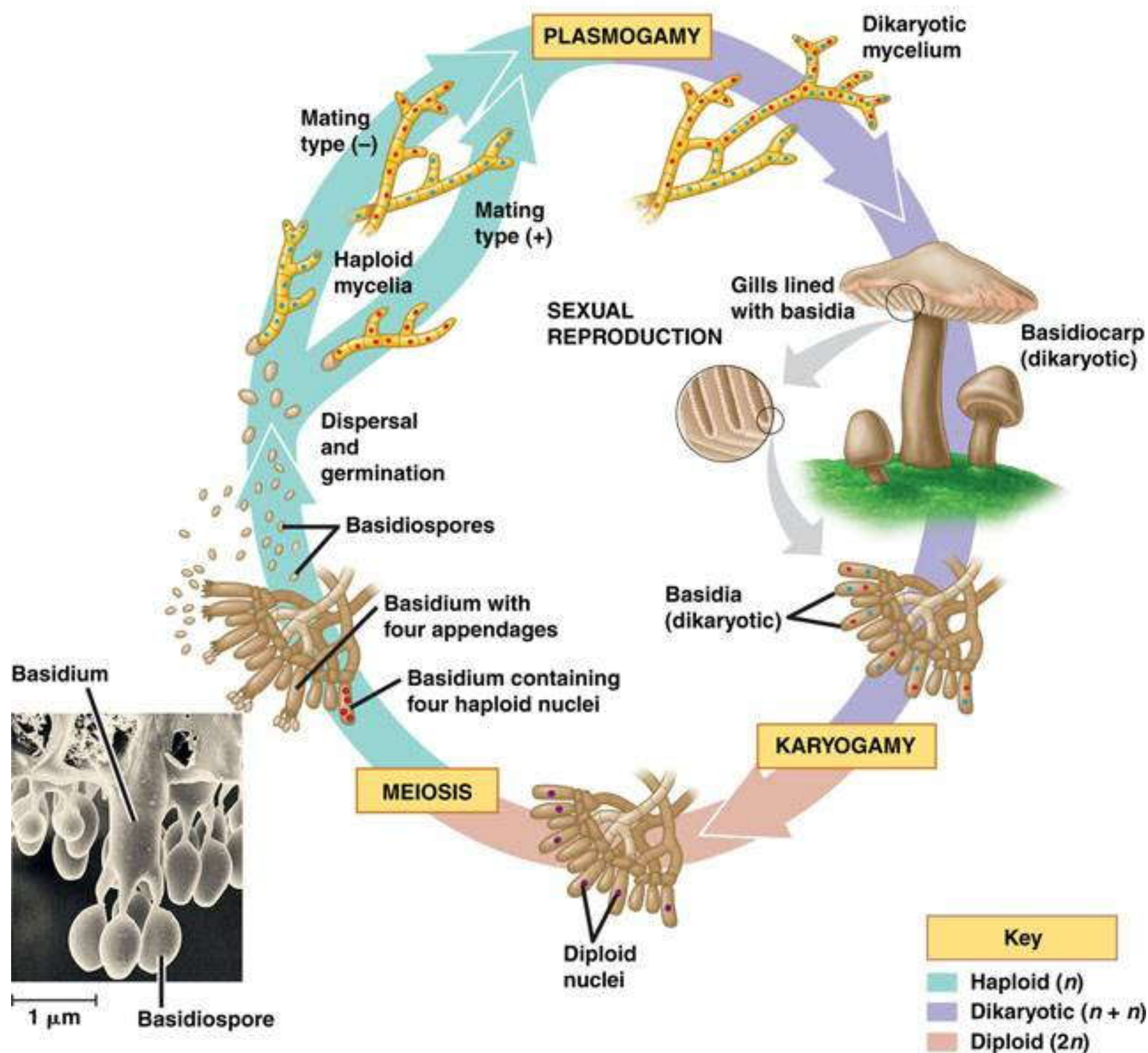

Fig. 0.1: life cycle of basidiomycete fungus from spore germination to fruiting body formation trough asexual and sexual development.

The principal pathways of secondary metabolites are discussed below.

Polyketide pathway seems to have no other role except in secondary metabolism [48]. The precursor is acetyl-CoA, which is carboxylated to form malonyl-CoA (a normal event in the synthesis of fatty acids), then three or more molecules of malonyl-CoA condense with acetyl-CoA to form a chain. This chain undergoes cyclization, then the ring systems are modified in different ways to give a wide range of products, including the antibiotic griscofulvin, the aflatoxins and the ochratoxins

Another important pathway in fungi is that of isoprenoid pathway, used normally for sterols synthesis [49]. Again, acetyl-CoA is the precursor, but three molecules of this condense to form mevalonic acid (a six carbon compound) which is then converted to a five carbon isoprene unit. The isoprene units condense head-to-tail to form chains of various lengths, then the chains undergo cyclization and further modifications. The products of this pathway include the mycotoxins of *Fusanum spp*. growing on moist grain.

The **shikimic acid pathway**, used normally for the production of aromatic amino acids, provides the precursors for the hallucinogenic secondary stools of *Psilocybe*,

and the toxin muscarine in toadstools of *Amanita muscaria* [50]. Yet other pathways lead from aliphatic acids to **eight carbon (C8) volatile compounds**, and especially to the aliphatic alcohol contributing to the typical mushroom flavour in fruit-bodies of several basidiomycota [51].

0.4 - Ecology in filamentous fungi: how they interact with other organism and with themselves

The mycelium is a very successful colony form capable of orchestrating the development of different cell types in response to the microenvironments which it encounters. This attribute in particular has led to understand that the mycelium is governed by a sophisticated chemosensitive system.
Many of the elements of this system have probably been already listed after several decades of intense research, but the functional connections between them remain largely undiscovered. On the one hand, many reported colony functions have been ascribed to as yet unidentified chemical signals, whilst on the other, many thousands of molecules have been purified and characterized, of which relatively few have been credited with a biological role [52].
Those signals recognized so far comprise two large categories. First, those coordinating relations with other organisms, such as hosts, and including elicitors and inhibitors, competitors involving antibiotics, and predators. Other complex symbiotic relationship between plant; animals will be not consider in this section. The second category consists of signals that are useful to coordinate mycelium colony in order to regulate sexual and asexual development, hyphal growth and other physiological processes.

0.4.1 - Relationship between basidiomycetes and bacteria

Bacteria play an important role in the functioning of lignocellulose-degrading basidiomycetes. They can have a negative effect on fungal growth and activity as they are potential competitors for low-molecular weight compounds released by extracellular fungal enzymes [53]. On the other hand, basidiomycetes may benefit from the presence of bacteria, in particular with respect to nitrogen supply and detoxification of mycotoxic compounds [54, 55]. During degradation of wood by basidiomycetes, the environmental conditions become very selective for bacteria because of rapid and strong acidification, production of reactive oxygen species and the presence of toxic fungal secondary metabolites [56]. Bacteria surviving these conditions must have special properties, but research on this is still in its infancy. Moreover, fruit body formation of several edible mushrooms is dependent on the presence of certain bacteria [57].

0.4.2 - Inter-relationships among basidiomycetes

Into the wild, life is a war to survive and fungi often have to fight not only against organisms of different kingdoms but also species of same genus. The ability of the interacting fungi to capture and defend territory and resources is achieved through antagonistic combative mechanisms [58]. Such interactions are realized by hyphal contact whereas some others can be mediated at a distance.
When two fungi grow in close proximity, changes in both mycelial morphology and secondary compound chemistry occur, resulting in the formation of characteristic 'barrages' and colour changes in the mycelia of both species. These changes are mediated by up-regulation of genes involved in antagonism [59], resulting in production of stress compounds, enzymes and low molecular weight secondary

metabolites, in the hyphae and into the surrounding environment. The production of such metabolites by one species may have deep effects on other species, mediating antagonism at a distance or after contact, or leading to attraction or stimulation of growth.
These chemicals may have differential effects on species attempting to colonize. Some species growth can be inhibited or slowed down, while other species can be apparently unaffected or positively effected [60]. Moreover, the composition of the secondary metabolites produced by a given species may alter when the presence of a potential competitor is detected [61, 62]. In ecology, these secondary metabolites must be produced in a sufficient quantity or they sufficiently persist in the environmental niche so that to effect growth on other organisms.
For example the antimicrobial compound methylbenzoates produced by the brown rot basidiomycete *Sparassis crispa* is present in occupied wood in large quantities, and persist for many years, even following death of the host tree [63].
Activity of these compounds against other fungi varies reducing growth of different fungi or stimulating other species.
In addition to diffusible secondary metabolites, fungi also produce volatile compounds [64], some of which may impact on potential competitors. Volatile compounds can induce changes in the behaviour of potential competitors at a distance from the producing organism. Changes appeared to be related temporally with pigment production, mycelial morphology and/or protein synthesis variations [65].
During fungal–fungal basidiomycetes interactions, up- and down-regulation of particular enzyme groups occurs. Enzymatic production of reactive oxygen species, ligninolitic enzymes and sometimes β-glucosidase increases [66, 67, 68, 69 70]. Laccase enzymes appear to increase in contact zones between interacting decay-causing species, with different temporal effects depending on the species tested. For example, laccase activities increased in cultures of *Trametes versicolor* and *Pleurotus ostreatus* following challenge with soil microorganisms, including fungi, bacteria and soil or soil extracts [66]. Laccase does not directly affect decay fungi [66]; its role is probably defensive [66].

0.4.3 - Fungal autoregulators: how fungi talk to themselves

In-house signals include pheromones, which facilitate the interaction of compatible gametes, and developmental hormones regulating the formation or maintenance of differentiated multicellular structures or protoorgans (ascomata or basidiomata) [71]. Excluding the above-mentioned, there is an array of endogenous signals which transmits information on environmental conditions or on the status of cells within the mycelium, thus ensuring a coordinated colony function. These signals are transmitted by extracellular metabolites and their signalling action results in the regulation of transitions between alternative morphogenetic and/or functional programmes of the colony as a living unit.
Signalling molecules performing equivalent tasks in other simpler microorganisms such as bacteria, have already been defined as autoinducers, autoinhibitors, *quorum sensing factors* and morphogens. Based on functional viewpoint, Ugalde proposes to term them collectively as autoregulators [72]. The concept of *quorum sensing* has become popular after the emergence of the existence of cellular communication among prokaryotic cells. Even though equivalent small molecular weight signaling agents are known to act as fungistatics for some time, the role of autoregulatory molecules may span beyond the *quorum sensing* concept in eukaryotic microbes,

such as fungi [73]. Autoregulators may indeed compose a sophisticated set of chemical cues which report on environmental conditions to the producing cell or cell cluster and help to determine colony growth, morphology and, more in general, relations with surrounding behaviours. Autoregulators differ from hormones and pheromones in that they do not necessarily target a different cell type and for the fact that the information they convey is not strictly related to mating.
Based on general traits of fungal life cycle, a correlation between morphology-physiology and autoregulators has been shown as follows. Starting from chemosensory mechanisms of fugal spores several autoregulatory signals are involved in indicating the potential success of germination. The first demonstration that spores "sense" and respond to an overcrowded environment through self-produced chemical signals - *autoinhibitors* or *self-inhibitors* – [74], came from studies with the rust *Puccinia graminis* [75]. These autoregulatory signals have been specifically identified and reported for several fungal species [76]: some of them are shown in figure.0.2.

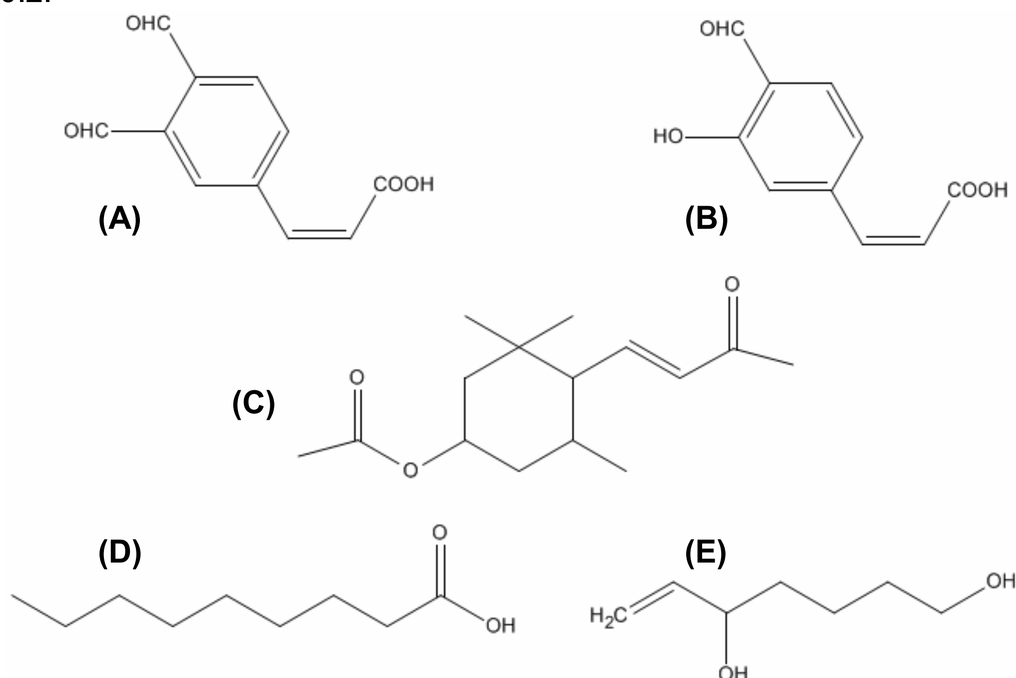

Fig. 0.2 autoregulator molecules involved in fungal germination: A cis-methyl 3,4 dimethoxycinnamate, B cys-methyl ferulate, C quiesone, D nonanoic acid, and E 1-octen-3-ol.

Germination autoinhibitors are produced at the time of sporulation, and deposited on the outer wall layers of spores, but their high partition coefficient in liquid and gas phases enables the signal to spread up to other spores in the surrounding behavior. Several fungi appear to use chemicals derived from the same pathway or from structurally related ones: some examples are methyl-cis-3,4-dimethoxycinnamate and methyl-cis-ferulate produced by the plant pathogenic fungi *Uromyces phaseoli* and *Puccinia graminis* [77, 78] respectively or 1-octen-3-ol produced in the cereal pathogen *Penicillium paneum* [79] related to nonanoic acid produced and "sensed" by spores of many soil fungi [80].
Conversely, in order to maximize the opportunity of compatible spores to form a dikaryon, some fungi can also produce germination autoinducers, such as 3-methylbutanoic acid produced by *Agaricus bisporus*.
In the subsequent phases of mycelium development, which contribute to form the structure shown in figure 0.3, the hyphal growth process is regulated by outward

aerotropism, chemotropism and autotropism. The formation of lateral branches in particular is induced by the presence of other hyphae and moreover, once formed, they are attracted to each-other [81] Other studies using *Aspergillus oryzae* have shown that conditioning fresh medium by spent medium (filtered medium obtained by harvesting the same fermentation after several days of growth) resulted in increased apical extension growth and branching [82]. However the chemical nature of these signals has still to be determined. The combined evidence indicates that autoregulators are involved in various stages of the colony morphogenesis, despite our current ignorance of their chemical identity.

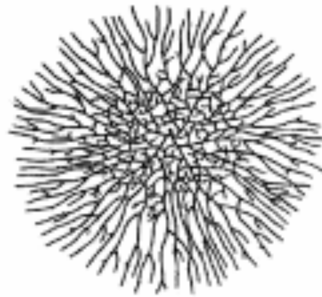

Fig. 0.3 mycelium pellet formed from a single fungal spore.

In addition to the mycelium formation, at least other two mechanisms are affected by autoregulators during the fungal life cycle: asexual sporulation, serving as short-term dispersal propagules, and sexual sporulation obtained by meiotic divisions and which is able to resist to enduring changes of environmental conditions.
Two molecules were reported to affect conidiation (asexual sporulation) in ascomycete species. Conidiogenone is a diterpene constitutively produced by *Penicillium spp.* [83]: when aerial hyphae emerge from the bulk medium, local concentration of the autoregulator molecule close to the hyphal surface increases up to threshold induction levels. In order to avoid non essential long-term signal accumulation, conidiogenone is converted to conidiogenol, the corresponding alcohol results in an inactive derivate. Another self-generated metabolite in *Aspergillus terreus* is Butyrolactone I, a small γ-butyrolactone derivative similar to N-acyl homoserine lactone (*quorum sensing* factor in gram-negative bacteria), stimulating hyphal branching and sporulation [84].
Examples of metabolites regulating asexual/sexual development balance are oxylipin called *Psi* factors A, B and C: the first molecule shifts the balance towards asexual spore formation whereas the other two chemicals stimulate sexual spore development [85]. The last autoregulator presented here is zearalenone. It is produced by *Fusarium graminearum* and induces ascocarp formation at a very low concentration in this strain [86]. As previously stated for what concerns another autoregulator, ketonic form is active whereas the relative alcohol results in an inactive derivative.

0.5 - Prospects of potential application of the white-rot basidiomycete *Pleurotus ostreatus* in green and white biotechnology

Fundamental and applied researches on basidiomycetes have significantly increased in recent years due to their potential use in a variety of biotechnological and environmental applications, particularly for the production of enzymes, dietary supplements, physiologically active compounds, and bioremediation [45]. The edible white-rot basidiomycete *Pleurotus ostreatus* is a biologically versatile mushroom

which could therefore serve as the basis for various biotechnological purposes [87]. It is the third most important cultivated mushroom for food market. Nutritionally, it has unique flavour and aromatic properties and it is considered to be rich in proteins, fibres, carbohydrates, vitamins and minerals. Moreover, it produces several polysaccharides showing medicinal proprieties, such as, antiviral, antitumor, antibiotic, hypocholesterolic and immunomodulation activities. The most important *Pleurotus ostreatus* features are related to the use of its ligninolytic system for a variety of biotechnological applications [87]. Wood-rotting fungi have evolved complex systems of lignin decomposition: they are catalyzed by a set of oxidases and peroxidases with auxiliary enzymes providing hydroxyl radicals, but they also include the provision of enzyme co-substrates such as organic acids or aryl alcohols. The *P. ostreatus* ligninolytic system is composed by different enzymes as reported below.

Manganese-peroxidases (MnP, EC 1.11.1.13) are heme proteins with molecular masses of 47–60 kDa, glycosylated, and have usually acidic pH optima. Although MnP is able to oxidize phenolic substrates, it more frequently oxidizes Mn2+ to Mn3+ which is stabilized by organic acids such as oxalate, malate, lactate or malonate. The chelated Mn3+ is diffusible and can oxidize a wide range of substrates including phenols, nonphenolic aromatic compounds, carboxylic acids, thiols and unsaturated aliphatic compounds (e.g. fatty acids). The initial oxidation can be followed by a sequence of radical-based or oxidative reactions leading to lignin decomposition and mineralization [88].

Versatile peroxidases (VP, EC 1.11.1.7) are heme containing enzymes, structural hybrid between MnPs and lignin peroxidase, since they can oxidize not only Mn2+ but also phenolic and nonphenolic aromatic compounds including dyes, in manganese-independent reactions [89]

Aryl-alcohol oxidase (AAO, EC 1.1.3.7) are other extracellular enzymes involved in wood lignin decomposition generating H2O2, and **aryl-alcohol dehydrogenases** (AAD, EC 1.1.1.91), are mycelium-associated enzymes reducing lignin-derived compounds [90].

Laccases (EC 1.10.3.2) typical proteins having a molecular weight of 50–70 kDa with acidic pH optima (3.0–5.5 for 2,6-dimethoxyphenol and 4.0–6.0 for guaiacol). *P. ostreatus* produces several isoforms of laccases, codified by a laccase gene family [91]. The produced related proteins are slightly different in their activities, which is probably associated with their physiological functions. Laccases are mainly involved in lignin transformation but they are also reported to be important virulence factors in many fungal diseases by protecting fungal pathogens from toxic phytochemicals [92]. Laccases can be also involved in the transformation of phenolic compounds having a low-molecular weight and which are produced in the cell. Moreover laccases located in the cell walls and the ones associated to spores can be involved in the synthesis of melanin and other substances protecting the cell walls [93, 94]. In the frame of mushroom ecology and physiology, compared to the complexity of ligninolytic enzymatic system, far less is known about its regulation [91].

A schematic representation of processes involved in the decomposition of lignin by *P. ostreatus* is reported below (Fig. 0.4).

Owing to their high and non-specific oxidation capacities, the lack of a requirement for cofactors and the use of readily available oxygen as an electron acceptor, laccases are useful biocatalysts with well-established or emerging biotechnological applications.

In food industry, the selective removal of phenol derivatives by laccases is exploited for stabilization and improvement of the quality of different drinks [95]. Laccases can be applied also in baking due to their ability to cross-link biopolymers. In pulp and paper industries the treatment of wood pulp with laccases, provides milder and cleaner strategies of delignification which are also respectful of the cellulose integrity [96]. The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibres in order to improve their chemical or physical properties. In textile industry laccases are used to bleach textiles and even to synthesize dyes [97]. Several applications are performed in the bioremediation field degrading xenobiotics contained in wastewaters, olive mill wastes and coffee pulp [98]. In the green biotechnology field, laccases have been suggested to be applicable for transformation of phenolic and related compounds, oxidative deprotection reactions, oxidative coupling reactions for the synthesis of the pharmaceutically compounds, oxidative cross-coupling reactions, polymerization reactions and oxidation of alcohols and other functional groups [99].

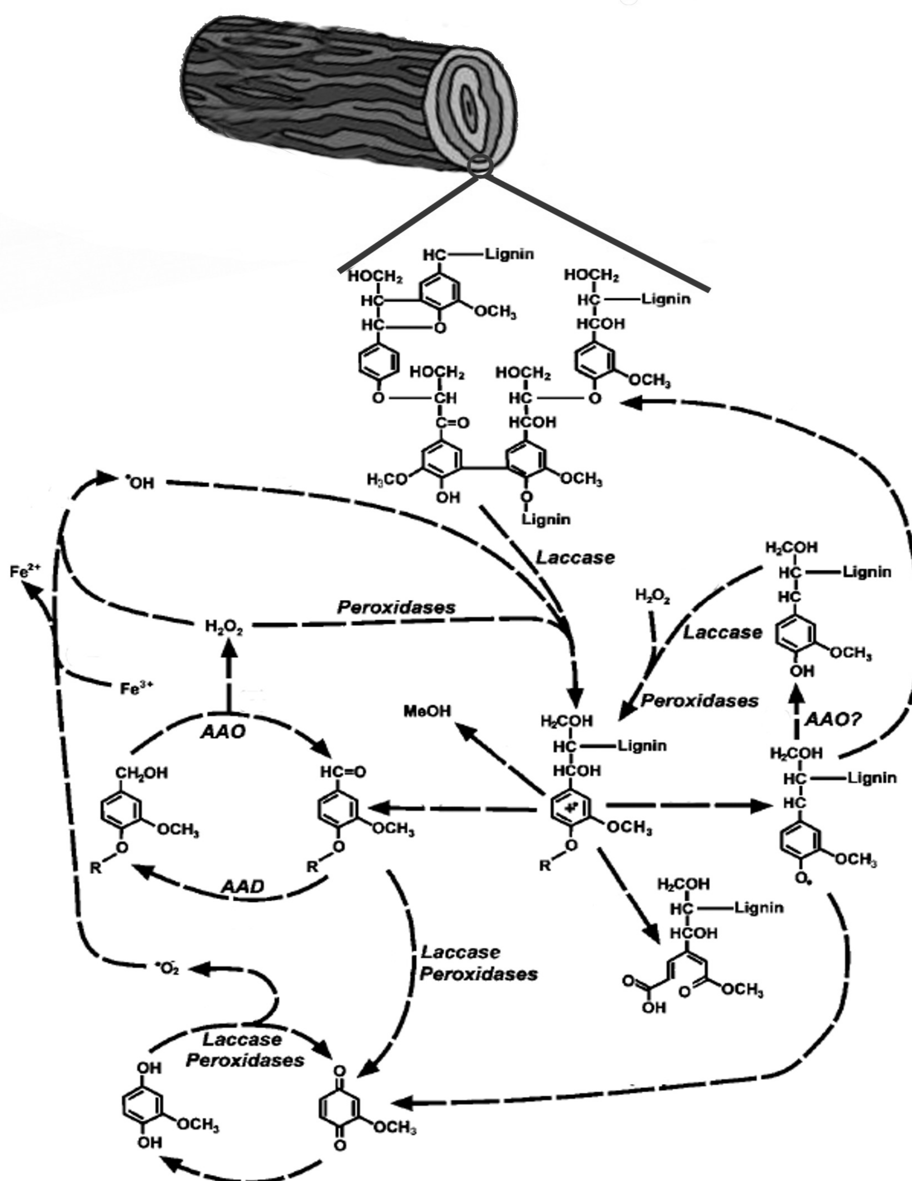

Fig. 0.4 representation of processes involved in the decomposition of lignin in *P. ostreatus.*

0.6 - Application of fungal autoregulative mechanisms to industrial biotechnological processes

A major problem for biotechnological industry involved in the production of microbial products is to be able to maintain the productivity at production-scale for what concerns successfully produced products in small-scale. This is an on-going problem which has been so far addressed through adopting traditional "rule-of-thumb" and engineering approaches. While these strategies have proved useful in the production of some bioproducts such as antibiotics and enzymes, successful production and overproduction of many novel useful products (e.g. a range of enzymes) has proved less than satisfactory [100]. The established approaches for overproduction and scale-up have serious limitations which have caused delays in marketing of some of the desirable products and in some cases, termination of otherwise promising projects leading to very useful products. This has lead to disadvantages for the consumers and loss of financial opportunities for the industry. The reason is that these strategies are time consuming and very expensive with only partial success.

While considerable attention has been devoted over the years to solve the above-mentioned problems, most of the strategies have arisen from approaching the problem from a "macro" scale view, very little attention has been devoted to investigating growth behaviour in terms of microbial communication in the community [100]. Moreover, production of many biocompounds is connected to a specific state of fungal physiology (e.g. spore production, sexual development, hyphal elongation, etc.) [101]. The question is how cell-cell communications could be exploited for designing economically feasible strategies for overproduction and successful scale-up of industrially useful microbial bioproducts.

As previously stated, laccases belong to an important class of enzymes with wide utility in many biosectors and, recently, particularly for environmental bioremediation purposes (section 0.5). However, the use of laccases in industry is limited because of high production cost. Yield and productivity are critical for the economics and viability of bioprocesses laccase-based.

Regulation by extracellular signal transduction correlated to the development of filamentous fungi and application of fungal autoregulators to industrial processes are new promising and exciting topics in the fields of molecular and industrial biotechnologies.

0.7 - Aim of the thesis

The white-rot fungus *P. ostreatus* belongs to a subclass of white-rot fungi that produces many ligninolitic enzymes such as laccases, MnPs and AAO. Isoenzymes with interesting structural and functional properties are expressed by this fungus and their production is differentially regulated by culture conditions. Conversely, nothing is known about properties of the fungus secondary metabolites and correlation between them and fungal morpho-physiological switch. In the frame of the "**QUORUM**: **Discovering Quorum Sensing in industrially useful Fungi, a novel approach at molecular level for scaling-up in white biotech"** Euorpean project (STREP-FP6-279 NMP4-CT-2006-032811 financed by sixth framework programme *Using nature as a model for new* 280 *nanotechnology-based processes*: NMP-2004-3.4.1.2-1), research has been targeted to understand autoregulation mechanisms in *P. ostreatus* and acquire knowledge for developing and improving fungal bioprocesses.

The description of work has been organised according to the following chapters:

1. Characterization of the endogenous exo-metabolomic profile on a submerged growth model of *Pleurotus ostreatus.*
2. Identification and characterization of *P. ostreatus* autoregulators affecting different morpho-physiological states.
3. Enhancement of eco-friendly enzymatic production combining classical breeding and autoregulation mechanisms: effects of autoregulators in different strains of *P. ostreatus*

0.8 - Reference

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Chapter 1:

Characterization of the endogenous exo-metabolomic profile on a submerged growth model of *Pleurotus ostreatus*

SUBMITTED TO INTERNATIONAL MICROBIOLOGY

The following chapter reports the description of experiments and theoretical study accomplished during investigation on *P. ostreatus* endogenous exo-metabolites produced in a model fermentation. The chapter consists of a paper concerning this research line submitted to *International Microbiology* journal for publication:

Improving fungal laccase production by autoinduction mechanisms: the influence of endogenous exo-metabolites on *Pleurotus ostreatus*

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SUMMARY: Metabolomics, is an emerging powerful approach to better understand microbial physiology and facilitate metabolic pathways in order to increase production of specific proteins and fine chemicals in industrially useful microrganisms. Secondary secreted metabolites are probably produced as a response to the microenvironment and to coordinate intra- and inter-species relationships. In this study, exo-metabolomics was used to elucidate the regulation of secreted protein production of the white-rot fungus *Pleurotus ostreatus* by extracellular signals. Time course samples of fungal basal submerged cultures were analyzed by GCMS and volatile compounds related to the shikimic acid pathway were identified. Laccase secretion was stimulated by supplementation of extracted spent media to the basal broth showing a threefold increase of laccase activity. This study has allowed new insights in improving laccase production by autoregolative endogenous compounds: the plasticity of fungal exo-metabolome related to excreted benzyl-derivatives compounds and fungal physiological state has been demonstrated.

Keywords: *Pleurotus ostreatus*; benzyl-derivatives ; shikimic acid pathway; laccase; autoregulators

INTRODUCTION

The white-rot basidiomycete fungus *P. ostreatus var. florida* is one of the most active micro-organisms degrading lignin, a complex aromatic biopolymer that is extremely recalcitrant to degradation [18]. This fungus produces different oxidative enzymes, with broad substrate specificity, which can also be used to degrade a vast range of toxic aromatic pollutants [14, 29]. Among these enzymes the production of several laccase (E.C. 1.10.3.2 isoenzymes is prominent [28]. The variety of laccase isoenzymes is related to the diversity of their roles: lignin synthesis/degradation [18, 26], fruit bodies development [19], pigment production [24], cell detoxification [8], etc [3]. Moreover, laccases are of extreme biotechnological interest because of their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants. These features are suitable for several different applications in fields such as detoxification of industrial effluents, medical diagnostics, bioremediation agent to clean up pesticides and explosives in soils, and

additive in cosmetics [20]. Costs of laccase production and downstream processes are economically not sustainable: this is the main reason why many researches are focused on understanding regulation and improving expression of laccases in fungal bioprocesses.
P. ostreatus is also known to produce many volatile compounds during fermentation on liquid and solid media. It has been previously suggested that extracellular aromatic compounds are produced as effect of lignin degradation through fungal secreted activity [6]. However, many evidences strongly indicate that *Pleurotus spp.* shows the ability to synthesize lignin-related chemicals [34]. The reasons why fungi produce secondary metabolites are still unknown and most of them have not been credited with a biological role [4]. Secondary secreted metabolites are probably produced as a communication pathway direct from or to the microenvironments which mycelium encounters, and are targeted against, or support actions on, receptor systems; others functions may include chemical signalling between organisms [32].
Many secreted metabolites have been identified from solid and liquid cultures of filamentous fungi and metabolic profiling has been used for fungal identification or to revise the taxonomy [33]. Volatile compounds produced by *P. ostreatus* were extensively analyzed and identified in fruiting body and mycelium and for some of them an antibiotic activity against moulds and bacteria has been demonstrated [25, 9]; however, no additional function of secreted chemicals indicated involvement in improving production and organisms survival fitness and in playing a role in autoregulatory mechanisms.
Although no comparative analyses about dynamic variation of exo-metabolites during fermentation in submerged culture was performed, it is evident that under conditions of nutrient limitation, morphological alterations and mycelium development variations in secondary metabolism dynamically occur.
This study aimed at characterizing the effects on growth and on secreted protein production profiles, mainly laccases, by conditioning fresh media with compounds extracted from spent media (obtained by harvesting cultures during fermentation time course) and at investigating production of major volatile compounds secreted at different growth days by GCMS. Regulation effects of excreted endogenous compounds on laccase expression in liquid fungal culture are reported. On the basis of fungal metabolomics data, available in literature [21], a common biosynthetic pathway is proposed.

MATERIALS AND METHODS

Organism and culture conditions

Dikaryotic strain of *P. ostreatus* (Jacq.: Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) was maintained through periodic transfer at 4°C on potato dextrose agar plates in the presence of 0.5% yeast extract (Difco).
Mycelium was grown in 1 l shaken flasks (125 rmp) containing 300 ml of GYM (Glucose, Yeast extract, Mineral solution) broth containing 10 g/l glucose; 3,8 g/l yeast extract (Difco) 2 g/l H_2KPO_4; 0,5g/l MgSO4 $7H_2O$; 0,1 g/l $CaCl_2$ $2H_2O$; biotin 10 mg/l; thiamine 10 mg/l and 10 ml of mineral stock solution (0,5 g/l $MnSO_4$ $5H_2O$; 1 g/l NaCl; 0,1 g/l $FeSO_4$ 7 H_2O; 0,1 g/l $CoCl_2$ 6 H_2O; 0,1 g/l $ZnSO_4$ 7 H_2O; 0,01 g/l $CuSO_4$ 5 H_2O; 0,01 g/l $AlK(SO_4)_2$; 0,01 g/l H_3BO_3; 0,01 g/l $NaMoO_4$ 2 H_2O); final pH5.
Except where indicated, all chemicals were obtained from Sigma Chemical Co. 5-day-old culture were homogenized by Ultra-Turrax® T25 Basic interconnected with S18N-19G dispersing tool (3 flashes of 30 seconds at 24.000 rpm separated by 30

seconds of stand-by) and 1 milliliter of homogenate was transferred in 1-l flasks containing 300 ml of GYM broth. The cultures were grown in shaken flasks at 125 rpm and incubated at 28°C in the dark for 18 days.

Conditioning by spent media

The procedure reported below in “liquid-liquid extraction” was used to prepare conditioning solution SM7, SM10 and SM16 (spent medium 7, 10 and 16 days old, respectively): 300 ml samples were concentrated up to 1000 times, sterilized by filter membrane (cut-off 0,22 µm, Millipore®) and used to condition 300ml of basal medium. GYM basal medium conditioning was performed using extracted spent medium solution supplemented at the time of inoculation.

Benzaldehyde, used to conditioning basal growth (1mM final concentration), was obtained from Sigma Chemical Co.

Protein, biomass and enzyme activity determinations.

Protein concentration was determined using the BioRad protein assay kit (BioRad, Hercules, California), following the manufacturer’s instructions, with bovine serum albumin as standard. Biomass was dried by drying oven at 65°C overnight and estimated gravimetrically. Spectrophotometric assays of laccase activity were carried out using 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [10]. The assay mixture contained 2 mM ABTS and 0.1 M sodium-citrate buffer, pH 3,0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}) for 1 minute. Each assay was done in triplicate.

Native PAGE

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

Glucose concentration determination.

Glucose concentrations were determined by the glucose oxidase method [22]. Each assay was performed in triplicate.

Liquid-liquid extraction

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

TMS derivatisation

The trimethylsilylation was carried out in 100 µl of N,O-bis(trimethylsilyl)trifluoro-acetammide (BTSTFA) (SIGMA) at 80°C for 20 min. Samples were dried down under nitrogen, dissolved in 10 µl of hexane and centrifuged to remove solid reagents excess. The hexane supernatant (1/10) was used for the GC-MS analysis.

GC-MS analysis

Analyses were carried out by using a Hewlett-Packard (HP) 6890 GC equipped with HP 5973 mass detector system. Chromatographic separation was performed with an Equity-5 (Supelco, Sigma-Aldrich, St. Louis, MO) capillary column (30 m length x 0,25 mm i.d. x 0,25 □m film thickness). Splitless injection was used. The carrier gas was ultra-pure helium at a flow of 1 ml/min.

The injector line temperature was set at 270°C. The oven temperature program was 120°C for 2 min, from 120°C to 250°C at ramp rate of 21°C/min, from 250°C to 280°C at ramp rate of 13°C/min and 280°C for 5 min. The parameters of the HP 5973 mass detector were: ion mass/charge ratio, 40-450 *m/z*; scan mode. All mass spectra were acquired in the electron impact mode (EI = 70eV, source temperature, 230°C). The identification is accomplished on the basis of theoretical electron impact mass spectra.
Semi-quantitative analysis was performed by evaluating chromatographic peak areas, where the relative amount of each compound is calculated as percentage of the total area.

RESULTS AND DISCUSSION

Growth profiling of *P. ostreatus* liquid fermentation in basal condition

To obtain a simple model of submerged fungal culture a basal growth medium was formulated. This formulation originated in a not very complex medium to be analysed by GCMS. Moreover, it was possible to easily monitoring all phases of fungal fermentation, from exponential growth to carbon starvation stress, in a related short time slot (18 days). Phenoloxidase was the only ligninolytic activity detected in the supernatants when *P. ostreatus* was grown in aerated cultures. Other ligninolytic activities, such as manganese peroxidases, were not detected under these experimental conditions (data not shown). A time course of phenoloxidase activity production in *P. ostreatus* culture broth is shown in figure 1. Laccase activity peaked on days 5 and 13, reaching 400 and 300 U/L respectively and decreased dramatically thereafter. Proteins in supernatants from different days were analyzed by PAGE in native gels and stained with ABTS. Analysis of samples withdrawn from the media at different growth times indicated that the activity is associated mainly to the production of three isoenzymes POXA3, POXa1B and POXC, as reported in figure 2. The same pattern of isoenzyme bands was observed in correspondence of the two maximum production levels, although the band relative intensities changed during the growth time course: detectable levels of POXa1B [9] and POXA3 [10] activity production were only found in the first peak of laccase activity (days 4, 5) while no significant difference in the relative amount of the POXC [27] isoenzyme was detected at different growth times (days 4, 5 and 13).
Laccases synthesis appeared not to be related only to the hyphal growth because the total activity does not parallel the biomass production, measured as mycelial dry weight (figure 1). The exponential growth extended from day 3 to day 5, while a lyses phase started at day 8, confirmed by light microscopy observations (figure 3). The beginning of exponential growth is related to the start-point of glucose consumption while cellular lyses in the last phase of growth is a clear consequence of carbon starvation.
Expression of total secreted protein appeared to be constitutive because total amount increased proportionally along the biomass production to a maximum of 7g/l. However, the increase in extracellular proteins concentration observed during the last days of growth could be consequent to intracellular proteins solubilisation after hyphal lyses.
Laccase activity production seems to be related to the stationary phase of mycelian growth and is triggered by nutrient limitation as previously reported for what concerns other fungi [18, 15, 4]. Conversely, another mechanism might govern the production of laccases during the exponential growth phase, as the first production peak in laccase activity appears during the exponential phase.

GC–MS analysis of spent media

Metabolomic profiling plays a key role in the understanding of metabolic state of different biological systems [23]. It let to obtain new insights in establishing the relationships between phenotype and metabolism, which is an important aspect to correlate metabolites distribution with biological functions. Mass spectrometry has been recognized as a 'Gold Standard' tool for the identification and analysis of a large number of metabolites simultaneously in complex systems as demonstrated by recent published papers [30, 12, 17]. MS analysis have therefore become a promising technique in deciphering functions and metabolic alterations.

In this study, MS based exo-metabolites profiling was performed on *P. ostreatus* spent medium by time course analysis. GCMS analyses were carried out on aliquots of samples collected at different growth days (3, 6, 9, 12, 15 and 18 days). All samples were subjected to a liquid-liquid extraction protocol as described previously. Raw data were processed through baseline correction, scaling, peak alignment, and matrix manipulation. The different analytes detected in the total ion current (TIC) profile were identified according to the standard mass spectra of the National Institute of Standards and Technology MS spectral library. All compounds were considered identified when their mass spectral fit values were at the default value of 90 or above. When available, some detected species were further confirmed by comparing their retention times with those of pure standards. All analyses were performed as triplicates and each sample was run twice. The corresponding relative standard deviation (RSD) ranged from 3.7% to 19.7%. The fluctuation of the retention time of all the identified peaks was < 0.05 min.

Figure 4 shows the TIC profile of all detected samples. Starting from the sample collected at 3rd growth day, the occurrence of few species is clearly visible: during the time course analysis, an increase in the complexity of the TIC profiles is observed and the changing trends are summarised in table 1, where the relative amount of each compound is reported as % of total area.

As shown by the chromatograms, almost similar profiles can be observed, however qualitative and quantitative differences can be appreciated. It should be noted that some species escaped direct mass spectral analysis. Samples were therefore derivatised with BTSTFA and TMS derivatives were analyzed by GCMS. Analyses essentially confirmed previous data. However, the chromatograms from 16 days sample showed the occurrence of a peak identified as benzoic acid by EI spectrum. The absence of such species in previous analyses performed could be related to the low abundance of benzoic acid in the sample and to its low ionization efficiency.

More than 20 different analytes were tentatively identified. Our data showed completely different profiles: some compounds were revealed in the control medium while others were specifically detected during the time course. Unassigned peaks were attributed to contaminants, such as phthalates and solvent components. As a whole we focused on the species differently excreted during *P. ostreatus* cell growth.

Among the identified species GCMS technique allowed us to identify several endogenous aromatic molecules reported in table 1, which are not present in the control medium (no growth), whose concentration strongly changes during the growth. As shown in table 1, 12 analytes resulted to be benzoic derivatives at different oxidation states, ranging from alcohol to carboxylic acid.

As it is possible to observe, their relative peak areas significantly changes during the growth. As an example, 4-methoxybenzaldehyde shows a significant increase in the relevant peak at the 7th day but it decreases subsequently until it disappears in the 16th day sample. As a consequence, the peak area of benzoic acid 4-methoxy,

ethylphenyl ester, increases gradually until the maximum amount at the 16th day, during the fungine death phase. this molecule can be probably derived from the correspondent benzaldehyde.
Cyclic aromatic compounds can be synthesized by fungi via either the polyketide or the shikimic acid pathway: the latter leads to the formation of the aromatic amino acids phenylalanine and tyrosine and to the formation of many other phenyl-C3 compounds as products of both cinnamate and phenylpyruvate pathways. Equilibrium of multienzymatic cyclic system between extracellular aryl-alcohol oxidases and intracellular dehydrogenases leads to the H_2O_2-oxidative and reductive reactions of these benzyl-derivatives [13]. Starting from metabolomic pathway reported for the white-rot fungus *Bjerkandera adusta* [21], these compounds are suggested as precursors of those detected metabolites which are more structurally complex (figure 5). The amount of any compound in the culture broth can be connected to the differential expression of enzymes of this pathway during fungal fermentation. Based on recently sequenced *Pleurotus ostreatus* genome (http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_1.home.html), the presence of genes coding for enzyme potentially involved in the reactions of aryl-compounds pathway has been confirmed by *in silico* analysis; moreover to the best of our knowledge, for the first time nitroderivatives of phenolic compounds have been identified as excreted endogenous metabolites in white-rot fungi.
Effect of endogenous secreted metabolites on ligninolytic enzyme production
In previous studies, veratryl alcohol, a volatile compound produced during mycelium growth in submerged and solid state cultures has been proved to induce stimulation of ligninolytic activity overproduction [1] and development of fruiting body in the white rot fungus *P. ostreatus* [31]. In order to test potential effects of molecules produced during starvation, spent medium solutions derived from liquid cultures, SM7, SM10, and SM16, were prepared harvesting fungal cells in correspondence of the stress beginning (day 7), after a long-time stress (day 10), and after a very long-time stress (day 16). Analyses were performed monitoring biomass growth, total secreted protein and laccase production profiles when these compounds were added to the basal medium. In figure 6 an increase of laccase activity in media conditioned by SM10 solution and SM16 solution is shown, in correspondence of the first peak at days 4 and 5, with no significant increase of the other parameters: a two and three times increase in laccase production was detected for SM10 and SM16 conditioned media respectively, whereas no relevant variation for media conditioned by SM7 solution was determined. Comparison of the zymogram patterns of laccases related to samples collected at 4th, 5th and 13th days growth relative to the basal, SM10 and SM16 conditioned growths did not show relevant differences (figure 2). Such data indicated that the presence of endogenous exo-metabolites in liquid culture affected general mechanisms of laccase expression and/or secretion and that the increase of laccase activity at day 4 and 5 is not dependent on overexpression of a single isoform. GCMS profiling as reported above show the presence of aryl compounds as overriding chemicals of exo-metabolome, mostly during starvation stress. In order to confirm if the presence of benzyl-derivate chemicals can induce same of the effects reported above, a time course analysis of laccase profile was carried out conditioning fungal basal growth by a precursor of aryl compounds pathway. Benzaldehyde, was mixed in the medium at 1mM final concentration before inoculation: these amount was decided taking into account high volatility of benzyl-compounds and general threshold of laccase inducer concentration to increase phenoloxidase activity production [7]. As reported in figure 7 increased laccase activity, similar to levels

detected in media conditioned by SM10, occurred with supplementation of benzaldehyde in the fungal culture.
Recently, it was reported that endogenous molecules excreted by *P. ostreatus* have antimicrobial activity against competitor microbes [25] as well as *quorum sensing* molecules produced by dimorphic fungi induce phenotype switches in filamentous fungi [16]. In a recent work, Beltran-Garcia [2] suggests a possible involvement of metabolites deriving from benzoic acid and other volatile compounds secreted by the Oyster Mushroom, in fungal antibacterial activities.
Reported evidences indicate a new physiological role for the molecules derived from shikimic acid metabolic pathway: these endogenous aromatic compounds can regulate the secretion pattern of industrially useful extracellular proteins, such as laccases. Benzaldehyde and its chemically related compounds are promising molecules for investigating possible applications useful in improving enzymatic production by self-producing strains and in controlling fungal bioprocess.

CONCLUSION

In the current study we have explored dynamic variation of metabolic profile associated to the state of fungal fermentation in the industrially useful white-rot basidiomycete *P. ostreatus.* Time course analysis were performed by GCMS and more than 20 different analytes were identified: 11 volatile compounds related to shikimic acid pathway were identified as differentially produced during submerged growth. By conditioning basal growth through endogenous exo-metabolites extracted from spent media an effect of these compounds on the general mechanisms of laccase expression liquid culture was demonstrated.
Reported data suggested that, in order to optimize fungal bioprocesses for extracellular enzyme production, autoinduction mechanisms might be used during submerged growth and a comprehensive metabolic frame should be known to design and control the required state of physiology.

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	RT		t=0	t= 3 days	t= 5 d	t= 7 d	t= 10 d	t= 13 d	t= 16 d
1	2.12	Benzaldehyde	Nd*	0.54	Nd*	0.32	0.15	Nd*	Nd*
2	4.10	Benzoic acid	Nd*	0.34	Nd*	Nd*	Nd*	Nd*	Nd*
3	4.24	benzaldehyde, 4-methoxy	Nd*	Nd*	3.01	5.05	2.59	2.33	Nd*
4	4.30	benzoic acid, phenylester	Nd*	Nd*	Nd*	Nd*	Nd*	Nd*	0.28
5	4.78	benzyl alcol	Nd*	Nd*	Nd*	Nd*	Nd*	0.20	0.25
6	6.06	4-quinoline carboxaldehyde	Nd*	1.58	1.35	0.38	0.51	0.96	0.16
7	6.38	benzoic acid,4-methoxy-4-ethylphenylester	Nd*	Nd*	Nd*	0.28	0.88	0.87	1.91
8	6.53	Benzeneacetic acid-4-hydroxy-3-methoxy	Nd*	Nd*	Nd*	0.56	0.33	0.87	0.40
9	7.16	5-amino-1-naphtol	Nd*	1.07	1.90	0.50	0.38	0.62	0.30
10	7.34	3-pyrrolidin-2yl-propionic acid	Nd*	1.70	1.74	1.02	0.42	1.56	0.75
11	7.55	benzenepropanoic acid,4-hydroxy	Nd*	Nd*	0.25	1.94	2.10	0.54	Nd*
12	7.91, 8.41, 8.50, 8.57	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	Nd*	11.59	13.41	1.51	2.03	4.48	1.31
13	8.04	phenol,3,5-dimethoxy	Nd*	1.00	1.24	0.41	Nd*	Nd*	Nd*
14	8.14	pyrrole-4-ethyl-2-methyl	Nd*	Nd*	Nd*	2.03	2.42	1.22	0.71
15	8.30	cyclobutane oxime	Nd*	Nd*	2.83	9.71	7.26	Nd*	Nd*
16	8.47	benzoic acid,3,5,-dihydroxy	Nd*	Nd*	Nd*	8.98	5.78	3.38	3.65
17	8.60	benzoic acid,2-hydroxy-4-nitro, benzoic acid,2-hydroxy-3-nitro, benzoic acid,2-hydroxy-5-nitro	Nd*	Nd*	Nd*	6.76	13.71	8.06	24.81
18	8.75	Phenylacetic acid-2-amino-6-methoxy	Nd*	Nd*	Nd*	7.24	3.28	3.34	2.58
19	8.78, 8.88	hexadecanoic acid	Nd*	1.10	0.62	Nd*	4.59	5.23	Nd*
20	9.85	octadecanoic acid	Nd*	0.20	0.69	Nd*	Nd*	Nd*	Nd*
21	10.62 10.83	pyrrolo-(1,2a)pyrazine-1,4-dione-hexahydro-3-(2-phenylmethyl)	Nd*	1.43	1.79	Nd*	0.97	0.66	1.21

*** Nd=Not Detected**

Table 1 – list of the metabolites identified by GCMS analysis (third column); in the second column the relative retention times (RT) are listed; the first column indicates the analyte on the TIC images;columns 4-10 indicate the relative amount of the compound, calculated as percent in relation to total volatiles.

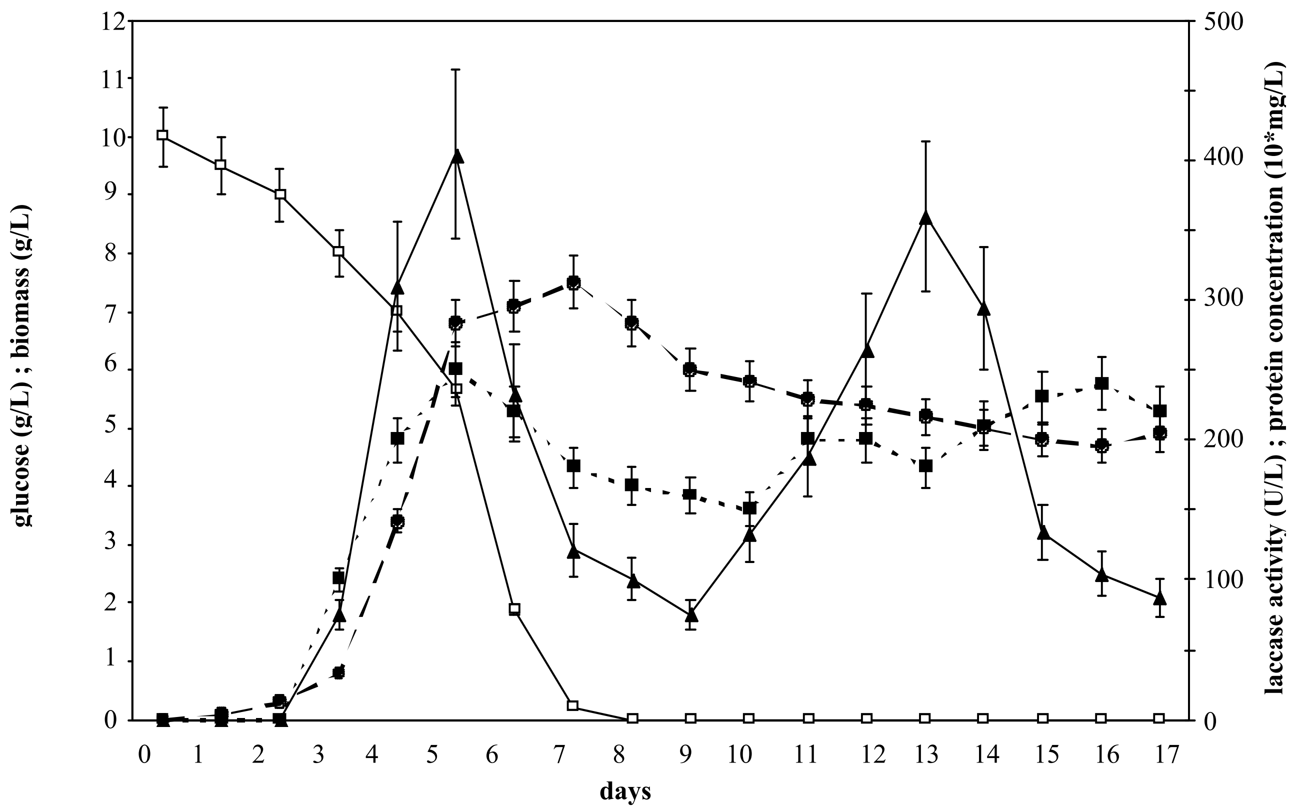


Figure 1- *P. ostreatus* 17 days growth profile in basal condition: extracellular laccase activity, secreted protein concentration, glucose consumption and biomass increasing are reported as U/ml (▲), 10* mg/l (■.); g/l (□), g/l(●.) respectively.

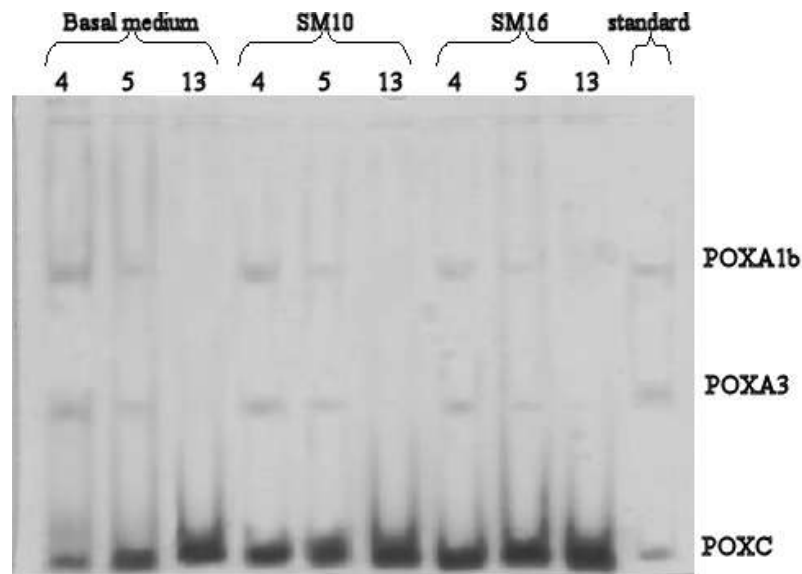


Figure 2- Zymograms of laccase isoenzymes in the absence and in the presence of conditioning solution derived from spent media old 10 and 16 days (SM10 and SM16). Samples containing 0.015 U of laccase activity collected at different times (4,5 , and 13 days) were used.

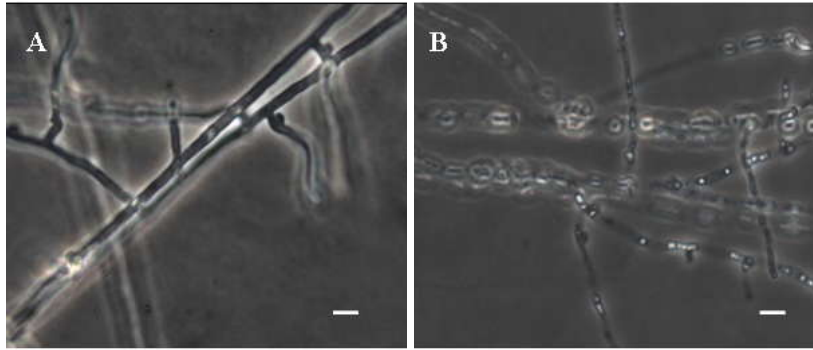
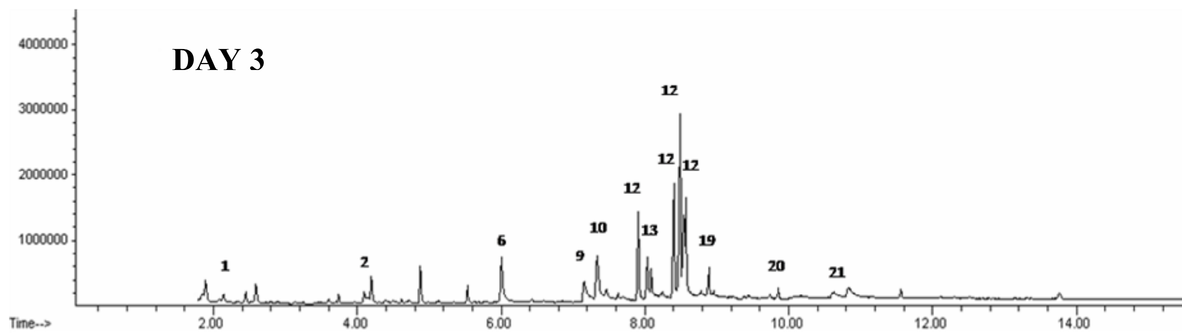
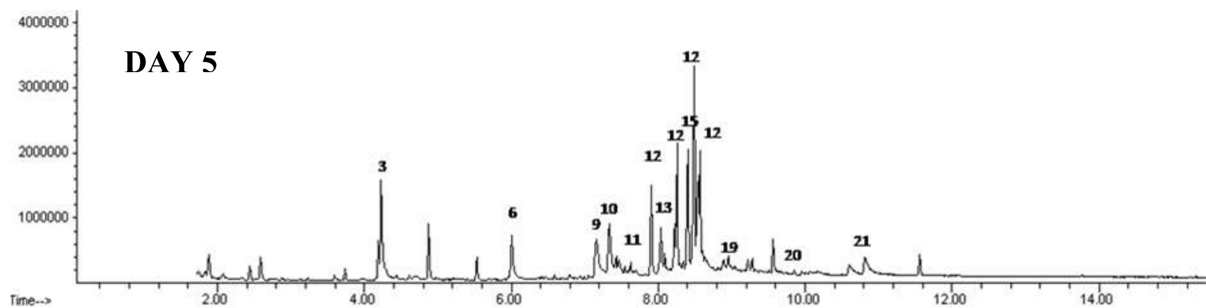
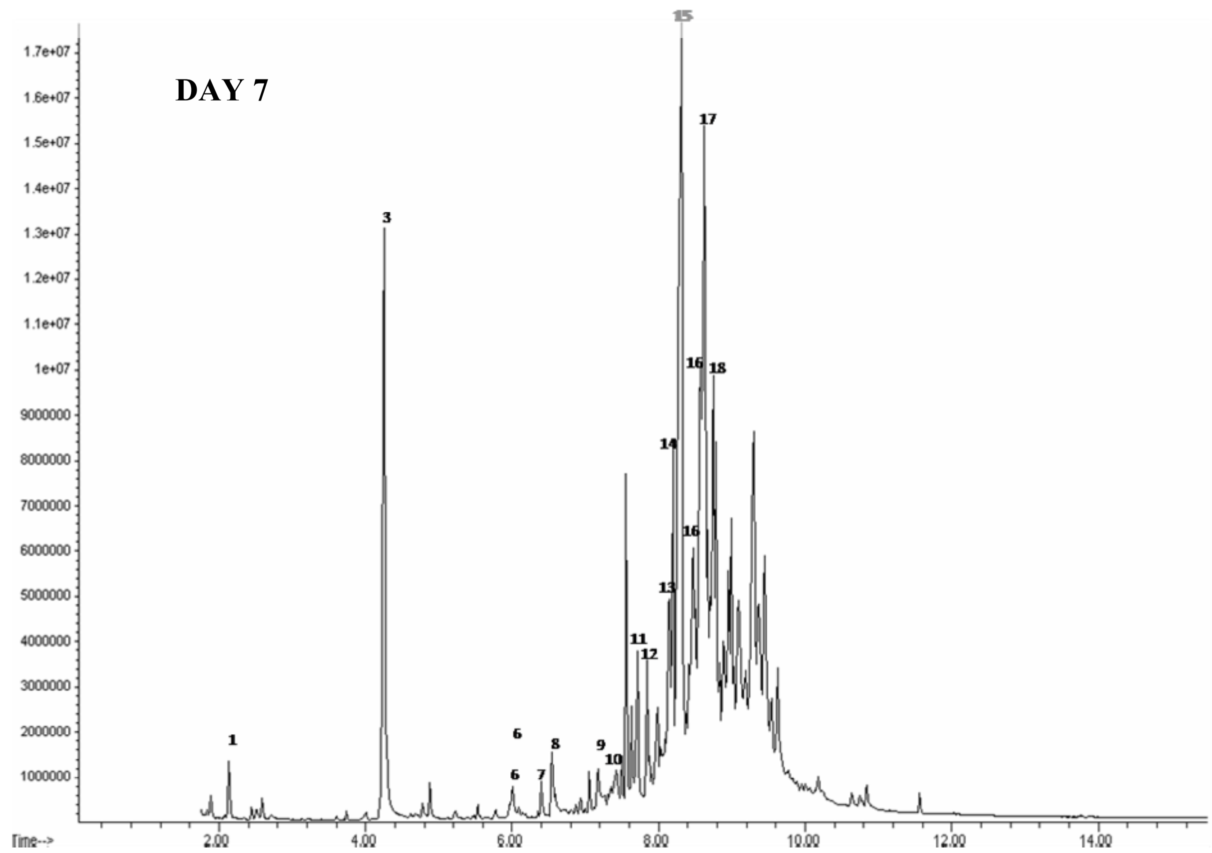


Figure 3- light microscopy observation of fungal pellet at 5th (A) and 12th (B) day of growth in liquid basal medium. In picture 3B it's possible to appreciate vacuolization and cell lyses. Bar, 12 µm.






DAY 7
1.7e+07
1.6e+07
1.5e+07
1.4e+07
1.3e+07
1.2e+07
1.1e+07
1e+07
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
Time-->
2.00
4.00
6.00
8.00
10.00
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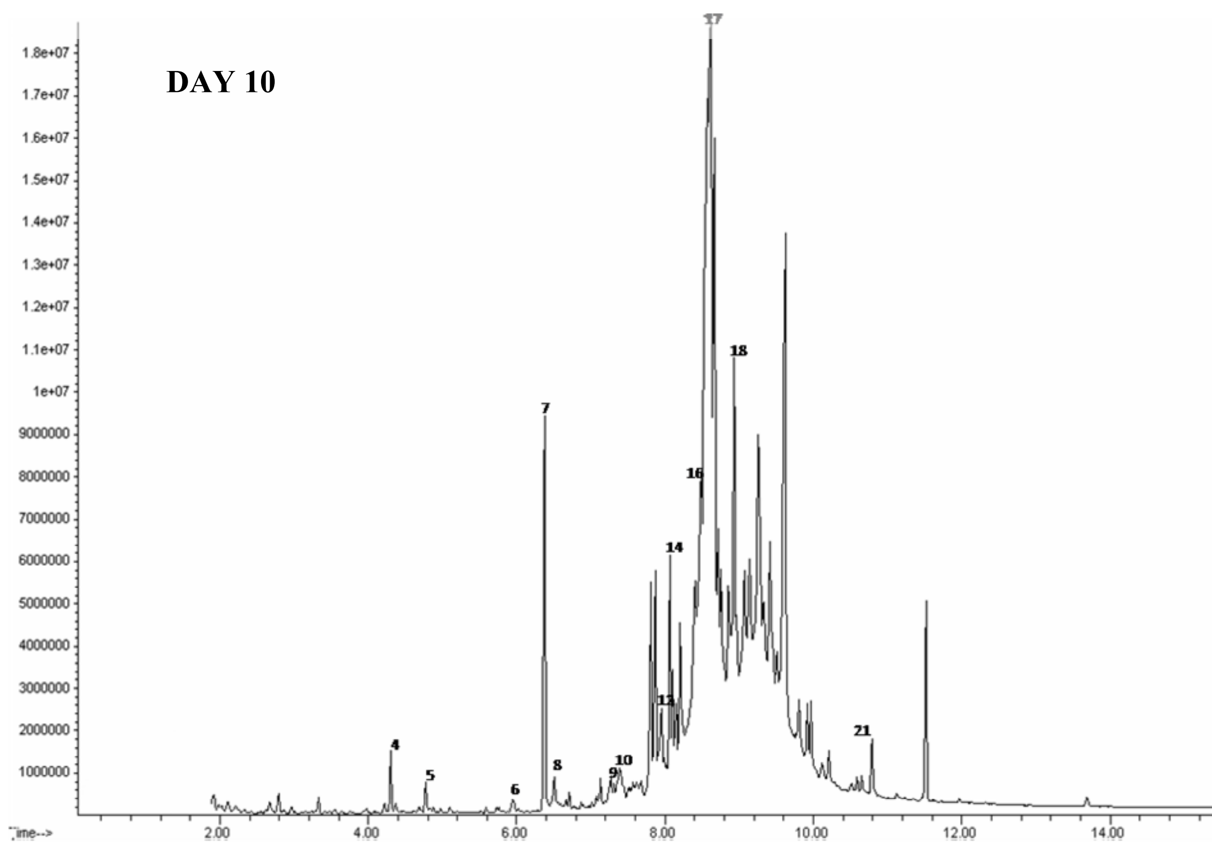
DAY 10
1.8e+07
1.7e+07
1.6e+07
1.5e+07
1.4e+07
1.3e+07
1.2e+07
1.1e+07
1e+07
9000000
8000000
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Time-->
2.00
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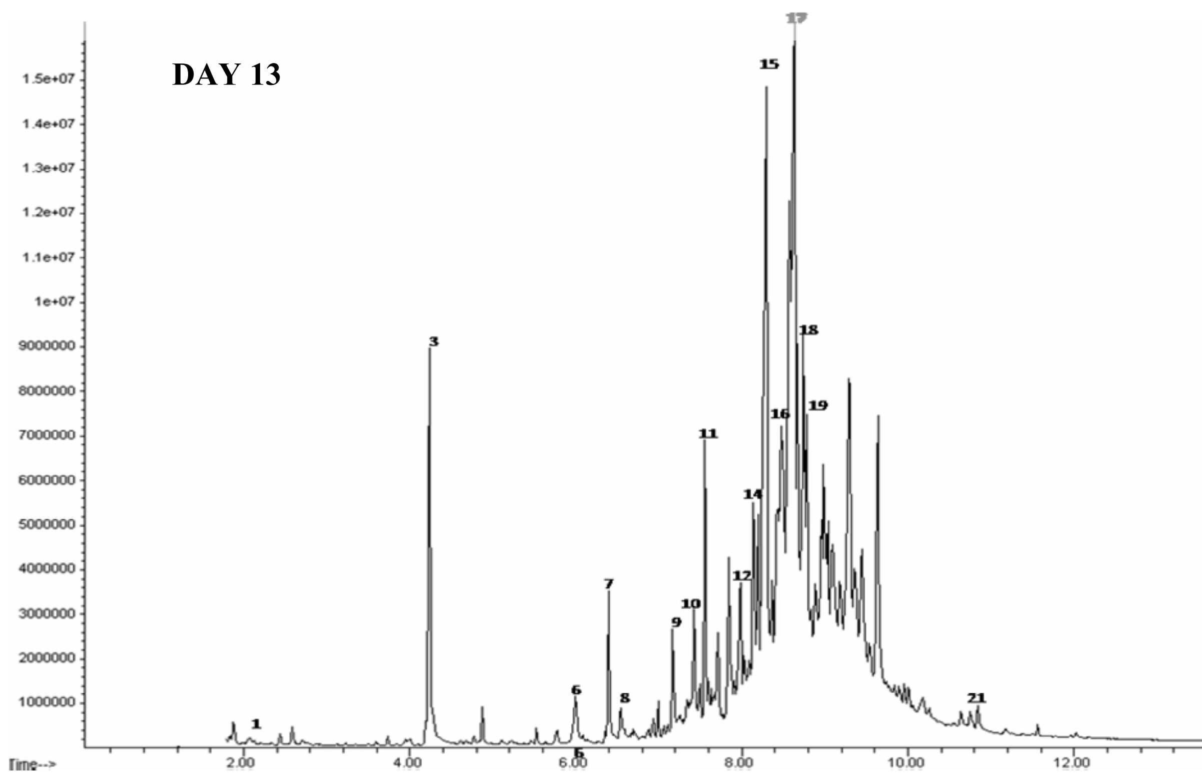


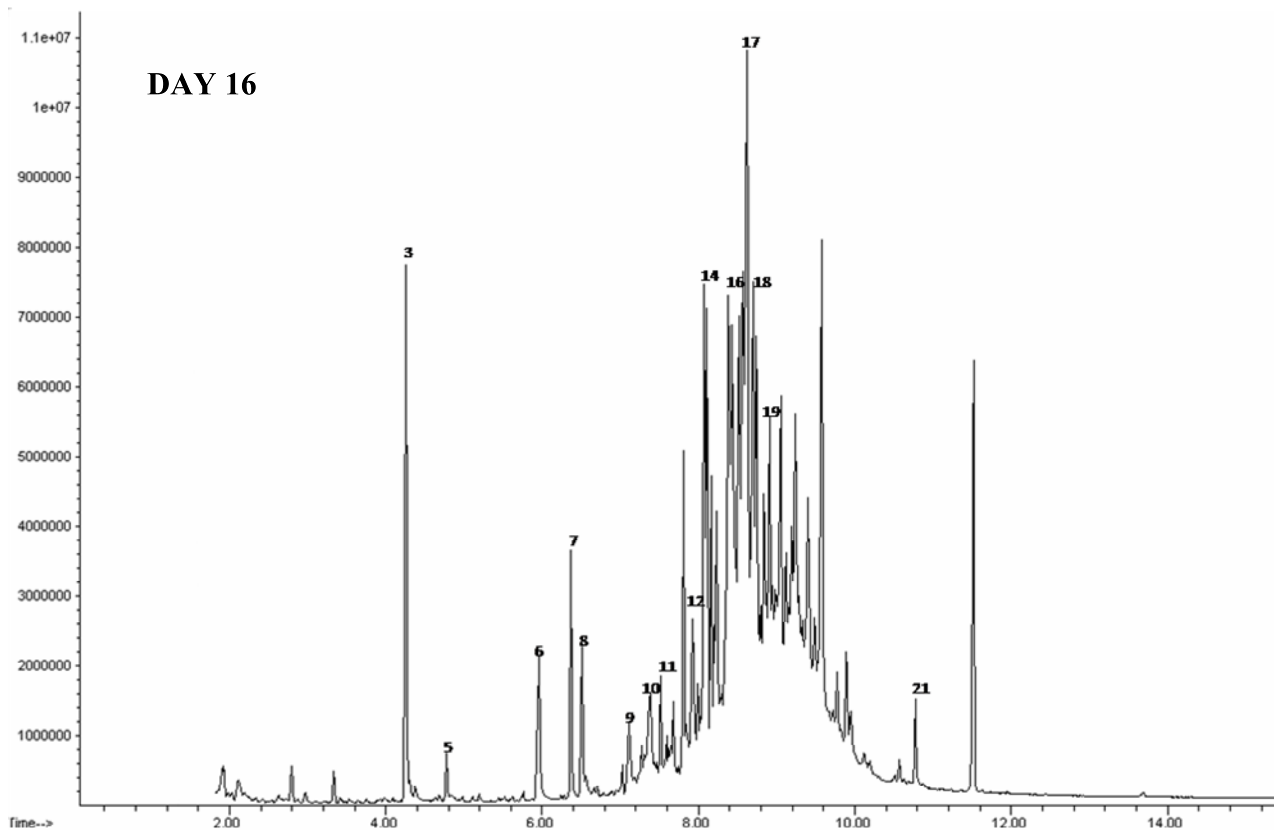


Figure 4- Chromatograms from six different samples during the time-course analysis: number of any identified metabolite is referred to table 1.

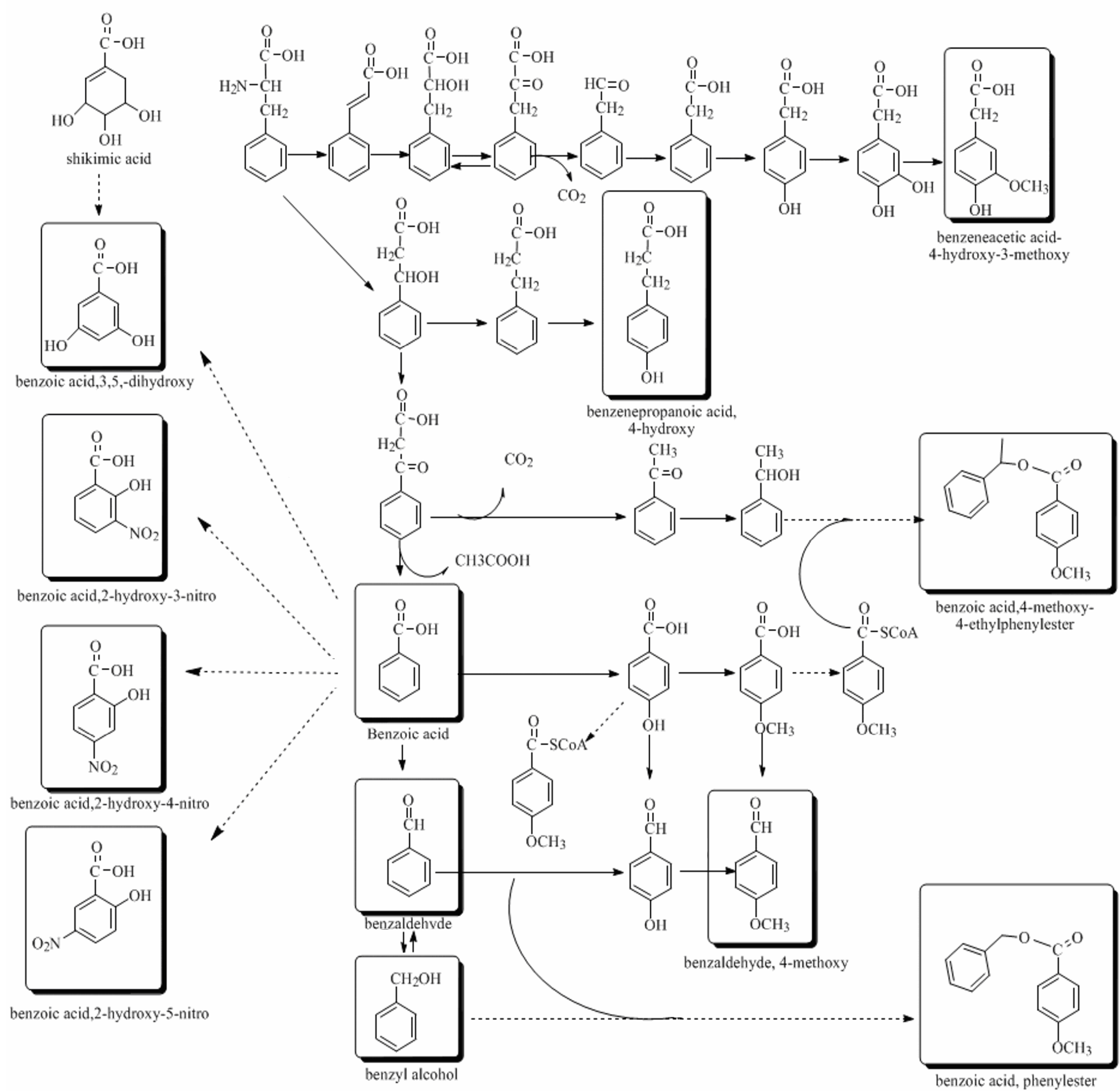


Figure 5- Adaptation of proposed pathways for biosynthesis of aryl metabolites in the fungus *B. adusta* [21]**. The intermediates in the frames has been detected in *P. ostreatus* and reported in table 1.**

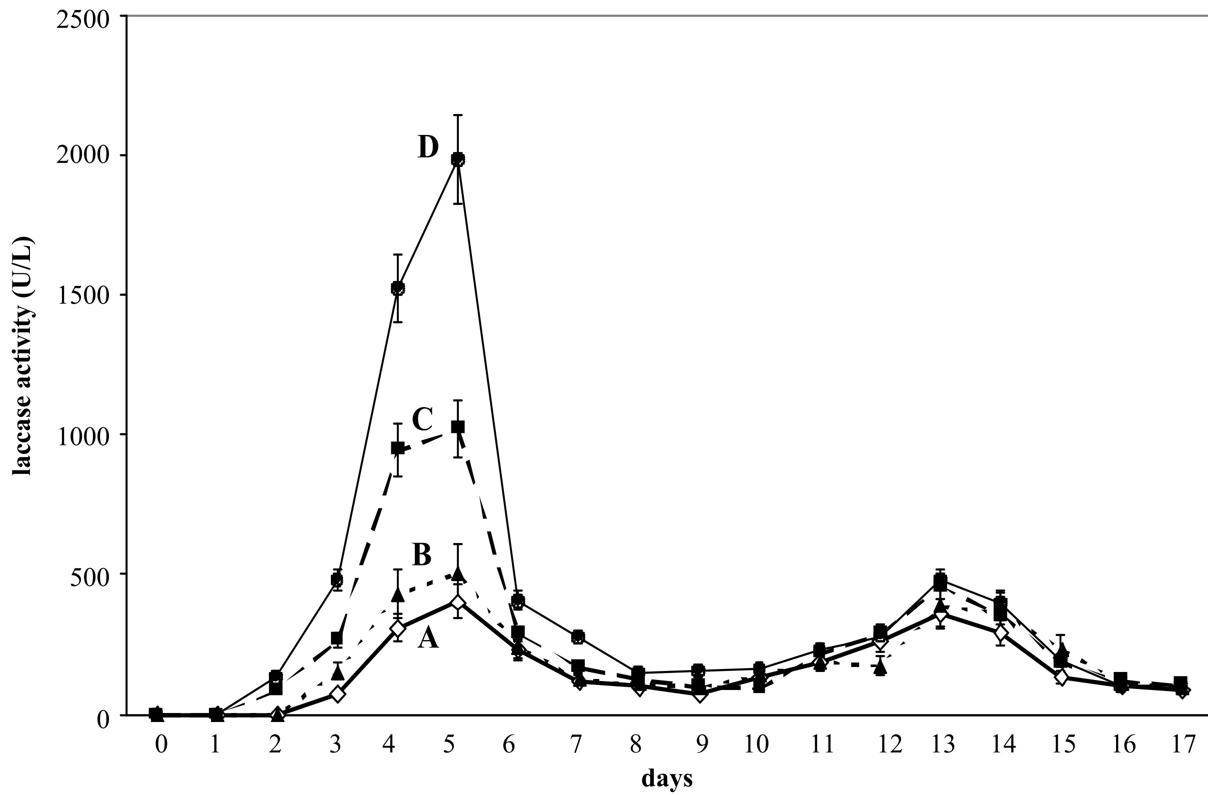


Figure 6- Laccase activity per litre of culture in absence (line A) and in the presence of SM7 (line B), SM10 (line C) and SM16 (line D) solution in *P. ostreatus* culture broths.

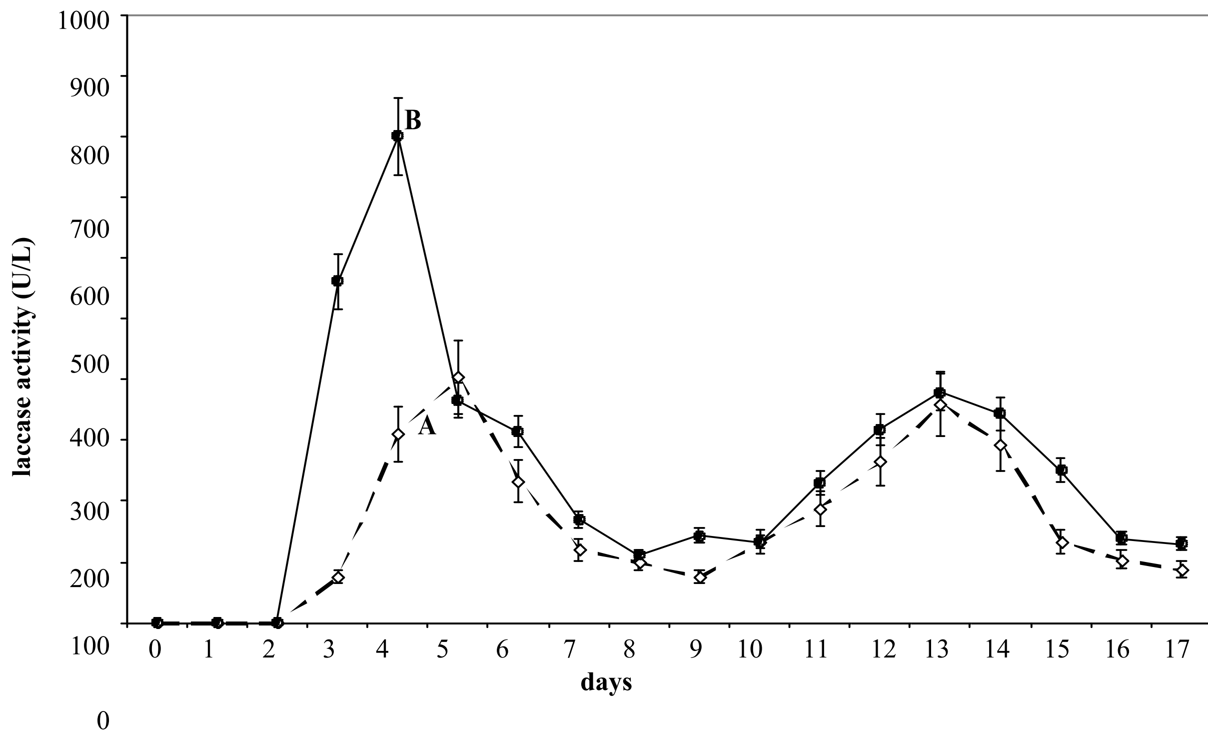
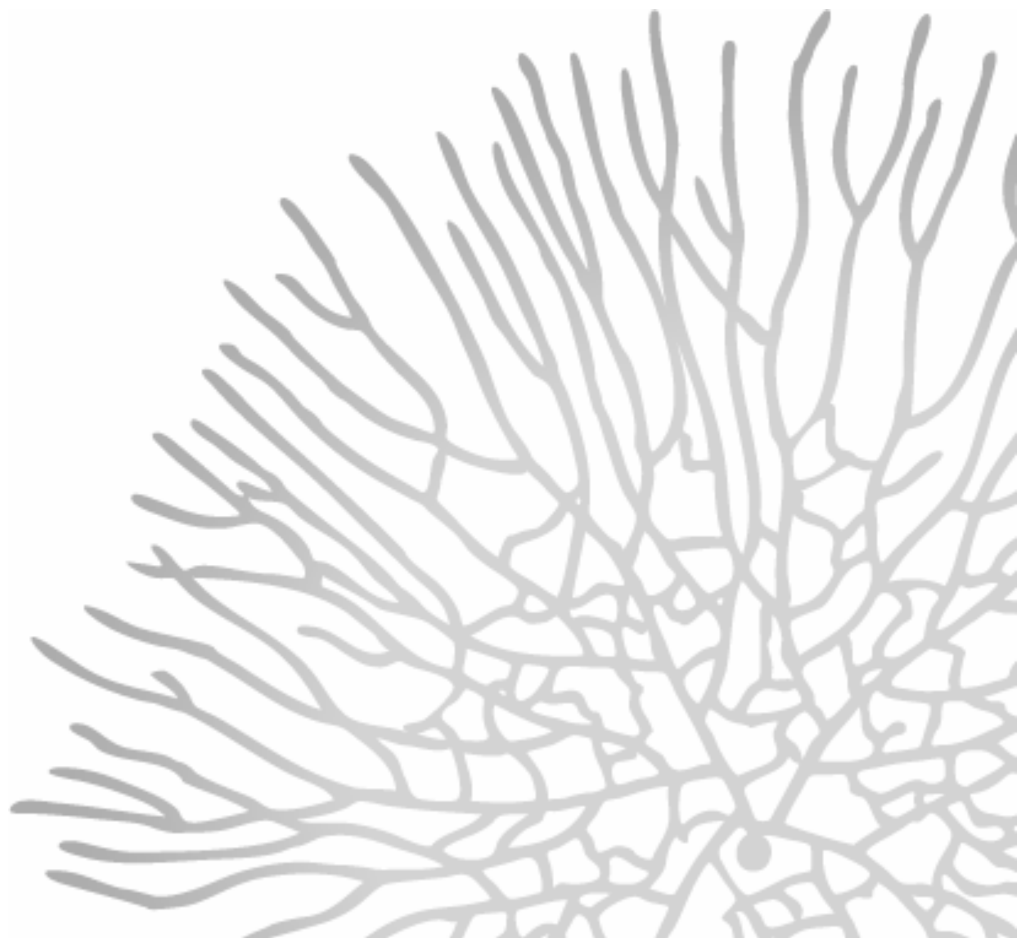


Figure 7- Laccase activity per litre of culture in absence (line A) and in the presence of benzaldehyde (line B) in *P. ostreatus* submerged growths .

Chapter 2:

Identification and characterization of *P. ostreatus* autoregulators affecting different morpho-physiological states



2.1 - Introduction

During the last decades fungi have attracted attention as potential sources of natural flavours. Production of these volatile compounds depends on the developmental phase of the fungus or on its physiological states. Because natural flavours are used in food and cosmetic industry, many efforts have been made in order to isolate and characterize volatile chemicals excreted in submerged cultures by mycelial pellets or produced during mushrooms fructification [1, 2]. The published literature reports evidences of a direct correlation of aroma compounds produced by a fungal species with synthetic growth media. Fragrance extracted from liquid broth largely differ from volatile compounds produced by mushrooms fruiting on a natural solid substrate [3]. *P. ostreatus* variant *florida* produce a sweet anise and almond-like odour when grown in synthetic liquid medium probably derived from benzaldehyde-related metabolites [3]. The major aroma compound in naturally grown fruiting bodies of *P. ostreatus* belongs to a family of short carbon (C8) chain chemicals [4]. However very few studies have been carried out to understand the physiological roles of these secondary metabolites in higher fungi in relation to specific states of fungal development [5] and even less efforts have been made to clarify whether these chemicals are involved in signalling and regulating processes in basidiomycetes [6].

Signalling molecules are secondary metabolites which are excreted and stimulate or inhibit, affecting their gene expression, microorganisms, such as fungi, during their physiological development. When the stimulus is produced by the same organism, or by a neighbouring organism of the same species, the signalling molecule is defined as autoregulator [5].

Some autoregulators, interacting with fungal spores, affect the emergence and initial growth of germtubes (the first hiphal structure arising from a spore) [7]; some others induce changes in hyphae morphology in comparison with the characteristic growth pattern of the fungal colony [8, 9, 10]. Thus, spores or hyphae sense not only the environmental conditions, but also the presence and the “state” of a neighbouring mycelium of the same species (cell density, fruiting body formation, starvation, etc.) [5].

In the present work the role and the effects of some of these selected communication molecules in *P. ostreatus* cultures were evaluated by morphological and physiological analysis. Fungal pellet morphology, sporal germination, radial growth in agar medium, and laccase production profiles in submerged cultures were monitored in presence and absence of each identified extracellular metabolite.

2.2 - Materials and Methods

Organism

Except where otherwise indicated, all chemicals were obtained from Sigma Aldrich.

Dikaryotic strain of P. ostreatus (Jacq.: Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) was maintained through periodic transfer at 4°C on potato dextrose agar plates in the presence of 0.5% yeast extract (Difco).

Culture conditions in GYM basal medium

Mycelium was grown in 1 l shaken flasks (125 rmp) containing 300 ml of GYM (Glucose, Yeast extract, Mineral solution) broth containing 10 g/l glucose; 3,8 g/l yeast extract (Difco) 2 g/l H_2KPO_4; 0,5g/l $MgSO_4$ $7H_2O$; 0,1 g/l $CaCl_2$ $2H_2O$; biotin 10 mg/l; thiamine 10 mg/l and 10 ml of mineral stock solution (0,5 g/l $MnSO_4$ $5H_2O$; 1 g/l NaCl; 0,1 g/l $FeSO_4$ 7 H_2O; 0,1 g/l $CoCl_2$ 6 H_2O; 0,1 g/l $ZnSO_4$ 7 H_2O; 0,01 g/l $CuSO_4$ 5 H_2O; 0,01 g/l $AlK(SO_4)_2$; 0,01 g/l H_3BO_3; 0,01 g/l $NaMoO_4$ 2 H_2O); final pH5.

5-day-old culture were homogenized by Ultra-Turrax® T25 Basic interconnected with S18N-19G dispersing tool (3 flashes of 30 seconds at 24.000 rpm separated by 30 seconds of stand-by) and 1 millilitre of homogenate was transferred in 1-l flasks containing 300 ml of GYM broth. The cultures were grown in shaken flasks at 125 rpm and incubated at 28°C in the dark for 16 days.

Culture conditions in PDY medium

Mycelium was grown in 1 l shaken flasks (125 rmp) containing 300 ml of PDY medium (Potato Dextrose 24 g/l + Yeast extract 5 g/l - Difco).

5-day-old culture were homogenized by Ultra-Turrax® T25 Basic interconnected with S18N-19G dispersing tool (3 flashes of 30 seconds at 24.000 rpm separated by 30 seconds of stand-by) and 4 millilitre of homogenate were transferred in 1-l flasks containing 300 ml of PDY liquid broth in presence of 150 µM $CuSO_4$. The cultures were grown in shaken flasks at 125 rpm and incubated at 28°C in the dark for 21 days.

Conditioning by endogenous compounds

All compounds were dissolved in ethanol 80% and sterilized by filtration (cut-off 25 µm). Conditioning solutions were added to the basal GYM broth after 3 days from the inoculation, whereas they were added to the autoclaved agar GYM broth after that temperature decrease until 50°C. 2hydroxy-4-nitrobenzoic acid was added to the rich medium PDY + 150 µM $CuSO_4$ after 4 days from the inoculation.

Biomass and enzyme activity determinations

Biomass was dried in a drying oven at 65°C overnight and estimated gravimetrically. Spectrophotometric assays of laccase activity were carried out using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [11]. The assay mixture contained 2 mM ABTS and 0.1 M sodium-citrate buffer, pH 3,0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M-1 cm-1) for 1 minute. Each assay was done in triplicate.

Native PAGE

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

Basidiospores preparation

the gills-side of *P. ostreatus* fresh sporocarps was overnight leaned on sterile glass surface of a humid sterile chamber. *P. ostratus* basidiospores were collected by sporal print formed on the surface. A spore suspension was prepared in 1ml sterile water. Concentration of spores was estimated by counting them in a *Thoma* chamber on optical microscopy.

Germinating spores determination

The basidiospores suspension was plated on solid GYM medium in *Petri* dishes (25 ml) after appropriate dilution (10^3 spread spores per plate) and incubated at 28°C. Germination was determined following incubated plates for 5 days and percent of germinating spores was calculated (% of spread spores per microscopic mycelial colony). The experiments were performed in triplicate.

Microscope silicon chamber

Autoclaved microscopy slide with Press-to-Seal silicone isolator® - 20 mm diameter, 1.0 mm deep- (Invitrogen) were filled up by 125 µl liquefied agar media. Slides were

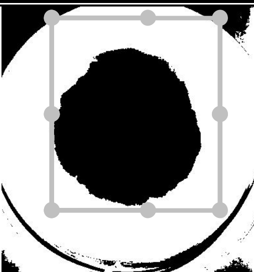
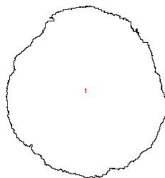
inoculated by hyphal fragment (3-4 hyphae per fragment), cutted from actively growing mycelia on water agar plate, and covered with sterile cover glass. Microscope silicon chamber were incubated at 28°C. Time course of mycelium growth (3 days) was performed acquiring pictures by means of a digital camera Leica DFC280 (Leica Microsystems) interfaced with under an optical microscopes Olympus BX-60 (Olympus Optical GmbH, Hamburg, Germany) at ×10 to x40 magnification. The camera was connected with a computer to acquire digital images. The digitalization process was performed using Leica IM software version 4 (Leica Microsystems).

Radial growth rate

Growth on solid media was carried out at 28°C in Petri dishes (90mm diameter) containing GYM medium. The plates were inoculated with mycelial plugs (11 mm diameter) cut from actively growing mycelia. Mycelial growth was followed acquiring the related image by HP Scanjet 5590P® (HP).

Image analysis system

Microscopic and macroscopic images were analyzed by Image j software (http://rsbweb.nih.gov/ij/). Hyphal length and branching angle were measured by the software comparing to the reference bar . Branching number were manually calculated. For the mycelial area calculation a methodological table is reported.

	Convert scanned color image of leaf to grayscale: ***Image → Type→8-bit*** • Set measurement scale: Draw a line over a 90 mm section of the ruler then ***Analyze → Set Scale*** In *Set Scale* window enter 90 into the 'Known Distance' box and change the "Unit of Measurement" box to mm , check "Global". • Draw a new line and confirm that the measurement scale is correct.
	• Threshold new image of the mycelium using manual settings: ***Image → Adjust →Threshold*** and play with sliders to include all of the mycelial area in red and click "Apply". The manual threshold setting includes all mycelial area.
	• Calculate area of entire mycelium: Enclose the black mycelial area with the rectangular selection tool. ***Analyze → Analyze Particles*** Use previous window settings and click "OK". Outline of entire leaf is automatically drawn. Data window reports the area A in mm^2. Average radium **r** is calculated → r = $\sqrt{\frac{A}{\pi}}$

Tab. 2.1 Area measurement procedure of a mycelium growing on agar medium.

The results were the means of three independent experiments. They were subjected to one-way analysis of variance (ANOVA). Bonferroni's analysis was conducted to identify significant differences (p≤0.05)

2.3 - Results and Discussion

2.3.1 - Benzyl derivatives

In chapter 1 a comparative analysis of dynamic variation of exo-metabolites during fermentation in submerged culture has been reported, and volatile compounds differentially produced were identified and related to the aryl-compounds pathway. Identity of enzymatic activities potentially involved in this pathway had already been reported for other basidiomycetes [12] and the corresponding enzymes was also proposed in *P. ostreatus* (Fig. 2.1). On the contrary, the presence of 2-hydroxy 4-nitrobenzoic acid, detected for the first time in white-rot fungi, could be due to a possible enzymatic nitration mechanisms that would occur during cell damaging: i.e. i) attach to a tyrosine structurally-correlated compound by peroxynitrite ($ONOO^-$) or by a different reactive nitrogen species produced by nitric oxide synthase or ii) monophenolic benzyl-derivatives nitration by nitrogen dioxide radical ($NO_2^{\cdot}$) produced by an oxydase activity [13].

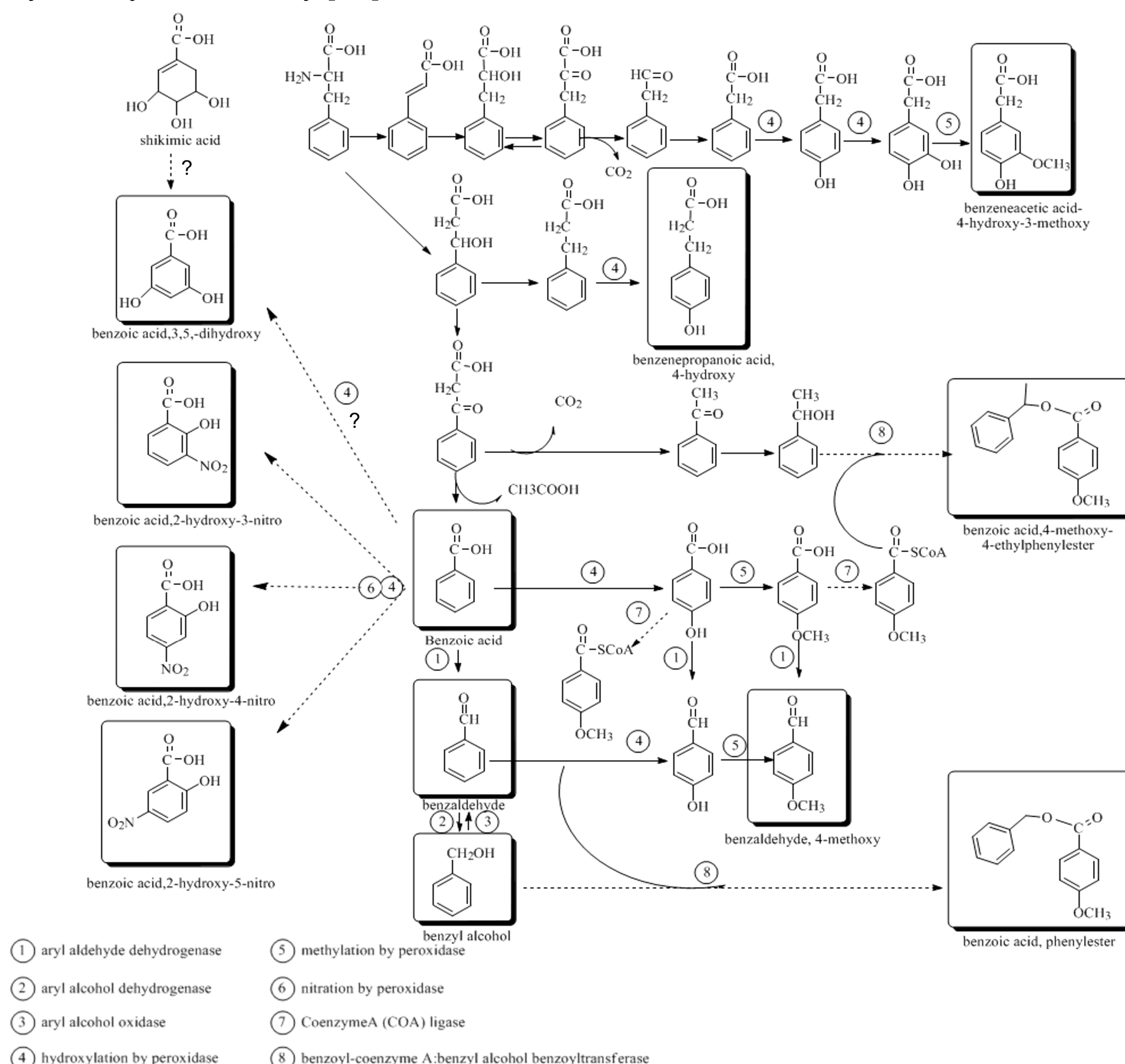


Fig. 2.1 hypothetical aryl-compounds pathway in *P. ostreatus* – integration of enzyme involved in bioconversion of benzyl-derivatives.

Data reported in Chapter 1 indicate that the presence of at least one benzyl-derivative metabolite increases laccase production in liquid fermentation.

In this work benzaldehyde, 4-metoxybenzaldehyde, benzoic acid, 2-hydroxy-4-nitrobenzoic acid and benzyl alcohol were used to condition solid and liquid growth media as already reported by previous analogue experiments [7, 14,]. Regulation of morphology, radial growth rate, sporal germination and laccace production were monitored in the presence of each one of these compounds, as it is described below.

2.3.2 - Hyphae morphology

Hyphal elongation and branching are fundamental processes defining the morphology of filamentous fungi. The effectiveness and success of these processes are essential for the acquisition of nutrients from the environment and in many cases, are part of the proliferative and reproductive cycle. Formation of the common cylindrical cell extension/branches by the polarized synthesis of new membrane and cell wall material must be both temporally and spatially regulated [15]. Though information concerning the regulation of hyphal growth is being accumulated, this aspect of hyphal cell biology is still in its infancy and a comprehensive picture of the processes network involved has yet to be obtained. A central goal of this subsection is to evaluate how the position and branching of fungal colonies is correlated to the presence of benzyl-derivatives. This objective will be reached by examination fungal colonies at microscopic level.

In order to analyse the mycelium morphological changes under optical microscope, sterile “microscopic silicon chambers” (Fig. 2.2) were filled up with basal agar medium conditioned by one of the putative autoregulators. Fresh mycelium of *P. ostreatus,* actively-growing on water-agar plate, was micro-dissected, and fungal pieces containing at least 3-4 hyphal fragments were sub-cultured into chamber centres. Fungal growth was monitored for 3 days acquiring pictures by means of a digital camera interfaced with the light microscope. The camera was connected to a computer to acquire digital images. The digitalization process converts colour information as well as spatial location onto a pixel grid. In order to simplify the model images, colours were converted from RGB to grey-scale (8-bit). In turn, each pixel possesses a numerical value which confers spatial coordination. These conversion in “numeric values” can than be statistically analyzed to extract information concerning the number and location of particular image features. Digital images therefore extend the value of the test from a purely subjective experience to a quantifiable object. For any acquired image of mycelial pellet, main hyphal length, number of branching and branching angle were measured using an image analyzer software (Image-j). The first two parameters are indicative of different aspects of the mycelium development. The main hyphal length indicates the necessity of the fungus to explore uncolonized neighbouring behaviours. Conversely, the branching frequency increases in function of concentration of nutrient sources in already colonized environments in order to maximize chemicals uptake and increase fungal fitness in favourable conditions. A further parameter can be derived from these two previously mentioned: it is defined as hyphal growth unit (HGU) and it is calculated as the ratio between the total hyphal length and hyphal branching number. HGU basically reports an average value of growth extension. Steele & Trinci (1975) [16] have shown that the main rate of extension ***(E)*** of hyphae in a fungal mycelium is a function of the HGU ***(G)*** and of the specific growth rate ***(μ).*** Thus,

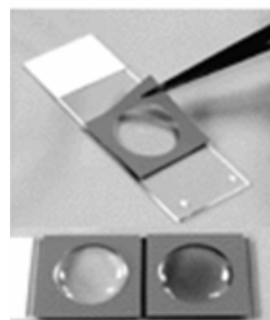


Fig. 2.2 microscope silicon chambers.

$$E=G*\mu \quad [a]$$

Equation predicts that, provided ***μ*** remains constant (it is strain-dependent), ***E*** will be directly related to ***G***. Thus, factors that reduce ***E*** without affecting ***μ*** will result in the formation of mycelia which branch more profusely and *vice versa*.
The last parameter to be considered, the hyphal branching angle, reports a specific direction affected from tropism processes. A tropism is a growth movement exhibited by part of an organism in response to an unidirectional stimulus.
Main hyphal length, branching numbers and their ratio (HGU) of *P. ostreatus* mycelium grown in presence and in absence of benzyl-derivatives are reported in figure 2.3.

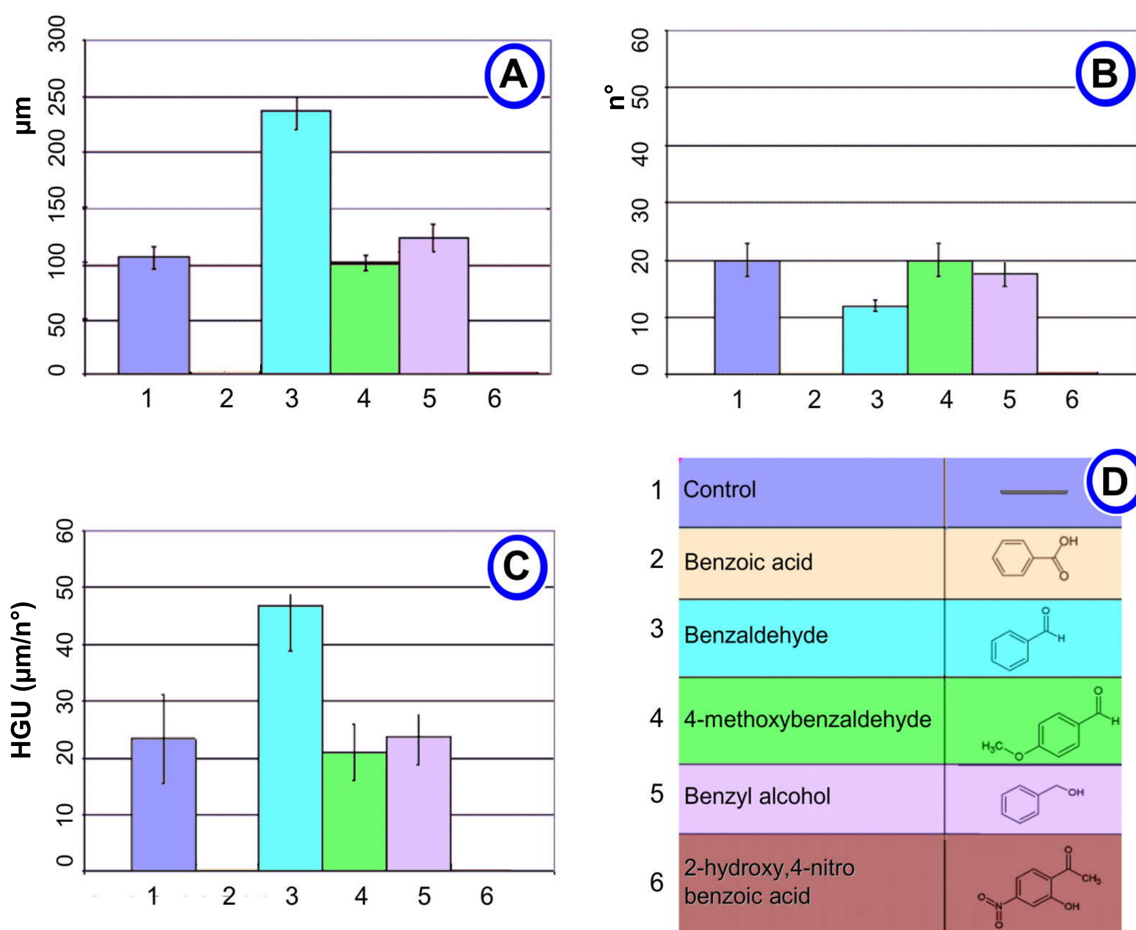


Fig. 2.3 Morphological parameters of *P. ostreatus* mycelium monitored during fungal development after 4 days of growth on microscopical silicon chambers in presence and in absence of benzy-derivatives. Any histogram bar is associated with one of the conditioning molecules by a number.
A. mean hyphal length reported in µm.
B. branching number (n°).
C. HGU (total hyphal length/ branching number - µm/n°).
D. table of tested benzy-derivative molecules with related chemical structure.

Some of the tested molecules induced different effects on fungal morphology. In the presence of benzaldehyde, main hyphal length increased, whereas hyphal branching was inhibited. Conversely, no evident effect was induced by the presence of benzyl

alcohol or of 4-methoxybenzaldehyde, a secondary metabolite closely related to benzaldehyde. Moreover, benzoic acid and 2-hydroxy-4-nitrobenzoic acid totally inhibited fungal development.
Concerning branching angles, no relevant variations were observed. However, after mycelium incubation with benzoic acid for 7 days, the branching angles was reduced to 20° ± 8° (in contrast to the 45° ± 5° grades of the control) showing that the neighbouring microenvironment was no more suitable for the development of the arising hyphae (Fig. 2.4).

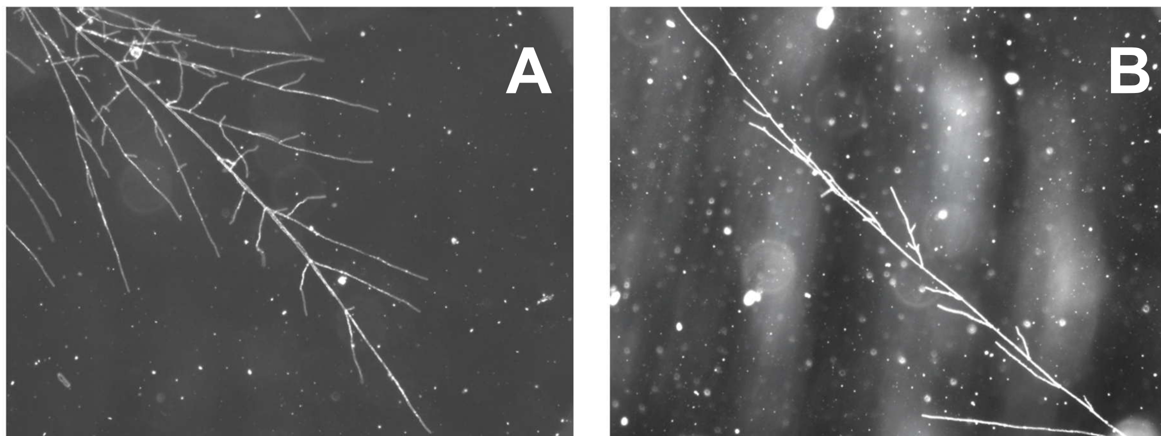


Fig. 2.4 hyphal fragments after 7 days of growth in absence (A) or in presence (B) of 1 mM benzoic acid.

2.3.3 - Macroscopical radial growth

P. ostreatus mycelium grows on agar medium as a circular colony. The radius of this colony initially increases in an exponential way, and becomes constant when individual hyphae extend at a linear rate. At this stage, colony radial growth rate reflects the average of maximum hyphal growth rate of any individual hyphae and is affected by the contribution of variation in the hyphal growth direction. Exponential growth ceases when conditions at the colony centre became unfavourable. However, the radial growth of the colony can indefinitely continue starting from the margin. The external margin ring is termed perhipheral growth zone (width of the zone is indicated as **W**). Within this zone mycelium grow exponentially at its specific rate [17].

$$\mu = K / W \qquad \text{[b]}$$

where K is a constant. Combining equation [a] and [b]

$$W = K * G / E \qquad \text{[c]}$$

W is related to the microscopical parameter (**E** and **G**) analyzed in the previous section. Thus, macro- and microscopical analysis can point out the same physiological state (active-growth, inhibition, etc.) in a different way.
In the present work radial growth was calculated measuring total area of the mycelium growing for 5 days, in presence or in absence of the tested putative autoregulators on agar media. Total area was approximated to the related circle area whose average radius was calculated. All scanned images (Fig. 2.5) were analyzed by Image-j tool as described in the subsection 2.1.1. As shown in the figure 2.6 the extension rate of fungal colony confirmed the previous microscopical analysis.

Benzaldehyde did not affect growth rate and resulted in lower hyphal density. Conversely, benzoic acid and 2-hydroxyl-4-nitrobenzoic acid inhibited mycelium development and positively affected hyphal density. Surprisingly, 4-methoxibenzaldehyde negatively affected macroscopic fungal growth, in contrast with data reported from microscopical observation.

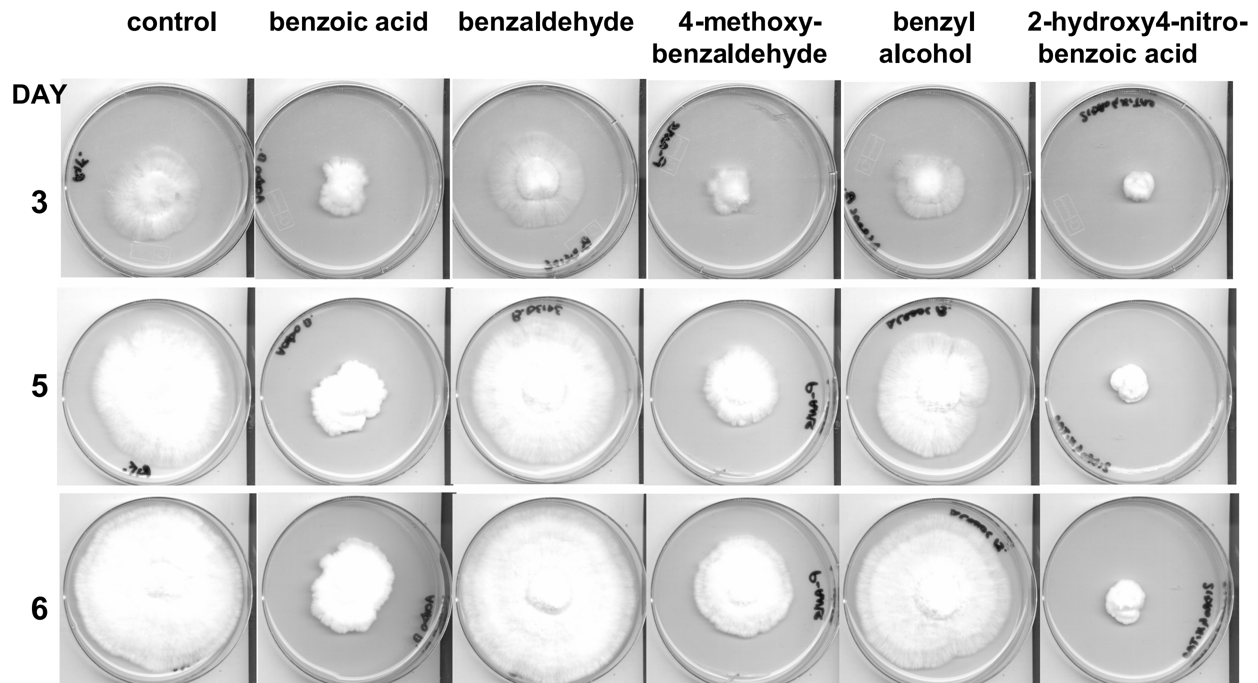


Fig. 2.5 Time course analysis of *P. ostreatus* radial growth on GYM agar media in presence and in absence of benzyl-derivatives (1 mM final concentration). Radial growth was monitored after 3, 5 and 6 day of growth, until mycelium confluence

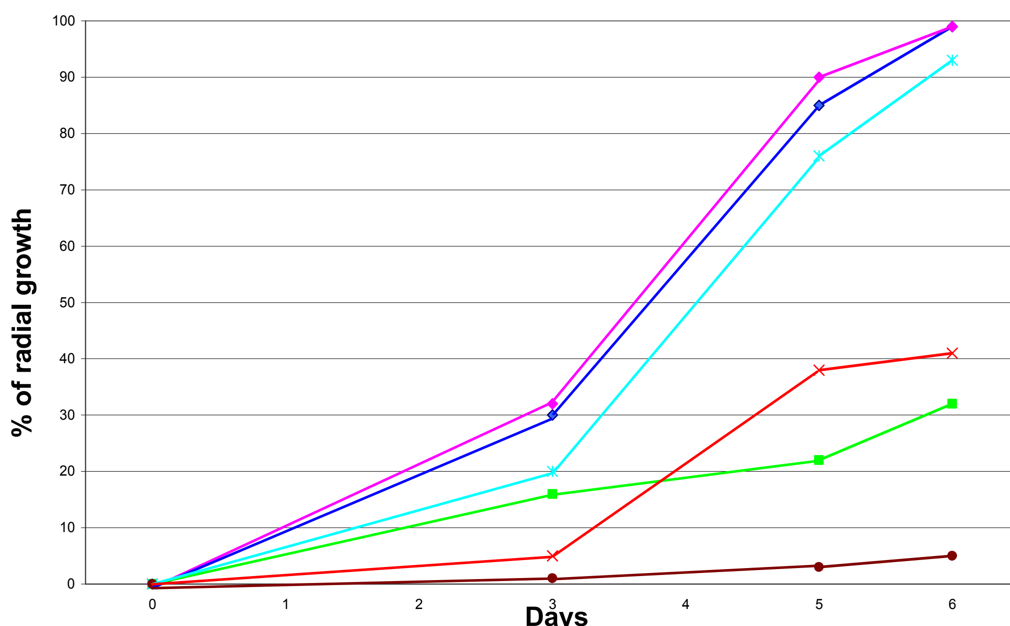


Fig. 2.6 mycelial radial growth in presence and in absence of benzyl-derivatives blue – control; green – benzoic acid; pink – benzaldehyde; red – 4-methoxybenzaldehyde; azureblue – benzyl alcohol; brown – 2-hydroxy,4-nitro-benzoic acid. The maximum extention (100%) is 4,5 cm, corresponding to the Petri dish radius. Standard deviations (< 10%) were omitted.

2.3.4 - Sporal germination

Spores play an important role in the life cycle of fungi, acting as dispersal or survival. Dispersal spores are produced in higher numbers than the survival spores and they are separated completely from the parent mycelium to facilitate migration to a new site. They have a moderate capacity for survival in the resting dormant state and are also capable to germinate readily under favourable environmental conditions including the presence of nutrients [18].

Several fungi produce substances, named self-inhibitors, which reversiblely inhibit germination and growth [19]. The major function of self-inhibitors is to prevent premature germination of spores directly after spore formation and before spore dispersion. The germination starts when self-inhibitors is removed from the spore or its environment. This mechanism guarantees that spores germinate only after dispersal into environments favourable to mycelium establishment [18].

In this paragraph, the effect of externally addition of the selected benzyl-deriveted compounds on *P. ostreatus* basidiospores is reported (Fig. 2.7). The basidiospores suspension was plated on solid GYM medium in *Petri* dishes and incubated at 28°C. Germination was determined following incubated plates for 5 days and percent of germinating spores was calculated (% of spread spores per microscopic mycelial colony). According to previously data, benzoic acid and 2-hydroxy-4-nitrobenzoic acid inhibited germ tubes formation. 4-methoxy-benzaldehyde did not significatively affect sporal germination. Surprisingly, benzyl alcohol partially inhibited sporal germination until the 5th day of incubation, whereas benzaldehyde displayed the same inhibiting effect only at the 3th day of incubation.

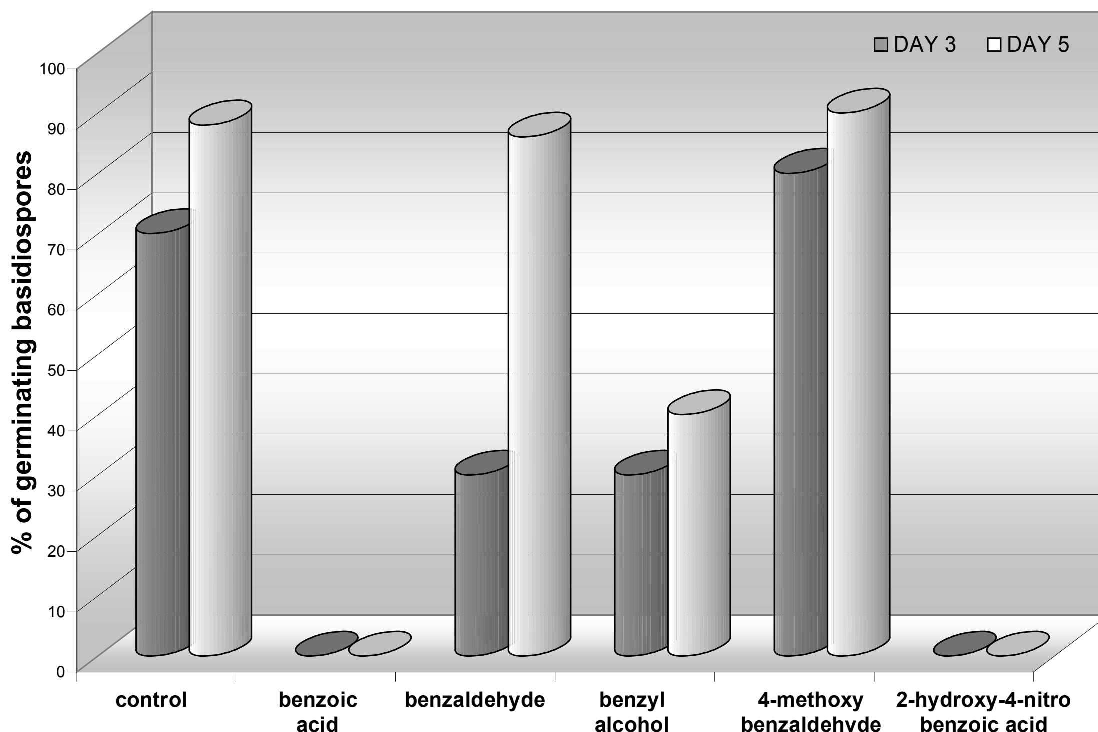


Fig. 2.7 percent of *P. ostreatus* germinating basidiospores. Spore were spread on GYM agar media in presence and in absence (control) of endogenous benzyl-derivatives compounds (1mM final concentration) and arising germtube were counted after 3 and 5 days of incubation at 28°C.

2.3.5 - Laccase production in liquid culture

In chapter 1, production of secondary metabolites related to aryl compounds has been shown to be triggered by starvation stress in the last phase of fungal growth. Furthermore, laccase production has been reported to be positively affected by the presence of extracellular endogenous compounds. In order to identify the benzyl-derivative able to induce laccase production, a time course analysis of laccase activity was carried out conditioning fungal basal growth with the above-listed aryl compounds. Results previously obtained with spent-media showed a 3-fold increase of the maximum of laccase production during the first phase of growth. Conversely, no tested molecule induced a similar behaviour. However, the presence of 2-hydroxy-4-nitrobenzoic acid (figure 2.8) led to a 7-fold increase of laccase activity in the last days of growth.

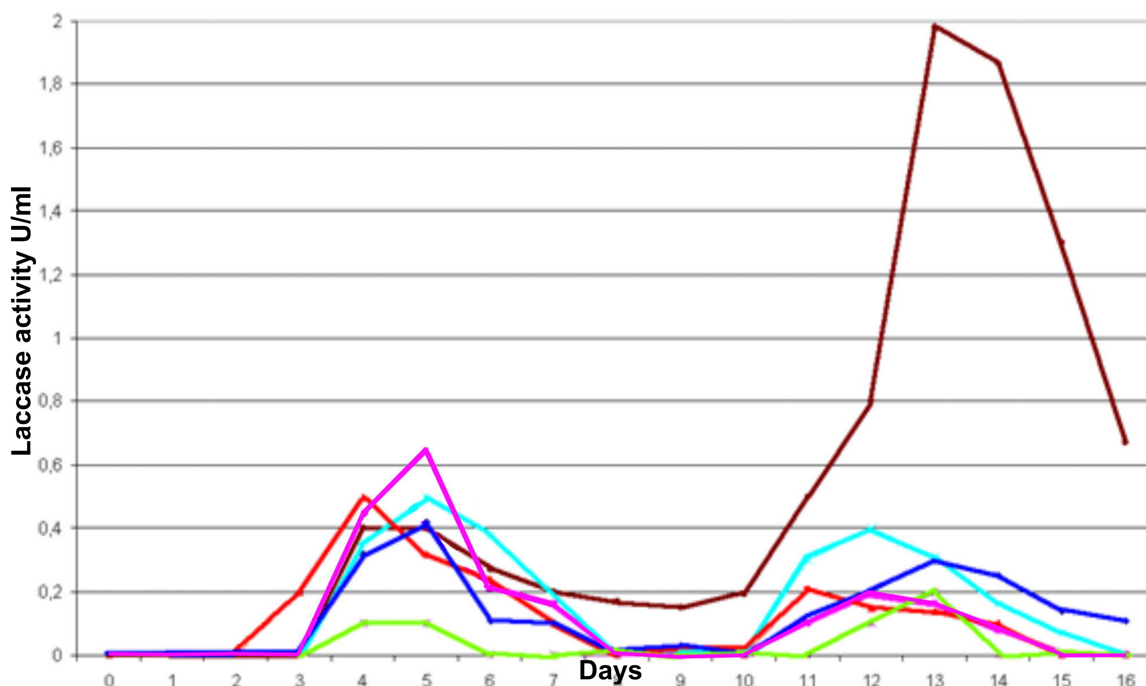


Fig. 2.8 Time course analyses of laccase production in PDY liquid culture in presence of 150 μM $CuSO_4$ and 1mM benzyl-derivatives: blue – control; green – benzoic acid; pink – benzaldehyde; red – 4-methoxybenzaldehyde; azureblue – benzyl alcohol; brown – 2-hydroxy,4-nitro-benzoic acid. Standard deviations (< 10%) were omitted.

In order to maximize laccase expression in *P. ostreatus*, fermentation parameters, such as inoculum size, induction time, and culture broth composition, were optimized (data not shown). 4 millilitre of inoculum containing fungal homogenate were transferred in 1-l flasks containing 300 ml of PDY liquid broth in presence of 150 μM $CuSO_4$. The cultures were grown in shaken flasks at 125 rpm and incubated at 28°C in the dark for 21 days. Submerged cultures were conditioned by 1mM 2-hydroxy-4-nitrobenzoic acid, added at the 4th day growth and time course analyses of laccase production was performed. As reported in figure 2.9, in the second part of fermentation, an increase of laccase production up to 10 times was detected. Moreover, high laccase production was observed in a temporal frame of 7 days. Increasing the laccase activity and enlarging the temporal frame of enzymatic production are two fundamental issues for the scale-up processing in the field of the industrial biotechnology. Conversely, a delay of biomass increase was detected in conditioned fermentation without affecting the highest value (Fig. 2.9 curve C).

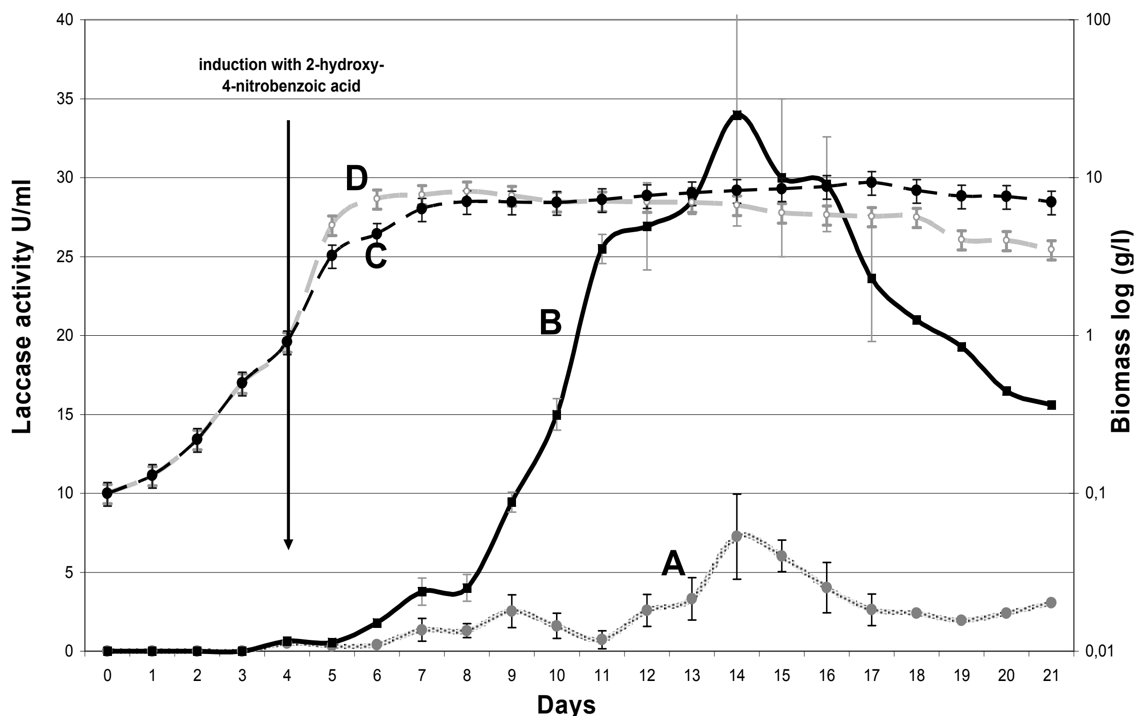


Fig. 2.9 Time course of *P. ostreatus* growths in PDY medium + 150 µM $CuSO_4$ in absence and in presence of 2-hydroxy-4-nitrobenzoic acid (1 mM). A and B curves indicate laccase activity of control and test growths, whereas C and D show the biomass increasing, respectively.

With the aim to fully characterize secretomic pattern in the presence of 2-hydroxy-4-nitrobenzoic acid, the supernatants of individual submerged cultures were separated from the mycelia harvesting fungal culture after 6, 9 and 13 days. In collaboration with the mass spectrometry laboratory of the BMA group (C. Giangrande and A. Amoresano - Department of Organic Chemistry and Biochemistry, Università degli Studi di Napoli "Federico II"), extracellular proteins were analysed and experimentally identified by means of 2-D gel electrophoresis and mass spectrometry. The highest level of protein secretion was detected at 9th day (Fig 2.10). The 2D-images were analysed for differential expression between control and test samples and proteins contained in any differing spot were identified. Several proteins co-migrated under the same spot. Table 2.2 reports the differentially produced protein detected in the secretoma of *P. ostreatus* growth after 9 days of growth in conditioned medium.

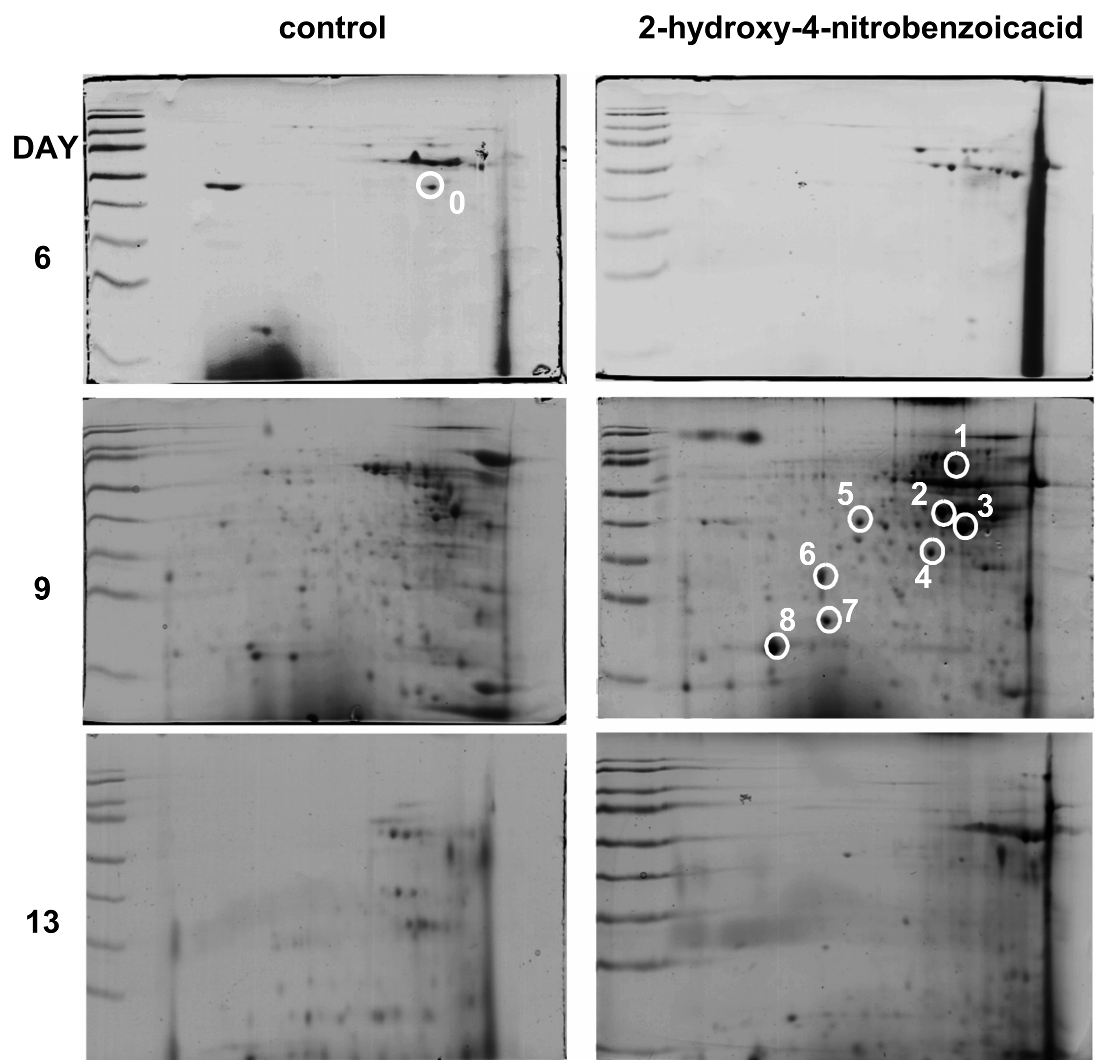


Fig. 2.10 Effect of 2-hydroxy-4-nitrobenzoic acid on protein profiles of *Postreatus* secretome. Two-dimensional gel electrophoresis of supernatant of submerged cultures harvested at 6, 9 and 13 day of growth in presence and in absence of the 2-hydroxy-4-nitro-benzoic acid.

SPOT							
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Q96TR4 Laccase POXA3	Tripeptidyl peptidase I	Aspartyl protease	Aryl-alcohol oxidase	Aryl-alcohol oxidase	Aspartyl protease	Unknown protein	Peptidyl-Lys-metallo peptidase
no predicted function	Q12739 Laccase POXC	Tripeptidyl peptidase I	Aspartyl protease				
Q12739 POXC	Q96TR4 Laccase POXA3	Q12739 Laccase POXC					
Glyoxal oxidase	Glycoside hydrolase family 47	Glycoside hydrolase family 15					
Copper radical oxidase	Extracellular triacylglycerol lipase	no predicted function					
Carbohydrate esterase family 4		Extracellular triacylglycerol lipase					
		Aryl-alcohol oxidase					
		Q96TR4 Laccase POXA3					

Table 2.2 spots related to the figure 2.9, analyzed by means of 2-D gel electrophoresis: differentially expressed protein at 9th day in *P. ostreatus* growth conditioned by 1mM 2-hydroxy-4-nitrobenzoic acid.

Even if *P. ostraetus* genome is available, identity and function of many of its secreted proteins remain to be elucidated. Some of the identified differentially expressed proteins are industrially relevant enzymes, such as oxidases (laccases and aryl alcohol oxidase), glycosidases, proteases and lipases (Fig. 2.11).

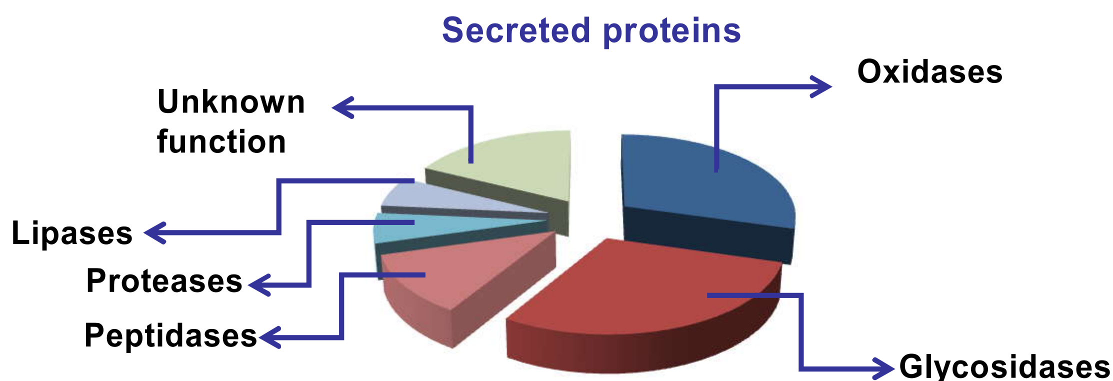


Fig 2.11 Pie chart categorizing the identified *P. ostreatus* secretome proteins differentially expressed in the conditioned medium conditioned by 1mM 2-hydroxy-4-nitrobenzoic acid according to their different gene ontology terms.

Based on the dynamic metabolic profile of the secondary metabolites reported in chapter 1 (Table 1), an autoregulative physiological role of some of the extracellular aryl-metabolites was proposed.

- BENZALDEHYDE

Starting benzaldehyde production has been related to the beginning of carbon starvation. Increasing hyphal length and HGU, induced by this chemical, indicate a well defined fungal cellular tropism through which hyphae sense a dramatic decrease of nutrient source (starvation). When nutrient becomes exhausted or fungal cell encounters nutrient-depleted regions, in fact, hyphae increase their extension rate in order to enhance the probability of finding new food sources. Because wood is the natural environmental of white-rot fungi, it is possible to imagine that in order to colonize a new region *P. ostreatus* increases production of extracellular ligninolytic enzymes, such as laccases. As reported in chapter 1, production of laccase activity in the first part of liquid fermentation may be related to the primary metabolism, and to biomass production: laccase increase, related to the presence of benzaldehyde, is a consequence of hyphal extension, not caused by metabolization of this chemical (fungal dry-weight does not change).

- BENZOIC ACID and 2-HYDROXY-4-NITROBENZOIC ACID

When cell-damaging begins, because of mechanical cell lyses or autolyses caused by a long starvation stress, a multicellular organism, such as a mushroom, needs to stop the growth in not favourable condition and protects its vulnerable parts from competitor microbes. Benzoic acid and 2-hydroxy-4-nitrobenzoic acid achieve both purposes. Actually, benzoic acid [20, 21] and nitro-derivatives (personal communication) in fact inhibit some bacterial and yeast strain and also fungal growth and sporal germination of *P. ostreatus*. Moreover, 2-hydroxy-4-nitrobenzoic acid (Fig. 2.12 A) induces, up to 10 times, laccase production in liquid culture. The reasons why 2-hydroxy-4-nitrobenzoic acid induces higher titres of laccases in white-rot fungi is to be speculated at this stage. In filamentous fungi induction of laccase is seen also as an activation of cellular defence mechanisms against competitor microbes [22] as well as against oxidative stress [22, 23]. (radical species can be produced during cellular lyses). Whether considering laccase production as a defence mechanism, this induction is also coherent with the hypothesized effect for 2-hydroxy-4-nitrobenzoic acid This specific elicitation effecting endogenous phenolic compounds has been also reported in the basidiomycete *Pycnoporus cinnabarinus*, producing the amino-phenolic compound 3-hydroxyanthranilic acid (Fig. 2.12 B) (Final Scientific Activity Report of Quorum sensing project, STREP-FP6-279 NMP4-

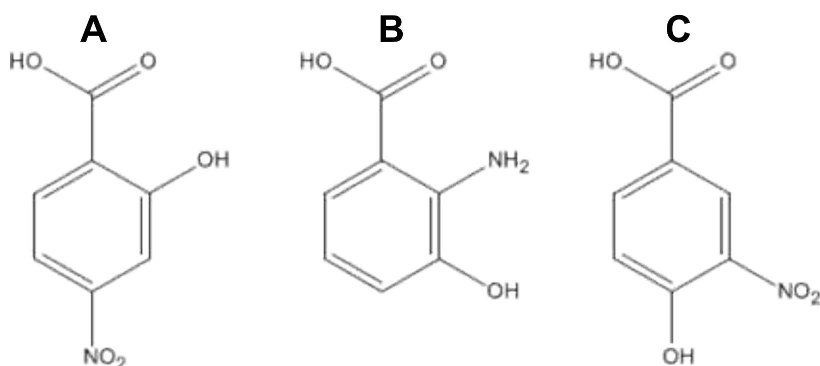


Fig. 2.12 chemical structure of autoregulator excreted by different organism: (A) 2-hydroxy-4-nitrobenzoic acid produced by P. ostreatus; (B) 3-hydroxyanthranilic acid produced by *P. cinnabarinus*; (C) 4-hydroxy-3-nitrobenzoic acid produced by *Salegentibacter sp. T436.*

CT-2006-032811). Moreover, several studies report that nitrophenyl derivatives are generally plant regulators. For example, 4-hydroxy-3-nitrobenzoic acid (Fig. 2.12 C) produced by *Salegentibacter sp. T436* promotes root elongation and shoot systems in plants [24].
Exposure to 2-hydroxy-4-nitrobenzoic acid was also observed to affect extracellular protein production. Analysis of protein patterns revealed striking differences between treated and not treated fungal cultures. Several industrially useful enzymes, as well as laccases, were more expressed in presence of 2-hydroxy-4-nitrobenzoic acid. Presumably, these proteins play a role in long-time stress adaptation. Further investigation could explain the mechanism of action of 2-hydroxy-4-nitrobenzoic acid in the frame of fungal physiology. However, reported data point out this chemical as autoinducer potentially suitable for biotechnological purposes.

2.3.6 - C8 compounds

Despite eight-carbon-atoms (C8) molecules involvement in several key biological processes little is known about their biosynthetic pathway(s) in fungi [7, 25, 26, 27,]. The biochemistry of 1-octen-3-ol formation is better documented, [28, 29, 30, 31]. Fungi and plants, utilize their fatty acids resources to produce volatile compounds; the fatty acid molecules are first oxidized and then cleaved to produce short-chain volatiles.
In this paragraph, the effect of externally addition of 1-octen-3-ol, 1-octen-3-one, 3-octanone and 3-octanol (Fig. 2.13) on *P. ostreatus* basidiospores is reported (Fig. 2.14). Experimental approach was similarly performed as for the previous subsection. In the presence of 1 mM 1-octen-3-one, the germination was totally inhibited after 4 incubation days at 28°C, whereas the other C8 chemicals do not affect germination.

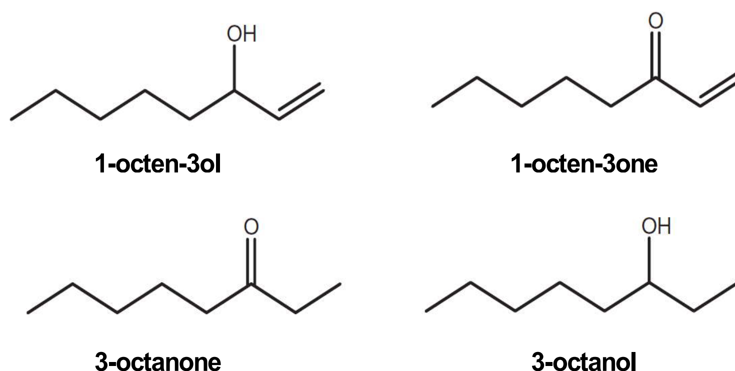


Fig. 2.13 Chemical structure of the main eight-carbon volatiles in *P. ostreatus.*

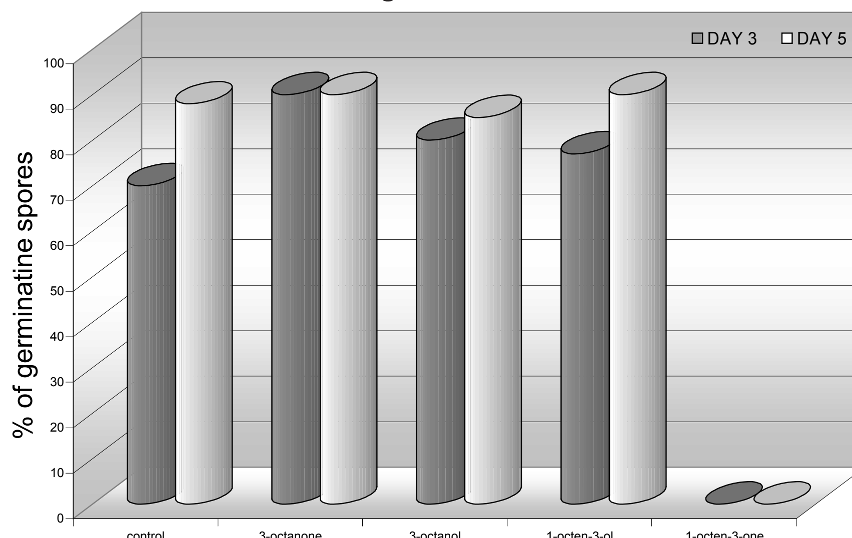


Fig. 2.14 percent of *P. ostreatus* germinating basidiospores. Spore were spread on agar media in presence and in absence (control) of endogenous C8 volatile compounds and arising germtubes were counted after 3 and 5 days.

In order to study the effect of the sporal inhibitors on a different developmental phase, C8 compounds were added to agar basal medium and a 13 mm plug of *P. ostreatus* mycelium was sub-cultured as previously described for radial growth rate measurements. 1-octen-3-one resulted in 30% inhibition of mycelium development, whereas no effect was induced by the presence of the other C8 compounds. Analysis performed in microscope silicon chambers and in liquid cultures, in the presence and in the absence of each C8 metabolite, showed no relevant effect. However, in a few cases, samples harvested from fungal fermentation, growing in presence of 1-octen-3-ol, resulted in a different laccase isoenzymatic pattern compared to the control. Analyses performed by native gel stained for laccase activity sometimes showed, in fact, a very faint new band among POXA3 [32] and POXC [33] isoforms (data not shown) (see paragraph 2.5).
Based on the present analysis and on the data available in literature a physiological role of one of the C8 compounds in fungal development is proposed.

- 1-OCTEN-3-ONE: a self-inhibitor of sporal germination

Germination autoinhibitors are produced at the time of sporulation, and deposited on the spores outer wall layers. Several filamentous fungi appear to use chemicals derived from the same pathway or from structurally related ones.
1-octen-3-ol is an autoinhibitor produced by conidiation of the ascomycete *Penicillium paneum* [7]: this C8 compound negatively affects germ tube formation and mycelial growth. Other alken alcohol are know to be involved in self inhibition in ascomycetes, such as the 3-hexen-1-ol excreted by *Colletrotrichum graminicola* [34].
Conversely, to the best of our knowledge, no relevant physiological function of C8 compounds have been reported in basidiomycetes. As a fact, the C-8 volatile compound 1-octen-3-ol, produced in gill tissue of basidiomycetes like *Agaricus bisporus* [35] and *P. ostreatus* [36], is known to be only responsible of the typical mushroom odour.
In the present study, the effect of some C-8 compounds on the basidiomycete *P. ostreatus* has been evaluated. 1-octen-3-ol did not show any effect on basidiomycetes spores, whereas the derivative 1-octen-3-one inhibited basidiospore germination. The ketonic form can be converted in the corresponding alcohol through a reduction step. Beltran-Garcia et al. [37] studied antibacterial activities of mixtures of volatile compounds (1-octen-3-ol, 3-octanol, octanol, 3-octanone, and 2-octanone) found in *P. ostreatus,* and reported that these mixtures inhibited growth of *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, and *Salmonella typhimurium*. Herein, for the first time, C8 compound produced by *P. ostreatus* has been demonstrated to be an autoregulator of sporal germination.
An analogue example of self-regulation equilibrium among ketonic and alcoholic form was found in *Fusarium graminearum* [38]: zearalenone affects sexual development whereas its conversion to zearalenol inactive the chemical.
The function of C-8 volatile compounds is evident in *P. ostreatus*: while 1-octen-3-ol inhibits pathogenic ascomycetes, 1-octen-3-one avoid premature sporal germination when basidiospores are connected to the gills.
Further investigations with fungal spore of different classes could demonstrate if 1-octen-3-ol and 1-octen-3-one can be used for a practical application as selective agent for the isolation of new wild-species of fungi.

2.4 - References

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2.5 - LACC12 a new member of *Pleurotus ostreatus* laccase family from mature fruiting body.

A new phenol-oxidase isoenzyme was previously sporadically detected in liquid fermentation conditioned by C8 compound. During sporocarps development the fungus mainly produces C8 compounds. In order to verify and possibly isolate the new laccase, investigations on laccase isoforms produced in *P. ostreatus* fruting bodies were was performed. The present paragraph reports the paper concerning this research line submitted to *Enzyme Microbial Technology* journal for publication.

Identification of a new member of *Pleurotus ostreatus* laccase family from mature fruiting body.

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Short running title: Laccase from *Pleurotus ostreatus* fruiting body.

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Abstract

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are blue multicopper oxidases, catalyzing the oxidation of an array of aromatic substrates concomitantly with the reduction of molecular oxygen to water. Most of the known laccases have fungal or plant origins, although few laccases have been also identified in bacteria and insects. These enzymes are implicated in a wide variety of biological activities and are being increasingly evaluated for a variety of biotechnological applications due to their broad substrate range. Most of the fungal laccases reported thus far are extra-cellular enzymes, whereas only few enzymes from fruiting bodies have been described so far. Multiple isoforms of laccases are usually secreted by each fungus depending on species and environmental conditions. As a fact, a laccase gene family has been demonstrated in the white-rot fungus *Pleurotus ostreatus*.
This work allowed identification and characterization of the first laccase isoenzyme from the fruiting body of *P. ostreatus*. Discovery through mass spectrometry of LACC12 proves the expression of a functional protein by the related hypothetical encoding transcript.
The reported data add a new piece to the knowledge about *P. ostreatus* laccase multigene family, and contribute to increase the meagre data regarding these enzymes from basidiocarp.

Keywords: fruiting body; laccase family; physiological role; fungi.

Introduction

Laccase is one of the very few enzymes that have been studied since the end of 19th century. It was first identified in the exudates of *Rhus vernicifera*, the Japanese lacquer tree [1]. A few years later its presence was also demonstrated in fungi [2]. Laccases are thought to be nearly ubiquitous among fungi, and their presence has been documented in virtually every fungus examined thus far [3]. More recently, proteins with features typical of laccases have been identified in insects [4] and prokaryotes [5].
Laccases are multicopper-containing enzymes belonging to the group of blue oxidases, along with ascorbic oxidases and ceruloplasmins [6]. The catalysis carried out by all members of this family is guaranteed by the presence of different copper centres. In particular, all blue multicopper oxidases (MCO) are characterized by the presence of one type-1 (T1) copper, together with at least three additional copper ions: one type-2 (T2) and two type-3 (T3) copper ions, arranged in a trinuclear cluster. These enzymes are being increasingly evaluated for a variety of biotechnological applications due to their broad substrate range.
Fungal laccases have been associated with delignification [7], fruiting body structuring [8], pigment formation during asexual development [9], pathogenesis [10; 11]. The majority of fungal laccases are extracellular monomeric globular proteins of approximately 60–70 kDa with acidic isoelectric point (pI) around pH 4.0; they are generally glycosylated, with an extent of glycosylation ranging between 10% and 25% and only in a few cases higher than 30% [6]. Only few laccases from fruiting bodies have been purified and characterized thus far. As a fact, laccases have been isolated from fruit bodies of *Lentinula edodes* [12], *Cantharellus cibarius* [13], *Hericium erinaceum* [14], *Albatrella dispansum* [15], *Tricholoma giganteum* [16], *Ganoderma lucidum* [17], *Pleurotus eryngii* [18], *Pleurotus cornucopiae*[19], and *Ganoderma sp.* MK05 [20].
Many fungi produce several laccase isoenzymes endowed with different catalytic properties, being the physiological significance of this multiplicity still unknown. Likewise, fungi belonging to *Pleurotus* genera display this feature [6]. As a fact, four laccase gene members have already been isolated in *Pleurotus sajior-caju* [21], two in *P. eryngii* [22], and seven in *Pleurotus ostreatus* [23]. In this latter fungus, the existence of a “laccase subfamily” consisting of three out of seven members has been postulated, based on sequence similarity, and intron-exon structure. Furthermore, a careful investigation of the recently released genome of this fungus by the DOE-Joint Genome Institute (http://www.jgi.doe.gov/sequencing/why/50009.html, DOE-JGI) suggested an even more complex MCO family with the identification of genes putatively coding for previously uncharacterized laccases, enriching the panel of laccase genes up to twelve members (unpublished data).
In this work the identification of a new laccase isoenzyme from the fruiting body of the white-rot fungus *P. ostreatus* has been achieved through the detection of six tryptic peptides by mass spectrometry. The identity of the functional laccase protein has been confirmed with the translated genomic sequence of *P. ostreatus* laccases. The new isoform has been analysed from the kinetic point of view. The acquired data contribute either to enlarge the assortment and knowledge of its laccase multigene family, and to increase the meagre data regarding laccases from fungal fruiting body.

Materials and methods

Organism and Culture Conditions. White rot fungus *P. ostreatus* (strain Florida) was maintained through periodic transfer at 4°C on potato dextrose agar plates (Difco) in the presence of 0.5% yeast extract (Difco).

Fruiting body production. P. ostreatus mushrooms were cultivated in 500 ml jars containing 400 g of humid wheat-straw, which were twice autoclaved for 1 h at 121°C separated by a period of 24 h at room temperature. Each jar was inoculated with 4 agar plug (13 mm diameter), and left to grow at 28°C for 30 days in the dark. Fructification were promoted by opening the jars, and placing them in presence of daylight in a chamber at 15 ± 5°C and 90% relative humidity. Primordia appeared after a further 15 days of growth, and basidiocarps were harvested 7 days later and weighed.

Laccase purification. Fungal proteins were extracted by homogenizing *P. ostreatus* fruiting bodies in a Waring blender in presence of 50 mM sodium phosphate buffer (pH 6.5) containing 1mM phenylmethanesulphonylfluoride (PMSF – Sigma). The homogenate was centrifuged at 13,000×g for 30 minutes, and the supernatant was filtered through cheesecloth. Proteins were precipitated by addition of $(NH_4)_2SO_4$ up to 80% saturation at 4°C and centrifuged at 10,000×g for 40 min. The ammonium sulphate precipitate was resuspended in 50 mM sodium phosphate (pH 6.5) and after extensive dialysis against the same buffer was loaded onto a Hi Trap DEAE Fast Flow column (GE Healthcare) in a fast protein liquid chromatography system (AKTA Explorer, Amersham Biosciences). Four fractions containing laccase activity were recovered with the equilibrating buffer and during the linear gradient from 0 to 0.4 M NaCl. They were separately pooled, concentrated and desalted on an Amicon PM-10 membrane. Fraction 3 was equilibrated in Tris–HCl 50mM pH 8.0 and loaded onto an anion exchange Resource Q column (Amersham Biosciences) in a fast protein liquid chromatography system (AKTA Explorer, Amersham Biosciences) equilibrated with the same buffer. Three fractions containing laccase activity were recovered during the linear gradient from 0 to 0.4 M NaCl. They were separately pooled, concentrated and desalted on an Amicon PM-10 membrane.

Protein determination. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard.

Assay of laccase activity. Phenol oxidase activity was assayed at 25°C using 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP) and syringaldazine as substrates, as previously described [24]. Enzyme activity was expressed in IU. K_M were calculated by nonlinear regression fitting with the GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com).

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

Protein identification by mass spectrometry. Slices of interest were destained by washes with 0.1 M NH_4HCO_3 pH 7.5 and acetonitrile, reduced for 45 minutes with 100 µl of 10 mM dithiothreitol in 0.1 M NH_4HCO_3 buffer pH 7.5 and carboxyamidomethylated for 30 minutes in the dark with 100 µl of 55 mM iodoacetamide in the same buffer. Tryptic digestion was performed by adding for each slice 100 ng of enzyme in 10 µl of 10 mM NH_4HCO_3 pH 7.5 for 2 hours at 4 °C.

The buffer solution was then removed and were added 50 µl of 10 mM NH_4HCO_3 pH 7.5 for 18 hours at 37 °C. Peptides were extracted by washing the gel slices with 10 mM NH_4HCO_3 and 1% formic acid in 50% acetonitrile at room temperature.
The peptide mixtures were filtered by using 0.22 µm PVDF membrane (Millipore) and analysed using a 6520 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed in 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at flow rate of 400nl/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% in 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan. Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (0.5 min) of ions from which definitive mass spectral data had previously acquired. Nitrogen at a flow rate of 3 L/min and heated to 325°C was used as the dry gas for spray desolvation. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50000 counts. Double and triple charged ions were preferably isolated and fragmented over single charged ions. The acquired MS/MS spectra were transformed in *mz.data* format and used for proteins identification with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA). Raw data from nanoLC–MS/MS analyses were used to query the *P. ostreatus* database and the Mascot search parameters were: trypsin as enzyme, allowed number of missed cleavage 3, carbamidomethyl as fixed modification, oxidation of methionine, pyro-Glu N-term Q, as variable modifications, 10 ppm MS tolerance and 0.6 Da MS/MS tolerance, peptide charge, from +2 to +3. Spectra with a MASCOT score < 25 having low quality were rejected. The score used to evaluate quality of matches for MS/MS data was higher than 30. Trypsin, dithiothreitol, iodoacetamide and NH_4HCO_3 were purchased from Sigma. Trifluoroacetic acid (TFA)-HPLC grade was from Carlo Erba. All other reagents and solvents were of the highest purity available from Baker.
*Denaturing PAGE***.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli [25], using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue and/or electroblotted.
Western blotting. Proteins were transferred to a PVDF membrane using an electroblotting transfer apparatus (Trans-Blot Semi-Dry Transfer Cell, Bio-Rad, USA). Proteins were detected by using anti-POXC antibodies (cross-reacting with the new identified laccase) at a 1:20,000 ratio and peroxidase-conjugated anti-rabbit secondary antisera (1:40,000) (Sigma). The membranes were developed by using SuperSignal West Femto Maximum Sensitivity Substrate detection kit (Pierce).
Analysis of protein sequence. The predicted protein sequence of newly identified laccase LACC12 was analysed by comparison with those of the previously defined *P. ostreatus* laccases. Alignments of amino-acidic sequences were generated with ClustalW2 (http://www.ebi.ac.uk/Tools/ clustalw2/index.html). Signal peptide were predicted with SignalP V2.0 (http://www.cbs.dtu.dk/services/). The amino-acid conservation scoring among the laccase signature sequences was performed by PRALINE (http://www.ibi.vu.nl/programs/pralinewww/).

Results

The supernatant of fruiting body extract was separated from the homogenized mycelium by filtration, concentrated by ammonium sulphate precipitation and dialyzed against 50 mM sodium posphate pH 6.5. Four bands corresponding to laccase isoforms were visible on a native electrophoresis gel when ABTS was used for the gel staining. Compared with the laccase isoenzymatic pattern usually produced by *P. ostreatus* in submerged cultures, mainly constituted by POXC [26], POXA1b [27] and POXA3 [28; 29], one new isoform with different electrophoresis mobility was detected (Fig. 1, lane A). Phenol-oxidases extracted from fruiting body were successfully separated into four distinct pools of protein fractions on a DEAE Sepharose column at pH 6.5. When analysed by native PAGE, a pool of active fractions, besides laccases POXC and POXA3, included the new isoenzyme (Fig. 1, lane B). The new isoform was successfully separated from POXC and POXA3 enzymes by anion exchange chromatography Resource Q column (Amersham Biosciences), as shown by the reported native page (Fig. 1, lane C).

The proteic band was excised from the native gel. Proteins in the band were reduced, alkylated and *in situ* digested with trypsin, The peptide mixture generated in the enzymatic hydrolysis was fractionated by capillary chromatography and analyzed on-line by tandem mass spectrometry, revealing information on the amino-acidic sequence beside the peptide molecular mass. The identification of tryptic peptides of the purified laccase was conducted using the MS/MS ion search on a MASCOT server against the partially annoted *P. ostreatus* genome. Six peptides (sequence coverage, 18 %; protein summary score, 282) could unambiguously be identified. The individual ion scores (>38) indicate identity or extensive homology ($p<0.05$). *P. ostreatus* database search with the raw MS/MS data unambiguously identified the predicted laccase transcript *lacc12*.

LACC12 predicted protein sequence contains N-terminal secretion signals (23 amino-acids), conform to the typical sequences of eukaryotic proteins [30], the conserved ten histidine and one cysteine residues of the copper-binding centres of laccases, the fungal laccase signature sequences L1-L4 (Fig. 2A), as defined by Kumar et al. [31]. Compared to the defined signature sequences, the only relevant substitutions observed in LACC12 are a phenylalanine to tyrosine change in L1, and a valine to isoleucine change in L4. Sequence of the substrate binding loops, previously described for the other isoforms of the same family, [23] are not conserved among the *P. ostreatus* proteins (Fig. 2B).

The molecular mass of LACC12 predicted from its whole deduced amino-acid sequence is 55 kDa, and it was confirmed by Western-blotting analysis (Fig. 3). Thus, even if three putative N-glycosylation sites (Asn-X-Ser/Thr) are present into the sequence, no glycosilation seems to occur.

Catalytic proprieties of LACC12 with respect to three substrates (ABTS, DMP, and syringaldazine) were determined and compared with those of the other known members of the isoenzyme family (Table 1).

Discussion

P. ostreatus is an active lignin degrader that lives as a saprophyte on dead or decaying wood [32]. *P. ostreatus* ligninolytic enzymes have been used in the bioconversion of olive mill wastewater [33], and in the biodegradation of industrial contaminants [34; 35], among other applications [36].

Seven members of the *P. ostreatus* laccase multigene family, have been isolated and sequenced [37; 38; 27; 28; 23]. Five laccase isoenzymes, representing a variegated

group of enzymes endowed with peculiar properties, have been purified from culture broth of the fungus and fully characterized [26; 24; 27; 28; 29]. Furthermore, up to twelve members of the laccase gene family were identified in the recently released *P. ostreatus* genome database (unpublished data). This redundancy raises the question about their respective functions *in vivo*, and this question is even more pertinent since cDNAs or proteins for some of these genes have not been found [23; 24; 37].
In the present work a new laccase protein was identified from *P. ostreatus* fruiting body. MS/MS analysis univocally identified the new laccase as the LACC12 from the corresponding encoding gene model in the *P. ostreatus* genome. This enzyme shares the highest sequence identity (68% identity) with a laccase from *Cyathus bulleri* (ABW75771) [39] and with laccase 2 from *Coprinopsis cinerea* (AAR01243) [40]. The protein contains all the L1-L4 consensus sequences and can be classified as a laccase in *sensu stricto*. Analyzing the LACC12 sequence, it displays the same substitution phenylalanine to tyrosine (F97Y), already found in POX3 [23] and in laccases belonging to *C. cinerea* subfamily [40], being this a conserved position among many basidiomycete laccase sequences. Substitutions of valine to isoleucine in loop 4 (position 490), are present in the laccase sequences of *Coprinus cinereus* [41], *Trametes versicolor* [42] and *C. cinerea* [40]. Based on sequence similarity, the new laccase LACC12 may belong to POXC sub-family (67% identity) (Table 2) [23]. As a fact, LACC12 is immuno-detected with antibodies against POXC (Fig. 3). The affinity (K_M value) of LACC12 towards typical laccase substrates follows the rank order syringaldazine > 2,6-DMP > ABTS (Table 2). Affinities of LACC12 towards common substrates are almost of the same order of magnitude respect to those of the other known members of the family and are in agreement with what reported for other known fungal laccases [43].
Discovery through mass spectrometry of *P. ostreatus* LACC12 proves the expression of a functional protein by the related hypothetical encoding transcript. LACC12 seems to be exclusively expressed in the fruiting body, thus suggesting a specific role for this isoenzyme, possibly involved in the development of fruiting body. As a fact, a particular physiological role in gill browning for the laccase LCC2 has been hypothesized in *L. edodes* [12]. On the other hand, transcript profiling has unveiled that in *Volvariella volvacea* [8] and *Laccaria bicolor* [44] laccase genes are differentially expressed as a function of the developmental stage.
The analysis of the LACC12 *P. ostreatus* transcript profile during fruit body development will be extremely helpful in better defining any possible role for this novel isoenzyme.

This study shows for the first time the identification of a laccase from the fruiting body of *P. ostreatus*. The reported data add a new piece to the knowledge about *P. ostreatus* laccase multigene family, and contribute to increase the meagre data regarding laccases from fungal fruiting body. As a fact, to the best of our knowledge only few laccases from fruiting bodies have been purified and characterized thus far [12-21] and their protein sequences is almost unknown. The enzymes - secreted and non secreted forms - even though coming form the same source might not share the same characteristics and/or may play different physiological roles. A further progress towards a more intelligible picture of the role(s) played by individual laccase isoforms in *P. ostreatus* has been attained with this work.

Acknowledgement
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Figure legends

Figure 1. Zymograms of laccase isoenzymes extracted from *P. ostreatus* fruiting body. Samples containing 0.015 laccase unities were loaded. Ammonium sulphate precipited (lane A); D3 fraction from cationic exchange chromatography on DEAE Sepharose column (lane B); isolated laccase after cationic exchange chromatography on Resource Q column (lane C). Known laccase isoforms POXA1b, POXA3a/POXA3b and POXC are loaded as standards.

Figure 2. A. Comparison of the laccase signature sequences from the *P. ostreatus* laccases POX1, POXC, POXA1b, POXA3, POX3 and POX4 with LACC12. The fungal laccase signature sequences (L1-L4) are shown together with the fungal laccase consensus sequence (Laccase Cons.) according to Kumar et al. [30]. The amino-acid conservation scoring is performed by PRALINE (http://www.ibi.vu.nl/programs/pralinewww/). Residue shading has been applied from scoring 10 (black) to 7 (grey). The Y97 residue in L1 is boxed. **B.** Sequence alignment of the potential substrate binding loops of the *P. ostreatus* laccases, according to Pezzella et al., [22]. Loops L1 sequence at positions 182-192, B4-B5 at positions 230-235, B7-B8 at positions 288-297, C1-C2 at positions 357-368, C4-C5 at positions 413-421 and C7-C8 at positions 479-488.

Figure 3. SDS–PAGE analysis. Lane A: molecular mass markers (Sigma), from top downward: Albumin bovin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), and a-lactalbumin (14.2 kDa). Lane B: *P. ostreatus* LACC12 stained with Coomassie brilliant blue; lane C LACC12 immuno-detected with antibodies against POXC.

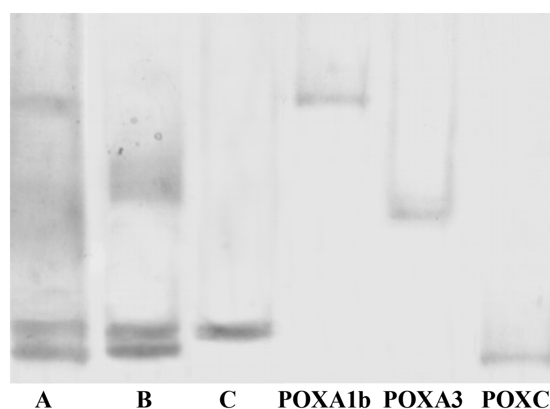
Figure/Table legends

			POXA1w	POXA1b	POXA3a	POXA3b
K_M ABTS (mM)	1.5×10^{-1}	3.9×10^{-2}	9.0×10^{-2}	4.7×10^{-1}	7×10^{-2}	7.4×10^{-2}
K_M DMP (mM)	2.7×10^{-1}	7.6×10^{-3}	2.1	3.8×10^{-1}	14	8.8
K_M Syringaldazine (mM)	5.5×10^{-2}	2.0×10^{-2}	1.3×10^{-1}	2.2×10^{-1}	3.6×10^{-2}	7.9×10^{-2}

Table 1. Affinity constants of purified LACC12 towards ABTS, syringaldazine and DMP substrates compared to the other members of the isoenzyme family

	POXA3	POX3	POXA1b	POX1	POXC	LACC12
POX4	50	58	60	77	80	68
POXA3		50	47	52	50	51
POX3			63	57	58	61
POXA1b				60	62	61
POX1					90	67
POXC						67

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



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A.

protein	L1	L2	L3	L4
POX1	HWHGFFQSGSTWADGPAFVNQCPI	GTFWYHSHLSTQYCDGLRGPF	HPFHLHGH	GPWFLHCHIDWHLEIGLAVVF
POX4	HWHGFFQAGTSWADGPAFVTQCPI	GTFWYHSHLSTQYCDGLRGAF	HPFHLHGH	GPWFLHCHIDWHLEIGLAVVF
POXC	HWHGFFQAGSSWADGPAFVTQCPV	GTFWYHSHLSTQYCDGLRGPF	HPFHLHGH	GPWFLHCHIDWHLEIGLAVVF
POX3	HWHGLYQEKTTWADGPAFVTQCPI	GTFWYHAHYGTQYCDGLRGPF	HPFHLHGH	GPWFLHCHVDWHLDLGLAVVL
POXA1b	HWHGLFVKGHNWADGPAMVTQCPI	GTFWYHSHLGTQYCDGLRGPL	HPIHLHGH	GPWFLHCHIDWHLDLGFAVVF
LACC12	HWHGFYQKGSNWADGPAFVTQCPI	GTFWYHSHLSTQYCDGLRGVF	HPFHLHGH	GPWFLHCHIDWHLELGLAIVF
POXA3	HWHGLFQHKTSGMDGPSFVNQCPI	GNYWYHSHLSTQYCDGLRGSF	HPFHLHGH	GAWFLHCHIDWHLEAGLAVVF
CONSENSUS	HWHG*********DG*****QCPI	GT*WYHSH***QYCDGL*G*F	HP*HLHGH	GAW*LHCHID*H***GL***F

B.

	Substrate binding loop					
	Loop I		Loop II	Loop III	Loop IV	
POX1	VVAP------QNAVLPT-	MSCDPN	ADPNL----GSTGF	MAFDVTNFELTI	IPALAVGGP	HIDWHLEIGL
POX4	VVAP------QNGPIPT-	LSCDPN	AQPNL----GTVGY	LNFDFTTFEMTI	MPALAVGGP	HIDWHLELGL
POXC	IVAP------QNAAIPT-	MSCDPN	ANPNL----GSTGF	MAFDFTTFELTI	MPALAVGGP	HIDWHLELGL
POX3	PTAE----ELVARGGPPQ	IACEPN	GNPSA----GPTGF	FSFDPMTSRFAT	MPGGVNGGP	HVDWHLDLGL
POXA1b	APSL------SLTGVPH-	TSCDSN	ANPNS----GDPGF	FAFDPATALFTA	MPALVFAGP	HIDWHLDLGF
LACC12	VPAP------SAGLVPV-	ISCDPN	ASPNL----GPQGF	IAFDEEKFKFSI	IPGLATGGP	HIDWHLELGL
POXA3	DLAPHAQNQFFQTGSVPI	ISCRPF	APLTGGNPAGNPNL	IAQPNPPF-FDI	IP---GAGA	HIDWHLEAGL

Figure 2. A. Comparison of the laccase signature sequences from the *P. ostreatus* laccases POX1, POXC, POXA1b, POXA3, POX3 and POX4 with LACC12. The fungal laccase signature sequences (L1-L4) are shown together with the fungal laccase consensus sequence (Laccase Cons.) according to Kumar et al. [30]. The amino-acid conservation scoring is performed by PRALINE (http://www.ibi.vu.nl/programs/pralinewww/). Residue shading has been applied from scoring 10 (black) to 7 (grey). The Y97 residue in L1 is boxed. **B.** Sequence alignment of the potential substrate binding loops of the *P. ostreatus* laccases, according to Pezzella et al., [22]. Loops L1 sequence at positions 182-192, B4-B5 at positions 230-235, B7-B8 at positions 288-297, C1-C2 at positions 357-368, C4-C5 at positions 413-421 and C7-C8 at positions 479-488.

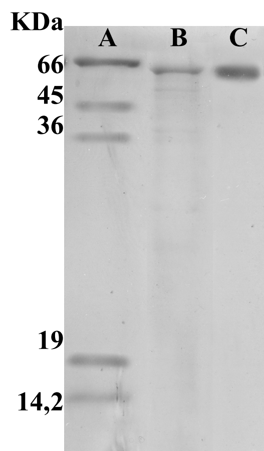

Figure 3. SDS–PAGE analysis. Lane A: molecular mass markers (Sigma), from top downward: Albumin bovin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), and a-lactalbumin (14.2 kDa). Lane B: *P. ostreatus* LACC12 stained with Coomassie brilliant blue; lane C LACC12 immuno-detected with antibodies against POXC.

Chapter 3:

Enhancement of environmental-compatible enzymatic production combining classical breeding and autoregulation mechanisms: effects of autoregulators in different strains of *P. ostreatus.**

*This section deals with the work carried out in the laboratory of Prof. Stéphane Declerck BCCM/MUCL Mycothèque de l'Universite catholique de Louvain (Louvain La Neuve, Belgium), under the supervision of Dr. S. Vanhulle, Dr. C. Decock and Dr. A. Bazes.

3.1 - Introduction

White-rot fungi belonging to basidiomycetes have been widely recognized as the most efficient lignin degraders. This capability is assumed to derive from the activities of numerous enzymes; among these, laccases and lignin and manganese peroxidases are some of the most intensively studied groups of lignin-modifying extracellular enzymes [1]. As a widespread and heterogeneous class of oxidases, ligninolytic enzymes and the organisms producing them are widely considered to have potential for industrial purposes [2]. In particular, interest in industrial and environmental applications involving laccase has increased in the last decades due to their potential use in biodegradation of environmental pollutants (e.g. polycyclic aromatic hydrocarbons), textile dyes or stain bleaching, bioconversion of lignin, biobleaching and biopulping of wood chips or delignification of agricultural plant residues [2]. However, large amounts of laccases are required for fundamental studies on laccase properties and, in particular, for their practical use. Nevertheless, laccases secreted from wildtype fungal organisms may not be suitable for commercial purposes mainly because of low yields or high costs of preparation procedures.

Because of the above-mentioned issues, with the constantly growing popularity of the edible *P. ostreatus* mushroom, as well as the tremendously increased knowledge of the relevant laccase families, there is a great incentive for obtaining strain improvement for phenoloxidases production at large-scale.

Several methods have been used for strain improvement in *Pleurotus spp.* including selection, hybridization and gene transformation [3, 4, 5, 6]. Based on current legislation (European Directive 2001/18/CE), genetic transformation and mutagenic treatments produce strains not suitable for natural or safe processes. In order to achieve improvements of this species, optimization of a specific traits of natural strains by classical breeding is required.

In contrast with the mating-type systems of other eukaryotes, including mammals, the basidiomycetes have not developed two sexes, but multiple mating types. *P. ostreatus* and its variants are homobasidiomycetes where mating is controlled by a bifactorial tetrapolar genetic system [7]. Factor A is known to control nuclear pairing, clamp cell formation, co-ordinate cell division and clamp cell septation, while factor B leads to nuclear migration, septa dissolution and clamp cell fusion.

Pleurotus spp. life cycle alternates between monokaryotic and dikaryotic phases [8]. Hyphae of two compatible monokaryons are able to fuse and give rise to a dikaryotic mycelium in which the two parental nuclei remain independent throughout vegetative growth and fruiting body development Diploidy occurs only in the basidia, where karyogamy takes place immediately before the meiotic division, followed by production of uninucleate basidiospores [8]. Inter-chromosomal genetic recombination can naturally occur only at this time.

It is well known that many *Pleurotus* spp., except *P. tuberregium* and few other, are interfertile [9]: in most species, these allow fertile crossing among over 90% of non-related individuals of a species while inbreeding among sibling strains is reduced to 25% [10].

Among the haploid meiotic basidiospores coming from the same fruit body (same parent strains), to have strain compatibility the allelic specificity of genetic locus A must to be different at least one of A subloci and the same for the B locus. In a tetrapolar mating behavior four different types of mating interactions can be observed amongst the progeny. Only one type of interaction is compatible, resulting in a dikaryotic mycelium, able to form fruit bodies (Fig. 3.1), whereas the other three

mating interactions are not fertile. Inter-crossing among evolutionary distant strains of the same species or genere having several different A and B subloci, allow fertile crossing in most of the cases.

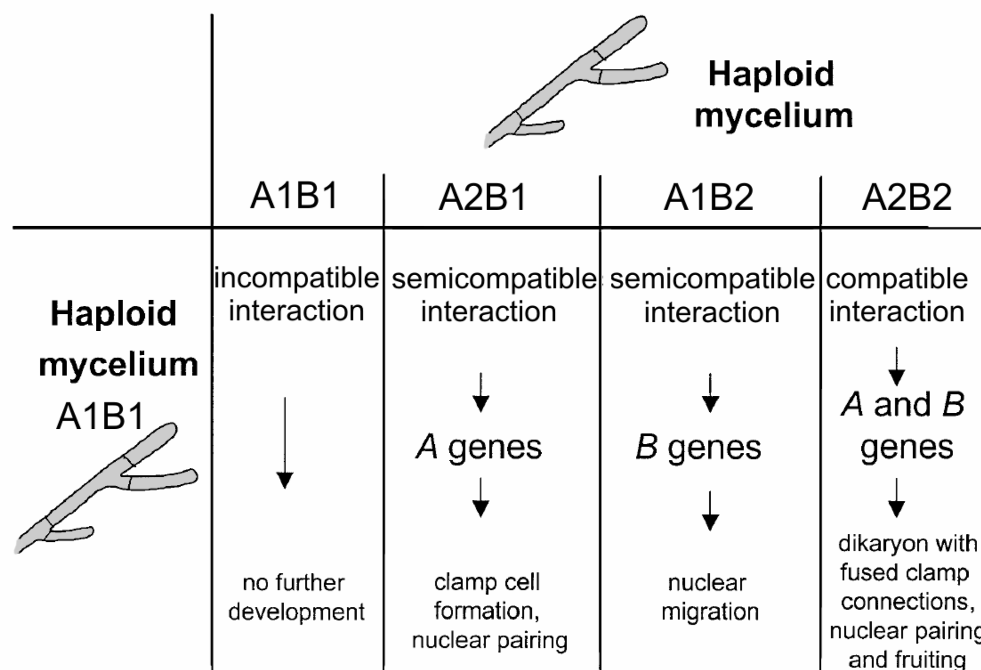

Fig. 3.1. Tetrapolar mating system governed by two different sets of mating-type genes.. Different mating-type genes (A1 and A2 alleles for A locus; B1 and B2 alleles for B locus) are able to induce specific development as indicated.

Formation of clam connection is used as presumptive evidence of this sexual compatibility. Clamp connection are formed during the conjugate division of the nuclei in the growing hyphae tip. When a binucleate hyphal tip is ready to divide, a short branch (the clamp-connection) arises between the two nuclei and begins to form a hook. The presence of clamp connection is therefore generally indicative of the dikaryotic condiction [11].

The expression of a set of allelic variants contained in the two different nuclei of the cross-hybrid fungus, results in a phenotypically different strain. As a fact, the major aim of breeding is to generate variability and combine desirable features from different monokaryotic strains. Chaudhary *et al*. [12] developed single spore isolates from *P. djamor, P. florida, P. citrinopileatus* and *Hypsizygus ulmarius*. The hybrids showed improved mycelial growth rate compared to that of the parent. In another work Sawashe and Sawant [13] developed hybrid cultures which required a significantly shorter period for spawn run as compared to the parents species.

The present work aimed at producing new *P. ostreatus* dikaryotic strains with improved efficiencies in laccase expression by inter-crossing different variants. The effects of the previously identified 2-hydroxy 4-nitrobenzoic acid autoregulator (see chapter 2) on laccase production was also tested on the best performing strains. Generally recognized as safe (GRAS) strains and elicitation by endogenous compounds are considered "natural" and environmental safety for bioproductions implementations.

3.2 - Materials and Methods

Organism and culture conditions

Two commercial dikaryotic strain of *Pleurotus ostratus*, *P. ostreatus* variant *Florida* (ATCC no. MYA-2306) and *P. ostreatus* D1208 (internal classification) were maintained through periodic transfer at 4°C on potato dextrose agar plates in the presence of 0.5% yeast extract (Difco). Mycelium was grown in 100 ml shacked flasks (125 rpm) containing 30 ml of PDY with 150 µM CuSO4. The flasks were inoculated with four agar plugs (8 mm diameter) cut from the actively growing part of the colony on a *Petri* dish and incubated for at least 15 days at 28° C in the dark.

Conditioning growths

PDY basal medium conditioning was performed using 2-hydroxy-4-nitrobenzoic solution (1mM final concentration - Sigma Chemical Co) supplemented at the time of inoculation.

P. ostreatus fruiting body induction

Spawn was prepared on 200 gr of boiled wheat grains and straw mixed with 20% w/w of CaSO4 and 20% w/w of CaCO3, autoclaved at 120°C for 20 min, cooled and inoculated with 7 plugs of mycelial agar aseptically in Erlenmeyer flasks at 27±1°C for 30–40 days until mycelium has completely covered the substrate.

Flasks were opened and incubated at 15°C and watered twice daily using mist spray. Fruit bodies appeared 2–3 weeks after the opening of the flasks, which were then harvested when their margin started rolling upwards (Fig. 3.2).

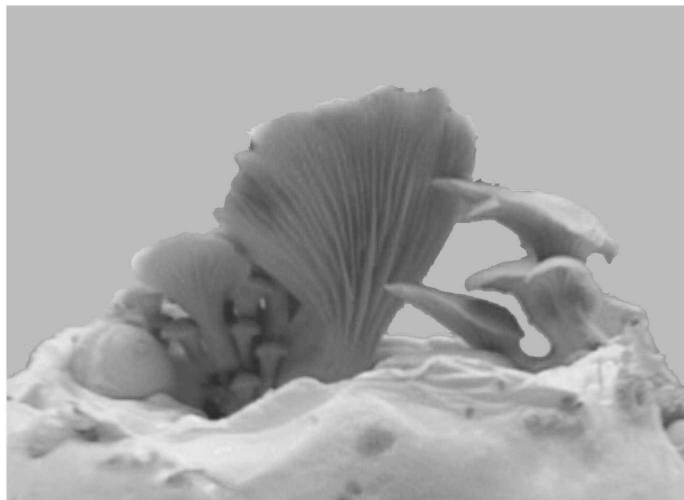

Fig. 3.2 - *P. ostreatus* mature fruiting bodies.

Basidiospores isolation

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

Mating test

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

Enzymatic assay
Spectrophotometric assays of laccase activity were carried out using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [10 in realtà13]. The assay mixture contained 2 mM ABTS and 0.1 M sodium-citrate buffer, pH 3,0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M-1 cm-1) for 1 minute. Each assay was done in triplicate.

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

3.3 - Results and discussions
Basidiospores-derivative monokaryons were obtained from the already studied *P. ostreatus var. florida* strain and from another *P. ostreatus* strain available on the market (D1208). Collected spores from the two different basidiocarps were successfully germinate in solid medium. Microcolonies progeny was microscopically analyzed and monokaryotic state was confirmed by the absence of mycelial clamp connections (Fig. 3.3 A).

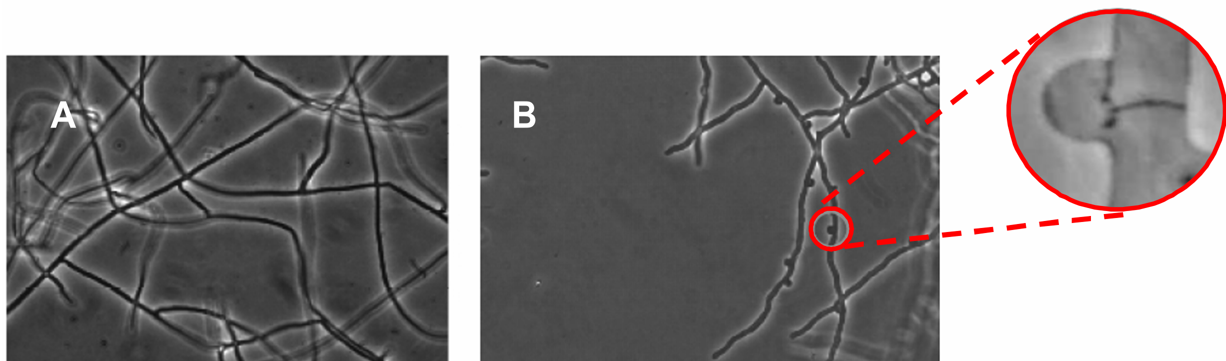

Fig. 3.3 – (A) *P. ostreatus* monokaryotic hyphae observed on optical microscope; (B) *P. ostreatus* dikaryotic hyphae having clamp connections. Clamp structure is zoomed in details.

Isolated monokaryons (called "A" and "D", for strains derived from the parental dikaryons **A**TCC MYA2306 or **D**1208, respectively) were inoculated in submerged culture in order to monitor laccase activity during fungal fermentation. The surprisingly high variability of laccase production indicated heterozygocity in genes responsible for synthesis and secretion. In the set of 26 randomly chosen germinating spores we founded 7 isolated with an higher or comparable laccase production whereas 16 strains showed a variable lower production in comparison with the two parent strains (Tables 3.1 and 3.2 for spores derivatives from MYA2306 and D1208, respectively). Time courses of secreted laccase production showed a maximumof production at 9 and 10 days of growth.

	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Parent strain MYA-2306	**0,27**	**0,79**	**1,54**	**2,5**	**3,53**	**17,3**	**15,8**
1A	0,38	0,28	0,43	0,58	1	3,87	6,22
2A	0,38	0,29	0,56	1,83	3,36	5,07	5,83
3A	1,35	0,92	0,73	0,61	2,6	4,21	5,23
4A	0,27	0,77	1,12	0,92	2,44	2,6	2,42
5A	**0,22**	**0,90**	**2,6**	**4,7**	**36,6**	**25**	**30,56**
6A	**0,29**	**0,65**	**0,91**	**5**	**14,06**	**13,92**	**14,08**
7A	0,24	0,77	2,69	17,06	17,11	13,04	12,23
8A	0,23	0,42	0,74	1,53	7,33	5,63	5,9
9A	0,31	1,54	3,24	2,2	3,18	2,13	1,98
11A	0,17	0,33	1,06	11,44	9,17	8,5	9,46
13A	0,16	0,33	0,38	0,89	2,66	2	1,99
17A	0,11	0,48	0,93	1,15	2,08	2	2,18
18A	**0,35**	**0,50**	**1,06**	**5,81**	**26,3**	**16,9**	**16,6**

Table 3.1 laccase production of monokaryons derivatives from parental strain A (ATCC MYA2306).

	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Parent strain D1208	**0,36**	**0,76**	**0,93**	**0,85**	**1,24**	**9,7**	**15,8**
D1	0,11	0,96	1,05	1,3	1,12	0,83	0,65
D2	0,16	0,61	1,1	2,2	2,8	4,5	5,1
D3	**0,25**	**1,21**	**2,3**	**4,9**	**6,7**	**8,72**	**5,1**
D4	0	0,006	0,007	0,018	0,01	0,02	0,015
D5	0,017	0,095	0,036	0,04	0,02	0,07	0,03
D6	0,08	0,6	1,2	2,7	4,2	4,31	5,09
D7	0,08	0,73	1,3	2,5	2,6	1,9	1,3
D8	0,16	1,54	2,6	4	3,7	3,2	1,02
D9	**0,26**	**0,93**	**1,4**	**2,9**	**3,4**	**9,8**	**16,6**
D10	0,008	0,24	1,18	2,5	3	2,16	1,21
D11	**0,15**	**0,93**	**1,54**	**4,2**	**6,2**	**8,9**	**7,8**
D12	0,05	0,69	1,34	2,9	2,6	0,77	0,83
D13	0,11	0,58	0,83	1,2	1,14	1,45	1,49
D14	0,03	0,5	1,31	2,3	2,4	1,83	0,38
D15	0,68	1,3	1,27	1,5	1,7	2,34	2,62

Table 3.2 laccase activity of monokaryons derivatives from parental strain D (D1208).

Analysis by electrophoresis on native gels stained for laccase activity revealed that monokaryotic strains produce during fermentation in submerged growth (data not shown). The isolates differ in morphology and growth rate when they grew on solid media in comparison with the controls. Some morphological patterns were transient and, in general, colony appearance could not be connected with laccase production.

Using mutual crossing, the compatible groups of all monokaryotic isolates were determined. Anastomosis induction followed by formation of clam connections (Fig. 3.3B) indicated compatibility among two strains and the formation of the corresponding dykarion. However, three types of incompatible reactions were observed in the contact zone of different mycelia: growth of a mycelium surrounding the other, mutual repulsion with a formation of a borderline and mutual inhibition with a barrage formation (Fig. 3.4A, 3.4B and 3.4C, respectively).

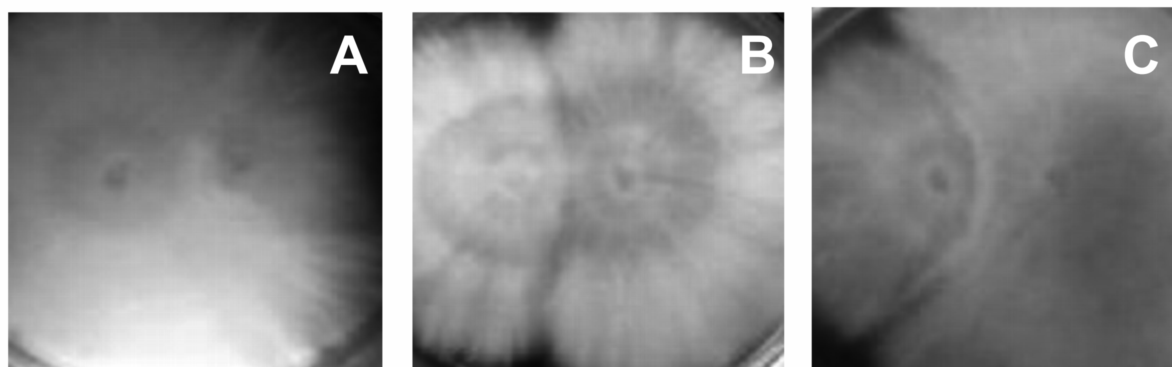

Fig. 3.4 Kind of interaction among two incompatible strains of *P. ostreatus:* (A) growth of a mycelium surrounding the other, (B) mutual repulsion, (C) mutual inhibition.

Because of laccase production is regulated by multifactorial and mutiallelic expression systems which are dependent on extra- and intra-cellular regulations [14, 15], inter-crossing of partially characterized monokarions was performed in order to obtain new high laccase-producers. All possible cross-hybridizations among the higher laccase-producers (table 3.3) were performed and relevant dikaryons obtained. 8 dikaryons resulted to be high-laccase producers compared to the parent strains and laccase production of three of them was significantly high (Fig. 3.5).

AXA

strain	1A	2A	4A	5A	6A	7A	8A	9A	11A	13A	15A	16A	17A	18A
1A														
2A	+													
4A	+	-												
5A	-	-	-											
6A	+	+	+	-										
7A	-	+	+	-	-									
8A	+	+	+	+	-	-								
9A	-	-	-	-	+	+	-							
11A	+	+	+	-	Nd	-	-	-						
13A	+	+	+	-	-	-	-	+	-					
15A	-	-	-	+	Nd	+	+	-	+	-				
16A	-	+	-	-	Nd	-	-	+	-	-	Nd			
17A	-	-	+	+	Nd	-	-	-	-	-	Nd	-		
18A	+	+	+	-	Nd	-	-	+	-	-	+	-	-	

AXD

strain	1D	2D	3D	4D	5D	6D	7D	8D	9D	10D	11D	12D	13D	14D
5A	Nd	Nd	+	Nd	Nd	Nd	Nd	Nd	+	Nd	+	Nd	Nd	Nd
6A	Nd	Nd	+	Nd	Nd	Nd	Nd	Nd	+	Nd	+	Nd	Nd	Nd
18A	Nd	Nd	+	Nd	Nd	Nd	Nd	Nd	-	Nd	+	Nd	Nd	Nd

Table 3.3. compatibility tables: A X A crossing and 5A/6A/18A X 3D/9D/11D.

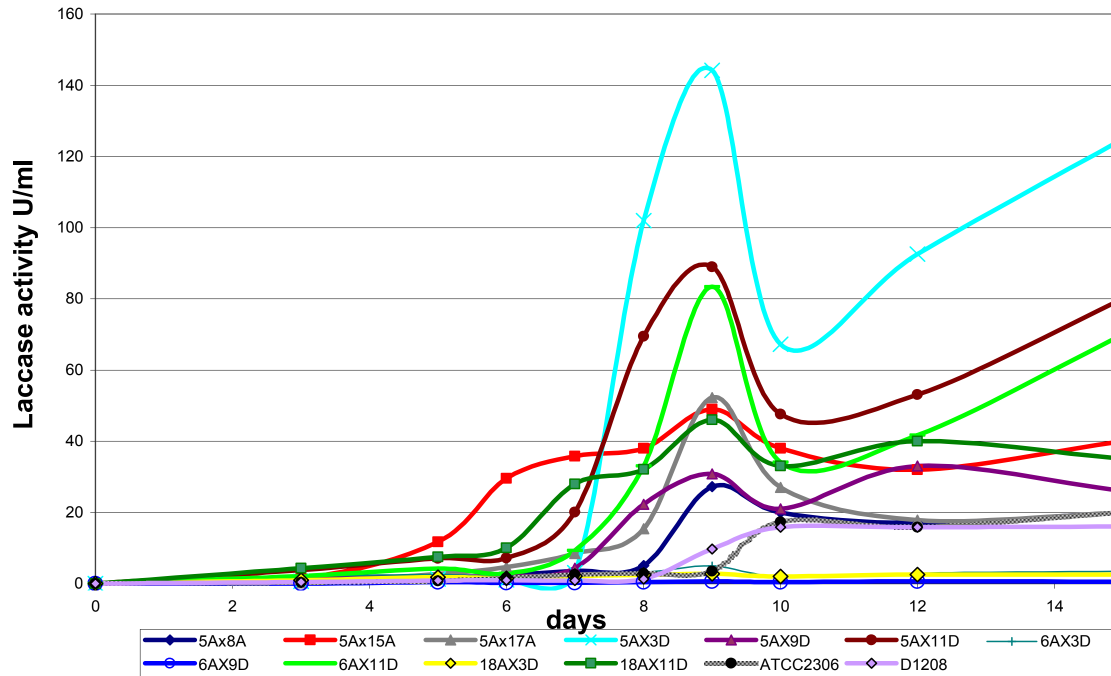

Fig.3.5 time course analysis on extracellular laccase activity produced by different natural strains.

Laccase production in these strains reached, in fact, levels of hundred of thousands units/liter of culture, a value suitable for industrial applications, as reported in the prospect of cost production by biotechnological industries (WETLANDS ENGINEERING S.P.R.L, personal communication). Moreover, analyses of phenoloxidase isoenzymatic pattern of any dikaryotic strain showed a differential expression of the POXA1b [16], POXA3 [17] and POXC [18] laccase enzyme during the time course (Fig. 3.6). In particular the POX A1b laccase resulted more expressed and for a longer time in the strain 6AXD11 in comparison with the parental strain ATCC MYA2306.

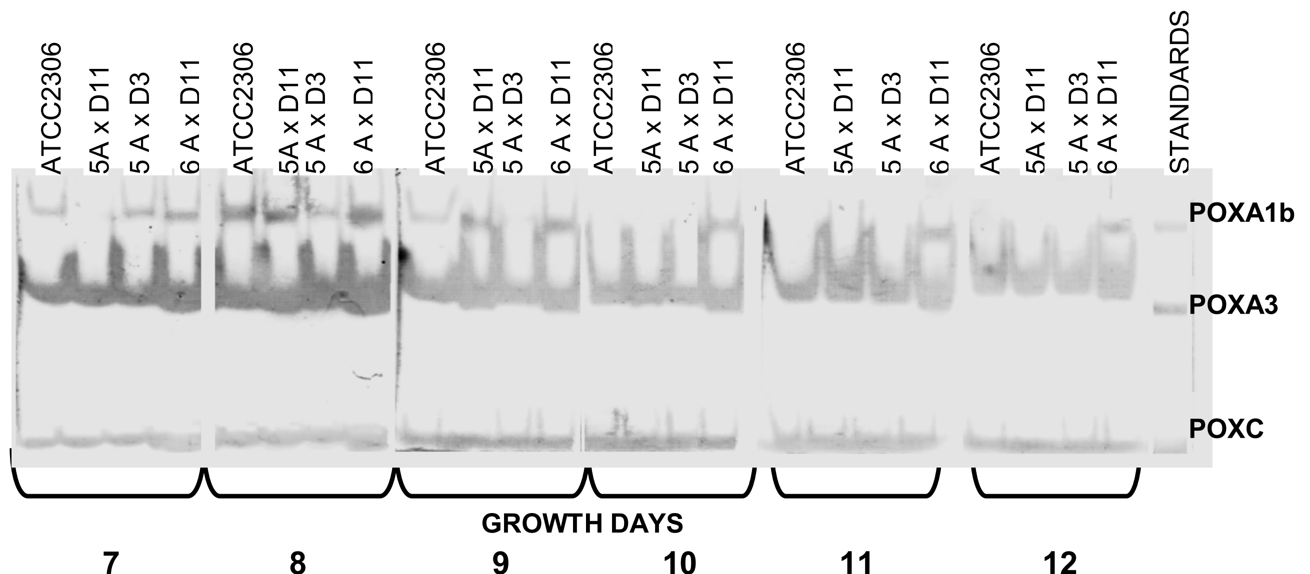

Figure 3.6 Zymograms of laccase isoenzymes produced by the highest producer dykariotic strains and by the parental strain ATCC MYA2306 in PDY culture broth. Samples containing 0.015 U of laccase activity collected at different times (7, 8 , 9, 10,11 and 12 days) were used. Known laccase isoforms POXA1b, POXA3 and POXC were loaded as standards.

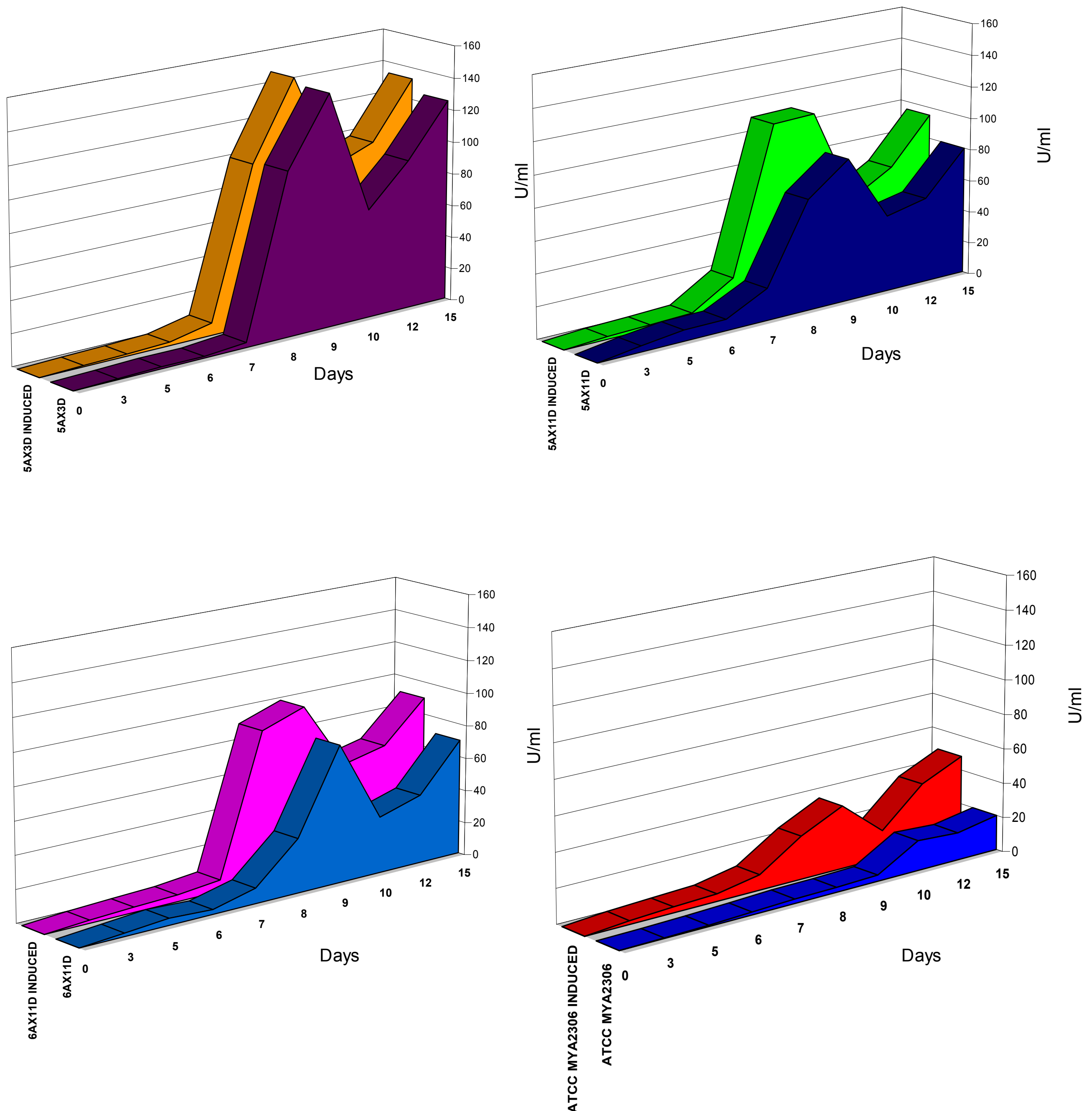

Fig. 3.8 comparisong of laccase production among new dikaryotic strains grown in liquid cultures in presence and in absence of 2-hydroxy-4-nitrobenzoic acid as natural inducer.

Fungal submerged growth of the improved dikaryotic strains was also conditioned with 2-hydroxy-4-nitrobenzoic acid (1mM final concentration). Analyses were performed in order to confirm autoregulatory properties of the nitrobenzil-derivative excreted by *P. ostreatus* on different related strains, and to verify the synergic effect produced by combining both "natural" approaches: endogenous autoinduction mechanisms and classical breeding. The signal molecule increased laccase production levels, for all but one of the new strains, up to 7 times, confirming that its signalling role is not strain-dependent (Fig. 3.8). No significant difference in the enzymatic pattern was reveled in the conditioned culture in comparison with the control. Conversely, the best 5AX3D dikaryon laccase producer (140.000 unit/L of laccase activity produced) resulted insensitive to the presence of the tested autoregulator. Different reasons can explain this exception: i) the strain can be

mutated in one or more genes involved in signal transduction of laccases expression and/or secretion that result therefore deregulated; ii) the natural mutant strain can have acquired the capability of excreted high level of 2-hydroxy-4-nitrobenzoic acid on its own resulting insensitive to the extra-concentration of this autoregulator; iii) finally mutations can have "overworked" protein synthesis system up to the maximum capability of hyphal cells resulting insensitive to any extracellular inducing factor. Later studies based on metabolomics analysis or transcriptomics profiles will be able to confirm one of these hypothesis.

3.4 - Conclusion

In this study strain improvement by classical breeding was performed in order to improve laccase production up to industrial level. 8 high-laccase producers were obtained and laccase production of two of them rose to hundreds of thousands units/litre of culture. Furthermore, some strains showed a different isoenzymatic pattern during liquid fermentation time course. Autoinducer regulation affecting laccase activity was applied to the new dikaryotic strains in order to further increase enzyme production. For all but one of the new strains, the autoregulator signal increased laccase production levels up to7 times.

Reported data suggest that, in order to optimize fungal bioprocesses for extracellular enzyme production, these approaches can be a valid option for obtaining isolates with interesting combination of enzymatic patterns and production capability. Classical natural methods also represent an advantage in comparison with other common methods, such as random mutagenesis, because undesiderable side-effects (pleiotropism) can be avoided. This approach exploits mechanisms so common in fungi that could hold for other industrially useful enzymes and/or basidiomycetes.

3.5 - References

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General Conclusions

In the current study 11 GC/MS-detected volatile compounds related to shikimic acid pathway (aryl compounds) were identified as differentially produced during *P. ostreatus* submerged growth. Endogenous exo-metabolites extracted from spent media positively affected the general mechanisms of laccase expression in liquid culture.
Some endogenous aryl-compounds and C8 volatile compounds (previously detected during *P. ostreatus* mushroom fructification) have autoregulatory features.
Morphological and physiological analysis showed, in fact, at least 3 autoregulators involved in different processes of *P. ostreatus* development.
Benzaldehyde signals a dramatic decrease of nutrient source (starvation) stress and stimulates the fungus to increase hyphal length, but not branching, in order to enhance the probability of finding new food sources.
Benzoic acid and 2-hydroxy-4-nitrobenzoic acid are involved in defence mechanisms overcoming during mechanical cell lyses or autolyses, inhibiting some bacterial and yeast strains and also fungal growth and sporal germination of *P. ostreatus*. Moreover, 2-hydroxy-4-nitrobenzoic acid induces extracellular enzymatic production in liquid culture (several of them are industrially useful enzymes). Presumably, these proteins play a role in long-time stress adaptation. Further investigation could explain the mechanism of action of 2-hydroxy-4-nitrobenzoic acid in the frame of fungal physiology. However, reported data point out this chemical as autoinducer that could be potentially suitable for biotechnological purposes.
Finally, 1-octen-3-one is involved in derivative inhibition of basidiospore germination.
Herein, the function of C8 volatile compounds in *P. ostreatus* is proposed: while 1-octen-3-ol inhibits pathogenic ascomycetes, 1-octen-3-one avoid premature sporal germination when basidiospores are connected to the gills.

In this study strain improvement by classical breeding was also performed in order to improve laccase production up to industrial level. 8 high-laccase producers were obtained and laccase production of some of them rose to hundreds of thousands units/litre of culture. Autoinducer regulation affecting laccase activity was applied to the new dikaryotic strains in order to further increase enzyme production. For all but one of the new strains, the autoregulator signal increased laccase production levels up to 7 times.
In order to optimize fungal bioprocesses for extracellular enzyme production, these approaches can be valid options for obtaining isolates with interesting combination of enzymatic patterns and production capability. Morover, these “natural” methods represent an advantage in comparison with random mutagenesis, because undesiderable side-effects (pleiotropism) can be avoided.

As parallel section the identification of LACC12, as a new laccase from the fruiting body of *P. ostreatus,* was performed. The reported data add a new piece to the knowledge about *P. ostreatus* laccase multigene family, and contribute to increase the meagre data regarding laccases from fungal fruiting body.

A progress towards a more intelligible picture of the roles played by endogenous secondary metabolites as autoregulators in *P. ostreatus* has been attained with this work.

Communications, Publications, Courses, Experiences in foreign laboratories.

Communications
Roggero U., Sannia G., **Lettera V.**, Varese G. C.
Intraspecific differences in laccase production within *Pleurotus ostreatus* species at transcriptional and translational level. 4th European meeting on oxizymes, 16-17-18 giugno 2008, Helsinki, Finlandia.

Roggero U., Luciano P., **Lettera V.**, Sannia G., Varese G.C.
Variazione intraspecifica in *Pleurotus ostreatus* nella produzione di laccasi in risposta a differenti condizioni di crescita. XVII Convegno Nazionale di Micologia, 10-11-12 Novembre 2008, Pavia, Italia.

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Napoli, Italia. Kick-off meeting. 7-8 dicembre 2006
Pècs, Ungheria. 3-4 Maggio 2007
Londra, Regno Unito. 18-19 Gennaio 2008
Praga, Repubblica Ceca. Mid-term meeting. 14-16 Maggio 2008
Marsiglia, Francia 2-3 Aprile 2009
Capri, Italia. Final meeting. 5-7 Ottobre 2009

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Lettera V., Del Vecchio C., Vanhulle S., Sannia G.
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Curses

- Protein Science Seminar on DIGE, Protein purification, Biacore Systems, INcell Analyzer. Napoli, 6 Maggio 2008.
- ESF Functional Genomics workshop su "Advanced large scale expression profiling – focus on miRNA, ChiP-on-chip/seq. and whole genome sequencing". Turku (Finlandia), 19-22 Maggio 2008.
- Corso Teorico-Pratico "Tecniche di Microscopia Elettronica a Trasmissione: dalla morfologia alla biologia molecolare in situ". Napoli, 19-20 giugno 2008.
- Seminario tecnico "Real Time PCR" dell' Applied Biosystems. Napoli, 23 febbraio 2009.
- Olympus workshop. "Digital Imaging in Microscopy: From basic image acquisition to real-time life cell imaging. Louvain La Neuve (Belgio), 29-30 ottobre 2008.

Experience in foreign laboratories

- 06/2008: Stage in the laboratory of the Dipartimento di Biologia Vegetale (Micoteca), dell'Università di Torino, under the supervision of Dr. C. Varese.
- 1/10/2008-31/01/2009: Stage in the laboratory of Prof. Stéphane Declerck BCCM/MUCL Mycothèque de l'Universite catholique de Louvain (Louvain La Neuve, Belgium), under the supervision of Dr. S. Vanhulle, Dr. C. Decock and Dr. A. Bazes. The stage was founded by the COST FPS Action FP0602, Biotechnology for Lignocellulose Biorefineries (BIOBIO).

- 1/10/2008-31/01/2009: Stage in the industrial laboratory of WETLANDS ENGINEERING S.P.R.L, Louvain La Neuve (Belgium), under the supervision of Dr.C.M. Bols and R. Nair. The stage was founded by the EU project "Discovering Quorum Sensing in industrially useful Fungi, a novel approach at molecular level for scaling-up in white biotech" (STREP-FP6-NMP4-CT-2006-032811).

ENHANCEMENT OF LACCASE PRODUCTION BY PLEUROTUS OSTREATUS BREEDING FOR BIOTECHNOLOGICAL PURPOSES

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Abstract

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

Introduction

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

Experimental

1. Organism and culture conditions

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

2. Enzyme activity determinations

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

Spectrophotometric assays of laccase activity were carried out using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate (12). The assay mixture contained 2 mM ABTS and 0.1 M sodium-citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M-1 cm-1) for 1 minute. Each assay was done in triplicate.

3. Native PAGE

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

4. *P. ostreatus* fructification at laboratory scale

Spawn was prepared on boiled wheat grains and straw mixed with $CaSO_4$ and $CaCO_3$, autoclaved at 120°C for 20 min, cooled and inoculated with 7 plugs of mycelial agar aseptically in Erlenmeyer flasks at 27±1°C for 30–40 days until complete mycelial covering of the substrate.

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

5. Basidiospore isolation

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

6. Mating tests

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

7. Estimation of radial growth rate

As a criterion to select better growing isolates mycelium extension rate on solid media was used. This was estimated by measuring the diameters of four individual colonies grown separately on solid agar PDY medium in *Petri* dishes inoculated with agar plugs (5 mm diameter) cut with an

injection needle from the actively growing part of colony on another *Petri* dish. All measurements were repeated three times and done in triplicate.

Results and discussion

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

It is well known that many *Pleurotus* spp., except *P. tuberregium* and few other, are interfertile (8): in order to produce oyster mushroom hybrids with favourable cultural characteristics, applicable in large-scale production, basidiospores-derived monokaryons, obtained from the laccase high-producer *P. ostreatus* va. Florida and from two hybrids currently available on the market (MBA-0001 and MBA-0002), were isolated an analysed. The surprisingly high variability of laccase production indicates heterozygocity in genes responsible for the synthesis and secretion.
In the set of 45 randomly chosen germinating spore we found 8 isolated with an higher or comparable laccase production whereas 16 strains showed a variable lower production. Time courses of secreted laccase production showed a maximum on the 9th day, two days before the peak of production of the *P. ostreatus* var. Florida dikariotic strain. Moreover, laccase zymogram revealed a different secretion pattern of isoenzymes for some monokaryotic strains during the growth; this should responsible of the different amount of total laccase production.
Estimation of radial growth rate on solid media showed that, after a short period of adaptation, monokaryons generally grow slowly than their dikaryotic parents. Growth rate was not directly correlated with laccase production, but more in general higher-producing isolated displayed lower growth rates.
Isolates differed also in morphological characteristics showing three main types of colony appearance:

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

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

2. Isolation of new hybrid dikaryotic strains

The above mentioned results stimulated us to study the behaviour of dikaryons prepared by crossing of characterized basidiospore-derived monokaryons in order to obtain fast growing higher-producing stable dikaryotic isolates.
Spore isolates from *P. ostreatus* var. florida and *P. ostreatus* were crossed within and between species for a total of 45 combinations. All compatible crossings were performed and four dikaryons were selected as higher-laccase producer. One of the selected dikaryons exhibits a radial growth rate significantly higher than that of all dikaryotic strains.

Conclusions

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

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