# DEVELOPMENT OF NEW BIOCATALYSTS TO IMPROVE PRODUCTION OF SECOND GENERATION BIOETHANOL

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*Alla mia famiglia*

*“Sapiente è soltanto chi sa di non sapere”*
*Socrate (470 a.C. – 399 a.C.)*

*Live, Love, Learn*

**Abstract**

Cellulases, hemicellulases (xylanases) and accessory enzymes (e.g. arabinofuranosidases, pectinases, mannanases) are needed for lignocellulose conversion into fermentable sugars for second generation bioethanol production. The high production costs of the above enzymes limit the bioethanol competitiveness, pushing towards the development of new biocatalysts, with improved catalytic properties, and new processes, whith lower costs. In this work, **industrial waste based compost** and **natural habitats** were investigated as a source of **new (hemi)cellulolytic bacteria and fungi** for discovery of new (hemi)cellulases. **Tomato processing waste** was shown a suitable substrate for the production of a new **α–L-arabinofuranosidase (PoAbf)** by solid state fermentation (SSF) with the basidiomycete *Pleurotus ostreatus*. Cellulolyitc activities of *Bacillus amyloliquefaciens B31C* and *Streptomyces* sp. *G12* isolated from industrial waste based compost were characterized. **An endoglucanase (CelB31C) from *B. amyloliquefaciens* B31C** was identified, purified and characterized. The enzyme follows a Michaelis-Menten kinetics towards carboxymethyl-cellulose ($K_M$ of 9.95 mg $mL^{-1}$; Vmax 284 µM $min^{-1}$). It shows a retention of 90% of its activity for at least 6 days of incubation at 40 °C and a range of optimal temperatures from 50 to 70 °C. Investigating the enzymes responsible for cellulase activity produced by ***Streptomyces* sp. *G12***, proteomic analyses led to the identification of three enzymes acting on cellulose. Gene coding for one of them, named **CelStrep**, was cloned and sequenced. Recombinant expression of CelStrep was carried out in *Escherichia coli* and the recombinant enzyme (rCelStrep) was characterized. rCelStrep follows a Michaelis-Menten kinetics towards carboxymethyl-cellulose ($K_M$ 9.13 mg $mL^{-1}$; Vmax 3469 mM $min^{-1}$). It exhibits a half life of 24 h and 96 h at 60°C and 50°C, respectively and a retention of around 80% of activity after 96 h at 40°C. **93 microorganisms** were isolated from 50 samples collected in the **Western Ghat region (India, Kerala).** Their screening for cellulases and xylanases production led to the selection of 7 xylanolytic and 14 cellulolytic microorganisms, identified by 16S rRNA sequencing as belonging to the species *Bacillus*, *Streptomyces*, *Lysinobacillus* and *Paenibacillus*. A further screening in liquid medium revealed the strain ***Lysinobacillus xylanolyticus* XR84** able to produce both cellulase and xylanase activities. Due to the interest in thermo-resistant cellulases and to the ability of filamentous fungi to grow on agro-industrial wastes by SSF, **150 thermophilic filamentous** fungi isolated from a “maâsra” were screened for cellulase activity production at 45°C, leading to the selection of four ***Aspergillus*** strains, a ***Malbranchea*** strain and a ***Myceliophthora thermophila*** strain, all representing a potential reservoir of thermostable enzymes to be further identified and characterized. Proteomic analyses led to the identification of an **α–L-arabinofuranosidase (PoAbf)** responsible for the xylanase activity produced by *P. ostreatus* SSF on tomato waste. cDNA coding for PoAbf was synthesized and sequenced. Recombinant expression systems were set up in *Kluyveromyces lactis* and *Pichia pastoris*, the latter being the best host for enzyme production (180 mg $L^{-1}$). Recombinant PoAbf (rPoAbf) follows Michaelis–Menten kinetics ($K_M$ 0.64 mM; $k_{cat}$ of 3,010 $min^{-1}$), it has a durable activity in a broad range of pH, particularly at pH 5 ($t_{1/2}$= 51 days), which is also its optimal pH. It shows high stability ($t_{1/2}$= 7 days) at 30°C and 40°C, the latter being its optimal temperature. It was shown to be a versatile enzyme, able to hydrolyze arabinooligosaccharides, arabinan and arabinoxylan. Glycosylation in position S160 positively affects the enzyme stability as demonstrated by site-directed mutagenesis experiments.

## Riassunto

### *Combustibili di nuova generazione*

Nell'ottica di uno sviluppo sostenibile, l'utilizzo di materie lignocellulosiche per la produzione di biocombustibili, bioetanolo *in primis*, è un obiettivo ambizioso.
La bioconversione di biomasse lignocellulosiche può dare un significativo contributo alla produzione di un ampio ventaglio di prodotti d'interesse industriale, quali polimeri, enzimi, additivi alimentari, coloranti, composti armatici, acidi organici, favorendo lo sviluppo della bioraffineria definita come la "co-produzione di zuccheri fermentabili e di un ampio spettro di bioprodotti a partire dalle biomasse lignocellulosiche" [1].
La produzione di biocombustibili risponde alla necessità di trovare nuove fonti rinnovabili di energia e abbattere l'inquinamento atmosferico. La Direttiva Europea 2009/28/CE, sulla promozione della produzione e dell'uso dei biocombustibili, stabilisce che questi dovranno sostituire del 20% i carburanti fossili (benzina e diesel) entro il 2020, fissando per tutti gli Stati membri un obiettivo del 10 % per la quota di energia da fonti rinnovabili nei trasporti. Nell'ambito della politica ambientale l'adozione del "Protocollo di Kyoto" (dicembre 1997) da parte della terza conferenza delle parti alla Convenzione Quadro delle Nazioni Unite sui Cambiamenti Climatici, (New York 9 maggio 1992) ha rappresentato una tappa molto importante. Il Protocollo impegna i paesi elencati nell'Annesso I della Convenzione (paesi industrializzati e paesi ad economia in transizione) a ridurre le emissioni annue di gas ad effetto serra del 5,2% nel periodo 2008-2012, con recente estensione al 2020, rispetto ai valori del 1990, con riduzioni differenti per ogni singolo paese, mediante azioni mirate, tra cui proprio l'utilizzo di fonti rinnovabili e la promozione dell'agricoltura sostenibile. In questa ottica, i biocombustibili rappresentano una forma di energia rinnovabile ed apportano una serie di vantaggi quali la significativa riduzione di emissioni di $CO_2$, la riduzione del particolato e delle emissioni di $NO_x$ e $SO_x$, l'aumento della sicurezza dell'approvvigionamento energetico e, non ultima, la diminuzione della dipendenza dall'energia importata. Tra i carburanti di nuova generazione il bioetanolo detiene il mercato più ampio. La domanda di etanolo nel 2011 è stata di circa 99.5-100 bilioni (bln) litri, di cui l' 83.5% è rappresentato da etanolo destinato ad uso come combustibile. Un ulteriore incremento della domanda di etanolo è stato previsto dall'IEA (International Energy Agency), secondo cui l'etanolo sostituirà del 5.3% la domanda totale di combustibili fossili entro il 2016. Attualmente, USA (62.8 %), Brasile (24.1%) e EU-27 (5.5%), seguiti da Cina (2.7%), detengono il mercato più ampio, con il 92% della produzione mondiale e l' 89-90% di consumo globale [2].

### *Bioetanolo di seconda generazione*

Il bioetanolo di prima generazione è prodotto attraverso la fermentazione di zuccheri ottenuti da materie agricole facilmente idrolizzabili. Il bioetanolo di prima generazione non è sufficiente a soddisfare la crescente domanda di biocarburanti ed il suo utilizzo non fornisce una consistente riduzione delle emissioni di gas serra. Inoltre, la sua produzione crea inevitabilmente un conflitto con la destinazione dei materiali agricoli alla produzione alimentare. Il bioetanolo di seconda generazione, prodotto a partire da biomasse lignocellulosiche, rappresenta una valida alternativa per il raggiungimento degli obiettivi fissati dalla Direttiva precedentemente citata. Le biomasse lignocellulosiche sono disponibili in grosse quantità, sono ben distribuite geograficamente e consentono di evitare l'impiego di biomasse destinate

all'alimentazione umana, riducendo il cosiddetto conflitto *"food versus fuel*". In particolare, è stato stimato che la produzione annuale di biomasse lignocellulosiche è di circa 100 milioni di tonnellate [3], dato che suggerisce quanto queste biomasse si prestino ad essere una fonte inesauribile di bioenergia e prodotti biocompatibili ad alto valore aggiunto. Le biomasse vegetali sono costituite dalla cellulosa, polimero di D-glucosio incastonato in una matrice di emicellulosa (polimero costituito prevalentemente da unità di xilosio), e dalla lignina, un complesso polimero costituito da molecole aromatiche. L'utilizzo di tali biomasse per la produzione di bioetanolo richiede la conversione delle componenti cellulosiche ed emicellulosiche in zuccheri fermentabili, previa rimozione della lignina che conferisce grande resistenza meccanica e rende inaccessibili le componenti polisaccaridiche. L'intrinseca complessità delle biomasse e la loro resistenza all'idrolisi sono i principali ostacoli al loro effettivo impiego su scala industriale.

Il processo convenzionale attualmente adottato per la produzione di bioetanolo di seconda generazione prevede un pretrattamento chimico/fisico per la rimozione della lignina. Diverse procedure di pretrattamento sono state investigate e, tra queste, l'idrolisi acida o basica e la *steam explosion* risultano largamente impiegate. Una volta rimossa la lignina, l'idrolisi della cellulosa è realizzata mediante trattamenti chimici o enzimatici. Questi ultimi sono basati sull'azione cooperativa di tre classi di enzimi: endoglucanasi, cellobioidrolasi, β-glucosidasi.

Una grande varietà di microrganismi, sia batteri che funghi, sono in grado di convertire le biomasse cellulosiche in zuccheri semplici. La produzione degli enzimi coinvolti nel processo di idrolisi è governata da fattori genetici e biochimici e, ad oggi, sono svariati in letteratura i casi in cui è stata applicata mutagenesi per incrementare la produzione di questi enzimi. Il sistema attualmente impiegato prevede l'utilizzo di una miscela di enzimi cellulolitici prodotti da *Trichoderma reesei* che, ad oggi, è il principale produttore di cellulasi.

Al fine di aumentare le rese di zuccheri fermentabili, diventa auspicabile l'utilizzo non solo della cellulosa, ma anche dell'emicellulosa che, pur rappresentando una minore percentuale, è ricca di zuccheri semplici fermentabili. La completa idrolisi della cellulosa e dell'emicellulosa richiede un cocktail di enzimi finemente progettato, costituito non solo da cellulasi ma anche xilanasi, mannanasi, pectinasi e tutti gli altri enzimi che agiscono sulle catene laterali di xilani e mannani.

I costi di produzione dei suddetti enzimi risultano ancora molto elevati, incidendo negativamente sui costi totali del processo di conversione biomassa-bioetanolo ed impedendo lo sviluppo del processo di produzione industriale del bioetanolo di seconda generazione a livelli competitivi. Recenti analisi tecno-economiche, infatti, attribuiscono circa il 20% del costo di produzione del bioetanolo di seconda generazione proprio alla produzione di enzimi cellulolitici coinvolti nell'idrolisi delle componenti macromolecolari per l'ottenimento di zuccheri fermentabili, per un costo industriale di $0.132 per litro di etanolo nel 2010 [1, 4]. Le principali sfide che la ricerca deve intraprendere per superare le limitazioni che impediscono una produzione competitiva di bioetanolo a partire dalle biomasse lignocellulosiche sono pertanto rappresentate dall'incremento dell'efficienza/resa dell'idrolisi della (emi)cellulosa e dalla riduzione dei costi di produzione degli enzimi (emi)cellulolitici. Un ulteriore sfida risiede nell'allestimento di una filiera in cui, mirando alla produzione di bioetanolo, si ottengano, a partire dalle stesse biomasse lignocellulosiche, altri prodotti di interesse industriale.

***Strategie per l'incremento della competitività del processo di produzione del bioetanolo***

La ricerca di nuovi microrganismi produttori di enzimi in grado di idrolizzare con alta efficienza le componenti polisaccaridiche delle biomasse lignocellulosiche rappresenta una delle principali strategie da seguire per ottimizzare il processo di conversione biomassa-bioetanolo. Microrganismi isolati da ambienti naturali che riproducono le caratteristiche delle biomasse possono costituire con la loro biodiversità una preziosa fonte di efficienti biocatalizzatori naturalmente evoluti per la conversione di cellulosa ed emicellulosa. La selezione di nuovi ceppi cellulolitici da ambienti anche estremi può consentire l'identificazione e la caratterizzazione di nuovi enzimi che assicurino rese di idrolisi competitive. Sono richiesti, infatti, enzimi che mostrino elevata stabilità in condizioni estreme (alte temperature, pH acidi) e che siano caratterizzati da una più alta efficienza catalitica.

Questo progetto di dottorato fornisce avanzamenti nel settore della produzione del bioetanolo di seconda generazione, attraverso l'identificazione di nuovi biocatalizzatori per la fase di conversione delle biomasse vegetali in zuccheri fermentabili e la riduzione dei costi associati alla loro produzione.

***Selezione e caratterizzazione di nuovi microrganismi cellulolitici e loro enzimi***

Nell'ambito del prgetto ENERBIOCHEM (Progetto di Ricerca Industriale "Filiere agroindustriali integrate ad elevata efficienza energetica per la messa a punto di processi di Produzione Eco-compatibili di Energia e Bio-chemicals da fonte rinnovabile e per la valorizzazione del territorio, PON01_01966), 180 ceppi isolati da compost agro-industriale sono stati sottoposti a screening funzionale per la produzione di attività cellulasica, usando un terreno di coltura solido contenente come unica fonte di carbonio la carbossimetilcellulosa (CMC). In base alle dimensioni degli aloni di attività rilevati attraverso colorazione con Congo Red, sono stati selezionati 19 microrganismi che presentavano un alone di attività di diametro superiore ai 6 mm. I microrganismi selezionati sono stati dunque caratterizzati a livello fenotipico e molecolare. La caratterizzazione fenotipica ha previsto l'osservazione della morfologia di colonia, la reazione di gram, la rilevazione delle attività catalasica e ossidasica e lo studio della morfologia cellulare. L'analisi molecolare, condotta attraverso l'amplificazione della regione 16S ribosomal DNA (rDNA) ha permesso di identificare quattro *Bacillus licheniformis*, quattro *Bacillus subtilis* subsp. *spizizenii*, cinque *Bacillus subtilis* subsp. *subtilis*, un *Brevibacterium linens*, un *Bacillus amyloliquefaciens* e quattro *Streptomyces* spp.. Per i 19 microrganismi selezionati, sono state allestite colture in mezzo liquido al fine di effettuare un ulteriore screening mediante dosaggio semiquantitativo dell'attività cellulasica, che ha portato alla selezione di 11 microrganismi. Per questi microrganismi sono stati valutati gli andamenti di produzione dell'attività cellulasica, attraverso dosaggio enzimatico con il substrato cromogenico AZO-CMC e, dal confronto dei livelli di produzione dell'attività cellulasica, è stato possibile selezionare due microrganismi con i più alti livelli di produzione, identificati come *Streptomyces* sp. G12 e *Bacillus amyloliquefaciens* B31C.

Per entrambi i microrganismi sono state ottimizzate le condizioni di crescita, al fine di massimizzare i livelli di produzione dell'attività cellulasica d'interesse. In particolare, è stato definito l'andamento della produzione al variare della fonte di carbonio e della temperatura, definendo la CMC e la paglia come migliori fonti di carbonio, per *Bacillus amyloliquefaciens* B31C e *Streptomyces* sp. G12, rispettivamente, e 37°C come temperatura ottimale per entrambi i microrganismi.

Attraverso analisi di zimografia accoppiate ad analisi di proteomica è stato possibile identificare le attività d'interesse prodotte dai due microrganismi selezionati. Nel caso di *Bacillus amyloliquefaciens* B31C è stata identificata in maniera inequivocabile un' endoglucanasi appartenente alla famiglia GH5 delle glicosil idrolasi. Nel caso di *Streptomyces* sp. G12 sono stati identificati peptidi appartenenti a tre diverse proteine che interagiscono con la cellulosa: una cellulasi da *Streptomyces xylophagus*, un'endoglucanasi da *Streptomyces halstedii* e una proteina di legame alla cellulosa da *Streptomyces griseoflavus*. Questi risultati hanno suggerito che le glicosil idrolasi prodotte dal microrganismo selezionato *Streptomyces* sp. G12 non sono ancora state depositate in banca dati.
D'altra parte, campioni prelevati nella regione "Western Ghat" (India), sono stati utilizzati per l'isolamento di nuovi batteri aventi attività cellulasica e xilanasica. I nuovi microrganismi sono stati sottoposti a screening su mezzo solido e liquido per la selezione dei migliori produttori di attività cellulasi e xilanasi.
Funghi filamentosi termofili, isolati da una "maâsra" (mulino per la lavorazione delle olive sito in Marocco) sono stati altresì sottoposti a screening su mezzo solido a temperature superiori ai 45°C, per la selezione di alto-produttori di attività cellulasica con caratteristiche di termo resistenza e termofilia.

- **Endoglucanasi da *Bacillus amyloliquefaciens B31C***

Al fine di caratterizzare l'endoglucanasi prodotta da *Bacillus amyloliquefaciens* B31C (CelB31C), è stato messo a punto un protocollo di purificazione consistente in una precipitazione in solfato d'ammonio, seguita da cromatografia ad interazione idrofobica. Il peso molecolare della proteina in condizioni denaturanti è stato stimato intorno ai 55,000 Da, un dato simile a quello di altre cellulasi purificate da ceppi del genere *Bacillus.* CelB31C segue una cinetica di Michaelis–Menten durante la reazione d'idrolisi della CMC, con $K_M$ pari a 9.95 ± 1.4 mg $mL^{-1}$ e $V_{max}$ pari a 710.7 ± 3.5 mg $mL^{-1}$ $min^{-1}$. L'enzima possiede un'alta termo-resistenza a 40°C, temperatura alla quale mantiene il 90% di attività dopo 6 giorni di incubazione. La temperatura ottimale di reazione è stata definita entro un ampio range, compreso tra 50 e 70°C.

- **Cellulasi da *Streptomyces* sp. *G12***

Le attività riguardanti il nuovo isolato *Streptomyces* sp. G12 sono state focalizzate sulla caratterizzazione della cellulasi ad alta similarità con la cellulasi da *Streptomyces xylophagus*.
Data la presenza di un'isoforma, con comportamento cromatografico simile alla cellulasi identificata, non è stato possibile ottimizzare la purificazione dell'enzima nativo. Pertanto, al fine di caratterizzare l'enzima d'interesse, è stato messo a punto un sistema d'espressione ricombinante in *Escherichia coli*.
Con lo scopo di sintetizzare il gene codificante la cellulasi prodotta da *Streptomyces* sp. G12, esperimenti di amplificazione (PCR) sono stati messi a punto sul DNA genomico del batterio utilizzando oligonucleotidi sintetici degeneri, disegnati sulla base delle sequenze amminoacidiche dei peptidi identificati tramite le analisi di spettrometria di massa. Le condizioni sperimentali sono state ottimizzate per la sintesi delle regioni centrale, 5' e 3' del gene, così da ottenere la sequenza codificante l'enzima d'interesse denominato CelStrep.
Analisi di bioinformatica della sequenza codificante CelStrep hanno dimostrato che il cDNA codifica una proteina matura di 342 aminoacidi, con peso molecolare predetto pari a 35,369.67 Da, avente un peptide segnale di 37 aminoacidi.

Tre potenziali siti di glicosilazione (Asn154, Asn192 e Asn 293) sono stati individuati nella sequenza aminoacidica dedotta.
Analisi di similarità effettuate attraverso il Basic Local Alignment Search Tool (BLAST) sulla sequenza aminoacidica dedotta, hanno rivelato che CelStrep appartiene alla famiglia GH12. In particolare, CelStrep mostra una significativa similarità con una β-1,4-endoglucanasi da *S. xylophagus* (EMBL Accession FJ44106; 90% identity) e una cellulasi (eglS) da *S. rochei* (EMBL Accession X73953; 93% identity). Studi di allineamento di sequenza hanno permesso di individuare in Glu156 e Glu240, i due potenziali siti catalitici, rispettivamente nucleofilo e acido/base.
Per allestire l'espressione ricombinante di Cellstrep in *E. coli*, l'intera sequenza del gene *celstrep* è stata ottimizzata e sintetizzata tenendo conto del codon usage di *E. coli*. Il gene *celstrep* è stato espresso sotto il controllo del promotore inducibile T7 RNA utilizzando il vettore di espressione pET28a, in cellule di *E. coli* ceppo BL21 CodonPlus (DE3) RP. L'espressione della proteina è stata indotta attraverso l'aggiunta di 1mM IPTG alle cellule, ottenendo una produzione di 1U $mL^{-1}$ di attività AZO-CMC nel brodo di coltura, dopo 6 ore d'incubazione a 37°C.
L'enzima ricombinante rCelStrep, purificato combinando precipitazione in solfato d'ammonio e cromatografia ad interazione idrofobica, è stato sottoposto ad analisi strutturali e funzionali.
rCelStrep ha un peso molecolare di 37,000 Da, come dedotto da analisi elettroforetiche in condizioni denaturanti (SDS-PAGE), e in accordo con le informazioni riportate in letteratura relative ad altre cellulasi da *Streptomyces* spp..
rCelStrep ha una temperatura ottimale di 50° C, presenta un'emivita di circa 24 ore e 96 ore a 60°C e 50°C, rispettivamente, e conserva l'80% di attività dopo 96 ore d'incubazione a 40°C.

- ***Nuovi batteri cellulotici e xilanolitici***

Nell'ambito del progetto BIOASSORT (318931_Improvement of technologies and tools, e.g. biosystems and biocatalysts, for waste conversion to develop an assortment of high added value eco-friendly and cost-effective bio-products; Marie Curie Action IRSES 2012), questo lavoro di dottorato ha previsto l'isolamento di nuovi microrganismi cellulolitici e xilanolitici, a partire da campioni prelevati nella regione Western Ghat del Kerala (India).
Da 50 campioni prelevati in diverse aree della regione d'interesse, sono stati isolati e purificati 93 microrganismi sottoposti a screening su mezzo solido per il rilevamento di aloni di attività celluliasica e xilanasica. Questo screening ha permesso di selezionare 7 microrganismi aventi attività xilanasica e 14 microrganismi con attività cellulasica. I microoranismi sono stati identificati attraverso parziale sequenziamento della regione 16S rRNA, mostrando alta percentuale di similarità alle specie *Bacillus* e *Streptomyces* spp..
Uno screening successivo effettuato in mezzo liquido ha permesso di individuare in R5A (*Bacillus* sp. 100%), R55A (*Paenibacillus xylanolyticus*, 100 %), XR50A (*Bacillus* sp., 100%) e XR84 (*Lysinobacillus xylanolyticus*, 100%), i migliori produttori di attività cellulasica e xilanasica. Particolare attenzione è stata focalizzata sul ceppo XR84 che, in condizioni basali di crescita in mezzo liquido, produce entrambe le attività di interesse. *Lysinobacillus xylanolyticu*s è stato recentemente identificato e solo parzialmente studiato, aumentando dunque l'interesse nello studio e la caratterizzazione del nuovo microrganismo XR84 isolato nell'ambito di questo progetto.

- ***Funghi cellulolitici termofili***

Nell'ambito di una collaborazione con il Dr. Sevastianos Roussos (IMEP-IRD, Université "Paul Cezanne", Marseille), 150 funghi termofili filamentosi sono stati sottoposti a screening su mezzo solido per la selezione di microrganismi alto-produttori di cellulasi termofile e resistenti ad alte temperature (> 45°C).
Due *Aspergillus fumigatus* (ceppi L-457 e L-521), due *Aspergillus niger* (ceppi L-382 e L-798), un *Myceliophthora thermophila* (ceppo L-433) e un *Malbranchea* (ceppo L-578) sono stati selezionati sulla base delle dimensioni degli aloni di attività cellulasica rilevati su piastra, e considerando la velocità di crescita apicale, quale indice della predisposizione del fungo alla colonizzazione di materiale lignocellulosico. I funghi filamentosi sono infatti i microrganismi maggiormente adatti all' allestimento di processi di fermentazione su stato solido su materiale lignocellulosico per la produzione di enzimi dì interesse industriale a basso costo.

**Identificazione e caratterizzazione di una nuova arabinofuranosidasi dal fungo *Pleurotus ostreatus***

Espermenti di zimografia accoppiati ad analisi di proteomica hanno permesso di identificare l'enzima responsabile dell'attività xilanasica prodotta dal fungo basidiomicete *Pleurotus ostreatus* durante esperimenti di fermentazione su stato solido (SSF) su scarti della lavorazione di pomodoro, precedentemente effettuati nel laboratorio dove questo progetto di dottorato è stato svolto.
Dal confronto con le sequenze dei genomi depositati in banca dati dei ceppi di *P. ostreatus* PC15 e PC9, l'enzima è stato identificato come una $\alpha$-L-arabinofuranosidasi, denominata PoAbf. Il cDNA e il gene codificanti l'enzima PoAbf nel ceppo ATCC MYA-2306 di *P. ostreatus* sono stati sintetizzati tramite reazioni di PCR. E' stato così isolato un cDNA codificante una proteina di 646 aminoacidi, con un peso molecolare stimato di 68,912 Da. Attraverso analisi bioinformatiche della sequenza aminoacidica dedotta si è rilevata la presenza di un peptide segnale di 20 aminoacidi e di due potenziali siti di O- glicosilazione (Ser160 e Thr187). Il confronto della sequenza amminoacidica dedotta di PoAbf con le sequenze amminoacidiche sia di enzimi appartenenti alla collezione del Prof. Bernard Henrissat (Architecture et Fonction des Macromolécules Biologiques, CNRS, Marseille France) sia di proteine presenti in banche dati di sequenze proteiche note, ha rivelato la sua alta similarità con $\alpha$-L-arabinofuranosidasi della famiglia GH 51 prodotte da *Meripilus giganteus* (63 % di identità)*, Postia placenta* (55 % d'identità), *Coprinopsis cinerea* (54 % d'identità) e *Schizophyllum commune* (52 % d'identità). Le analisi di similarità hanno inoltre permesso di riconoscere in E364 e E471 i potenziali siti catalitici acido/base e nucleofilo, rispettivamente.

- **Caratterizzazione dell'enzima ricombinante rPoAbf wt**

Al fine di sviluppare un sistema per la sovra-produzione di PoAbf sono stati messi a punto e paragonati sistemi di espressione ricombinante eterologa del cDNA *poabf* nei lieviti *Kluyveromyces lactis* e *Pichia pastoris.* Quest'ultimo è risultato essere il miglior ospite per l'espressione ricombinante dell' $\alpha$-L-arabinofuranosidasi con una massima produzione, dopo 15 giorni, di $30\times10^{-2}$U $mL^{-1}$, corrispondente a 8.3mU $O.D.^{-1}$, nel coltura mezzo extracellulare, e 1.2 mU $O.D.^{-1}$ nell'estratto cellulare. Il clone più produttivo è stato utilizzato per ottimizzare la produzione di rPoAbf variando la concentrazione di metanolo nel terreno di coltura e la temperatura durante la crescita di *P. pastoris*. Il massimo livello di produzione dell'enzima ricombinante secreto (180 mg $L^{-1}$) si è ottenuto quando la crescita del lievito è stata condotta alla

temperatura di 20°C in presenza della più bassa concentrazione di metanolo analizzata (0.5% v/v). L'enzima ricombinante prodotto in *P. pastoris* è stato purificato e caratterizzato. Esso è risultato essere un monomero glicosilato con un peso molecolare di 81,500 Da in condizioni denaturanti. Analisi di spettrometria di massa hanno consentito la localizzazione di un unico sito di O-glicosilazione sull'aminoacido Ser160. Quando coinvolta nella reazione di idrolisi del substrato sintetico p-nitrophenil-$\alpha$-L-arabinofuranoside, rPoAbf segue la cinetica di Michaelis-Menten con una $K_M$ di 0.64 mM e una $k_{cat}$ di 3010 $min^{-1}$. Il valore di pH ottimale della reazione è 5, mentre la temperatura ottimale per la conversione enzimatica è pari a 40°C. L'enzima possiede un' elevata stabilità in un ampio intervallo di pH, mostrando una emivita massima di 51 giorni a pH 5.
rPoAbf possiede entrambe le attività eso- ed endo- xilanasica, ed è in grado di idrolizzare arabino-oligosaccaridi e substrati naturali, quali arabinani e arabinoxilano, dimostrandosi un biocatalizzatore versatile.

- **Preparazione e caratterizzazione dei mutanti rPoAbf S160G, rPoAbf E471G e rPoAbf E364G**

Al fine di elucidare il ruolo della glicosilazione individuata a livello del residuo Ser160, e per dimostrare il coinvolgimento dei residui E471 ed E364 nel meccanismo di reazione come residui catalitici nucleofilo ed acido/base rispettivamente, sono stati allestiti esperimenti di mutagenesi sito-diretta, con la preparazione dei mutanti PoAbf S160G, PoAbf E471G, PoAbf E364G.
L'enzima rPoAbf mutato nelle tre diverse posizioni è stato prodotto nel lievito *P. pastoris*, sistema previamente ottimizzato per la produzione dell'enzima *wild-type* (wt). Una notevole riduzione dei livelli di produzione dei mutanti E471G e E364G è stata rilevata, suggerendo che le mutazioni apportate inficiano l'attività d'interesse e confermando il ruolo ipotizzato per i residui mutati.

**- rPoAbf S160G.** Il mutante è stato purificato e caratterizzato. Analisi di spettrometria di massa hanno confermato l'assenza di O-glicosilazione sull'aminoacido Ser160. Quando catalizza l'idrolisi del substrato sintetico p-nitrophenil-$\alpha$-L-arabinofuranoside, rPoAbf S160G segue la cinetica di Michaelis-Menten con $K_M$ di 0.89 mM e $k_{cat}$ di 2,590 $min^{-1}$, valori simili a quelli previamente misurati per l'enzima wt. Come per l'enzima wt i valori ottimali di pH e temperatura sono 5 e 40°C, rispettivamente. Tuttavia, è stato dimostrato che rPoAbf S160G ha un'emivita ($t_{1/2}$) a pH 8 e alla temperatura di 50°C notevolmente inferiore a quella di rPoAbf wt, come riportato nella tabella 1.

**Tabella 1:** rPoAbf wt *versus* rPoAbf S160G

| | **rPoAbf wt ($t_{½}$)** | **rPoAbf S160G ($t_{½}$)** |
|---|---|---|
| **Temperature 50 °C** | 17 ore | 3 ore |
| **pH 8** | 38 giorni | 2,5 ore |

Questo dato suggerisce che la glicosilazione in posizione S160 favorisce la stabilità dell'enzima rPoAbf, come corroborato anche dalle analisi di dicroismo circolare (CD) da cui si è rilevata una parziale perdita della struttura secondaria nel mutante in

esame. In particolare, per rPoAbf S160G si è misurata rispetto all'enzima wt una diminuzione della struttura beta dal 35 al 24%, ed un aumento della struttura disordinata dal 36 al 44%, rispetto all'enzima wt.

***Conclusioni***

In sintesi questo progetto di dottorato ha previsto:

- lo sviluppo di nuovi biocatalizzatori, due cellulasi batteriche ed una $\alpha$-L-arabinofuranosidasi da fungo, di cui sono state studiate le proprietà catalitiche. Le abilità idrolitiche dei suddetti enzimi saranno testate allestendo cocktail enzimatici con enzimi commerciali attualmente utilizzati per la conversione della lignocellulosa in zuccheri fermentabili;

- l'isolamento e la selezione da campioni prelevati nella regione Western Ghat (India, Kerala) di 14 batteri cellulolitici e 7 batteri xilanolitici, il cui sistema (emi)cellulolitico sarà studiato seguendo un approccio uguale a quello definito per lo studio delle cellulasi già caratterizzate in questo progetto di dottorato;

- la selezione di 7 funghi termofili a partire da una collezione di 150 funghi isolati da una "maâsra", aventi attività cellulolitica. Questi saranno usati per l'allestimento di processi di fementazione su stato solido su biomasse lignocellulosiche, al fine di produrre a basso costo, e caratterizzare, cellulasi termofile e termoresistenti.

***List of Abbreviations***

**AF:** Arabinofuranosidase
**AFEX:** Ammonium Fiber Expansion
$a_w$: water activity
**AZO-CMC:** Azo-CarbossiMetilCellulosa/Azo-CarboxyMethylCellulose
**AXE:** Acetyl Xylan Esterase
**bdt:** bone dry ton
**BG:** β-Glucosidase
**BI:** Billion liters
**BLAST:** Basic Local Alignment Search Tool
**BX:** β-Xylosidase
**CAZY:** Carbohydrate-Active EnZYmes
**CBD:** Cellulose-Binding Domain
**CBH:** Cellobiohydrolases
**CBM:** Carbohydrate-Binding Module
**CBP:** Consolidated BioProcessing
**CD:** Dicroismo Circolare/ Circular Dychroism
**cDNA:** complementary DNA
**CE:** Carbohydrate Esterases
**CMC:** CarbossiMetilCellulosa/CarboxyMethylCellulose
**EG:** endoglucanase
**EX:** Endo-β-Xylanase
**FAE:** Feruloyl Esterase
**FOLy:** Fungal Oxidative Lignin Enzymes
**FPU:** Filter Paper Unit
**GE:** Glucoronyl Esterase
**GH:** Glicosil idrolasi/ Glycosyl hydrolases
**LCA:** Life Cycle Analyses
**LDA:** Lignin Degrading Auxiliary enzymes
**LO:** Lignin Oxidases
**PCR:** Polymerase Chain Reaction
**PL:** Polysaccharide Lyases
**pNPA:** para-Nitro Phenyl Arabinofuranoside
**PoAbf:** α-L-arabinofuranosidase from *Pleurotus ostreatus*
**R&D:** Research&Develpoment
**rPoAbf:** recombinant α-L-arabinofuranosidase from *Pleurotus ostreatus*
**rRNA:** Ribosomal Ribonucleic Acid
**SDS-PAGE:** Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
**Smf:** Submerged fermentation
**ssf:** simultaneous saccharification and fermentation
**SSF:** Solid State Fermentation
**wt:** wild-type

## 1.1 Bioethanol as an alternative to fossil fuels

Fossil fuel reserves depletion, global warming, costly and problematic waste recycling and population growth induce to find new strategies for energy production, such as the use of biofules which are produced from renewable sources and can support a sustainable development. From this point of view, it is worth noting that European Directive 2009/28/CE establishes national renewable energy targets that result in an overall binding target of a 20% share of renewable energy sources in energy consumption by 2020 and a binding 10% minimum target for renewable fuels in transport to be achieved by each Member State.
Adoption of Kyoto protocol has also a key role in this contest, setting binding targets for 37 industrialized countries and the European community for reducing greenhouse gas (GHG) emissions of at least five per cent against 1990 levels over the five-year period 2008-2012, with a recent extension up to 2020.
Among biofuels of new generation, bioethanol is mostly produced all over the world.
Global bioethanol production in 2011 has been estimated at 84.6 billion liters (Bl). The United States is the leading producer with 52.6 Bl (62%), while Brazil produced 21.1 Bl (25%). The EU-27, with a production of 5467 Ml (4.6%), ranks third behind these two majors producers [2].
It is worth notice that ethanol use in Brazil has started in 2003, with the introduction of the so called “flex fuel” cars, able to use petrol, ethanol or any mixture of the two fuels. As far as Europe is concerned, the major stakeholder Abengoa Bioenergy (Spain), a biofuels subsidiary of the Abengoa group, has been registered as the leader with a fuel production capacity of 3,125 millions of litres (Ml), in 2011. In the same year, the French industrial group Tereos registered a total production capacity of 543 Ml while the German group CropEnergies reached a total production capacities of 692 Ml/year [5].
Generally, liquid biofuels can be classified into "first-generation" and "second-generation", where the main distinction is the characteristic of the feedstock used.
First-generation bioethanol is made from sugars, grains or seeds, using only a specific portion of the above-ground biomass produced by a plant, often edible such as sugarcane in Brazil and corn in USA. In these cases, simple processing of the biomass is required to produce the finished fuel. In producing bioethanol from sugar crops, the latter are first processed to extract the sugar (e.g. through crushing) and the sugar is then fermented to yield ethanol. A final step of distillation is needed to purify the ethanol and reach the desired concentration, usually removing all water and producing "anhydrous ethanol" that can be blended with petrol. Bioethanol production process from starchy materials is more difficult compared to sugar crops because an additional step of feedstock hydrolysis is required. Starch is in fact a polysaccharide consisting of long chains of glucose molecules. Through hydrolysis, starch is broken down to fermentable glucose molecules. Hydrolysis, also known as saccharification, can be either enzymatic (using a mixture of enzymes, known as amylases) or acid-based [6]. Once the starch is broken down to glucose syrup, the process is similar to that for sugar crops with fermentation of sugars followed by distillation. Both processes of ethanol production from sugarcane and starch yield several by-products, such as bagasse (used as solid fuel and burned for heat and electricity) and protein-rich animal feed, respectively.
However, many concerns are still associated to first generation bioethanol such as feedstock insufficiency related to the increased demand; food *versus* fuel conflict, due to the use of edible material for fermentable sugars recovery; greenhouse gas

emissions not low as required. Thus, it is needed pushing towards alternative systems, such as second-generation liquid biofuels based on lignocellulosic biomass. Various life cycle analyses (LCAs) have shown that lignocellulosic ethanol, produced through biochemical conversion, performs significantly better than first-generation, sugarcane/corn-based ethanol, following various environmental and energy security criteria [4]. For instance, second generation ethanol offers a more attractive greenhouse gas emissions profile than other biofuels, with a 86 % reduction in comparison to gasoline (Figure 1) [7].

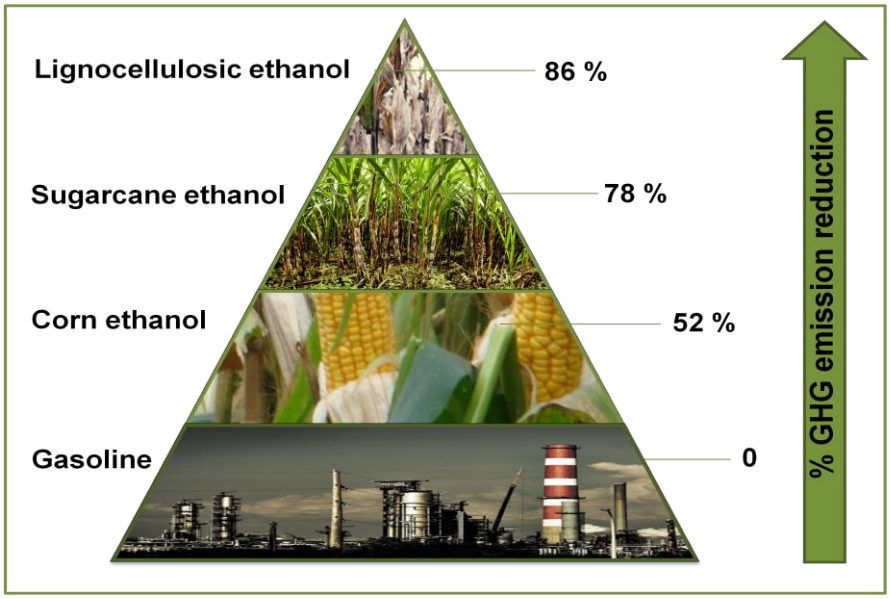


**Figure 1:** Net Green House Gas (GHG) emission (adapted from Wang et al., 2007 [6])

### 1.2 Lignocellulose as raw material for second generation bioethanol production

By comparison to feedstock used in first-generation bioethanol production, lignocellulosic biomass has many advantageous characteristics: (a) it is generally not edible and therefore does not compete with food production; (b) it can be bred specifically for energy purposes, thereby enabling higher production per unit land area; (c) it represents most of the above-ground plant material, thereby further increasing land-use efficiency. Moreover, lignocellulosic biomass is geographically well distributed and its annual production has been estimated at around 100 billion tones organic dry matter of land biomass and 50 billion tones of aquatic biomass. Food use is only 1.25% of the entire land biomass, the rest of the biomass being unused or recycled into the earth system [2].

These properties of lignocellulosic materials can be easily translated into substantial economical, energetic and environmental benefits aimed at a sustainable development.

Lignocellulose (Figure 2) is mainly composed of cellulose, hemicellulose and lignin.

Cellulose, the most abundant biological material on earth, is a polysaccharide consisting of glucose residues. It makes up about 40-50% of the weight of dry wood and it is largely responsible for the plant cell wall mechanical strength. In higher plants it is organized into microfibrils, each containing up to 36 glucan chains having thousands of glucose residues linked trough β-1,4-glycosidic bonds [8].

Hemicellulose accounts for 25-35% of dry wood [6]. It is a very heterogeneous and ramified polymer, consisting of a mixture of various monosaccharides, such as xylose

and arabinose (both 5-carbon sugars), glucose, mannose and galactose (6-carbon sugars), and glucoronic acid. Its composition depends on the characteristics of the tissue from which it is recovered. Lignin, instead, is a highly branched polyphenolic macromolecule, strongly resistant to chemical and biological degradation. The relative proportions of these three materials in lignocellulose feedstock vary, depending on the specie. Table 2 shows the composition of some second generation bioethanol feedstock.

**Table 2:** Cellulose, hemicelluloses, lignin content* in common lignocellulosic biomass

| | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|
| Switchgrass | 45 | 30 | 12 |
| Corn stover | 40 | 25 | 17 |
| Wheat straw | 38 | 27 | 20 |
| Poplar | 45 | 21 | 24 |
| Eucalyptus | 45 | 25 | 24 |
| Douglas fir | 44 | 21 | 32 |
| Miscanthus | 48 | 30 | 12 |

* percentages of dry weight

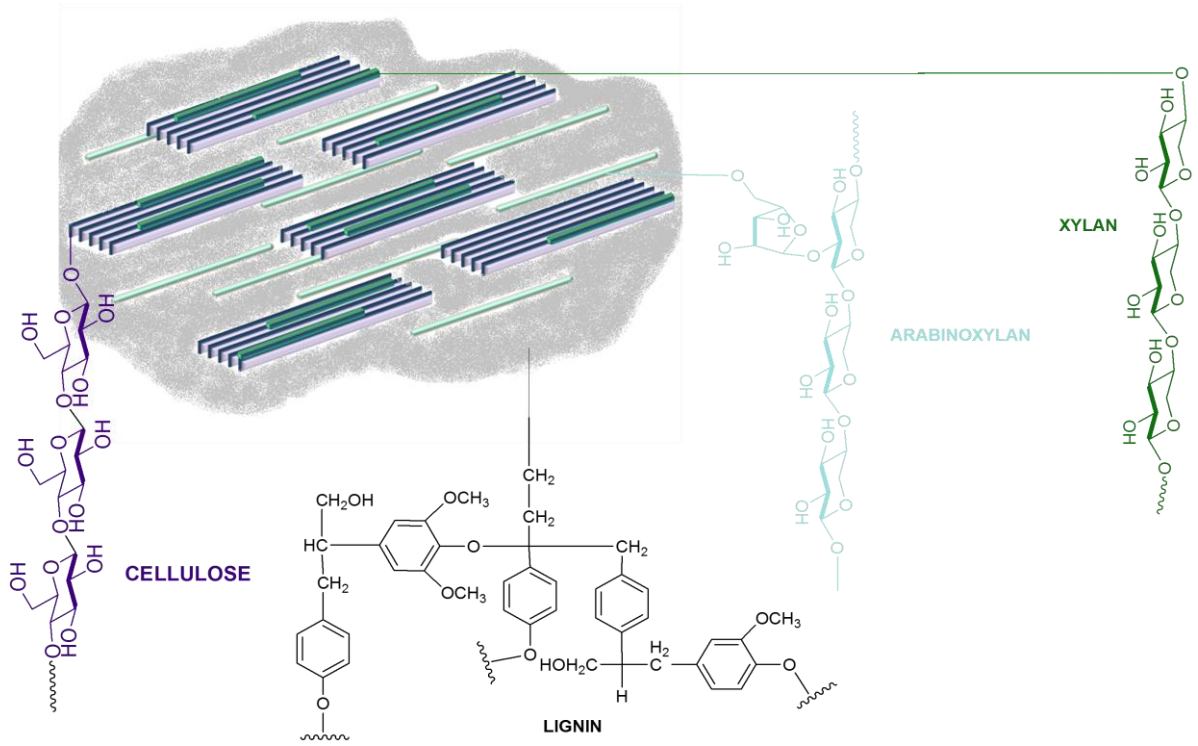


**Figure 2:** Native cell walls of lignocellulosic biomass

### 1.3 Process of production of second generation bioethanol

To produce second generation bioethanol, cellulose and hemicellulose conversion into fermentable sugars is required after removal of lignin. Intrinsic complexity of lignocellulosic biomass and its resistance to breakdown are the major factors which hamper its effective use for bioethanol production. This is the reason why industrial and competitive production of second generation bioethanol has not been developed yet, as demonstrated by the only presence of demonstration/pilot plants currently working: Iogen plant in Canada, where cellulosic ethanol is produced from wheat-straw or Abengoa plants, a pilot scale in York (Nebraska, US) and a demonstrative plant in Salamanca (Spain). Clariant (Germany) has recently inaugurated a new cellulose ethanol pilot plant, the biggest of its kind in Germany with a production rate of around 1,000 tonnes/year, using 4,500 tonnes/year of locally sourced agricultural waste as a feedstock. In italy, Mossi&Ghisolfi (M&G) group has recently started the building of a semi-industrial plant for bioethanol production from dedicated cultures of *Arundo Donax*. M&G is also entering the Brazilian ethanol market, with an agreement for building the first country's second-generation plant, with production of the fuel from the residue of sugar cane on a commercial scale. A lot of American companies are exploiting facilities for first generation bioethanol production to be upgraded for second generation bioethanol production from lignocellulosic biomass [9]. Second-generation, biochemically derived ethanol is generally produced through a process involving four primary steps (Figure 3): (i) pre-treatment, (ii) hydrolysis to sugars, (iii) fermentation, (iv) products/co-products recovery. Conventional process currently adopted foresees a chemical/physical pre-treatment of lignocellulose in order to disrupt the fibrous matrix and remove lignin which can be recovered and used as a fuel for heat and electricity. Several techniques have been explored for removing lignin, steam explosion, dilute acid hydrolysis and ammonia fiber expansion (AFEX) being the most studied [10].

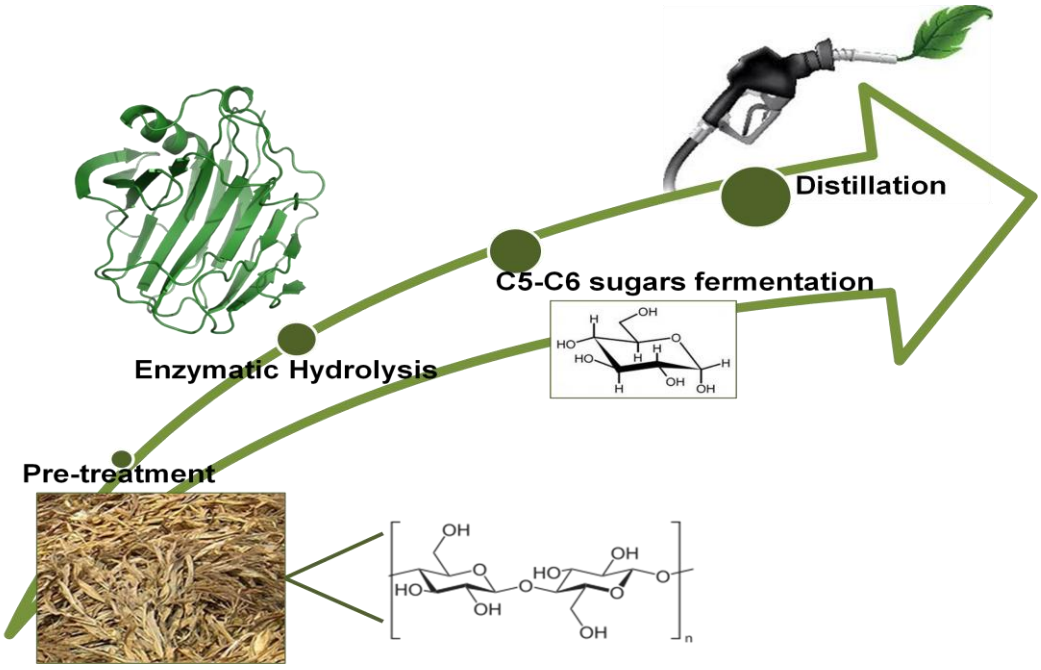


**Figure 3:** Main steps of bioethanol process of production from lignocellulosic biomass

Once lignin has been removed, saccharification of the free accessible (hemi)cellulose portions of the biomass is carried out. A variety of thermal, chemical and biochemical methods are being developed to carry out it in an efficient and low-cost manner [11]. However, enzymatic hydrolysis has been so far demonstrated to be the best way to achieve (hemi)cellulose depolymerization (Figure 4).

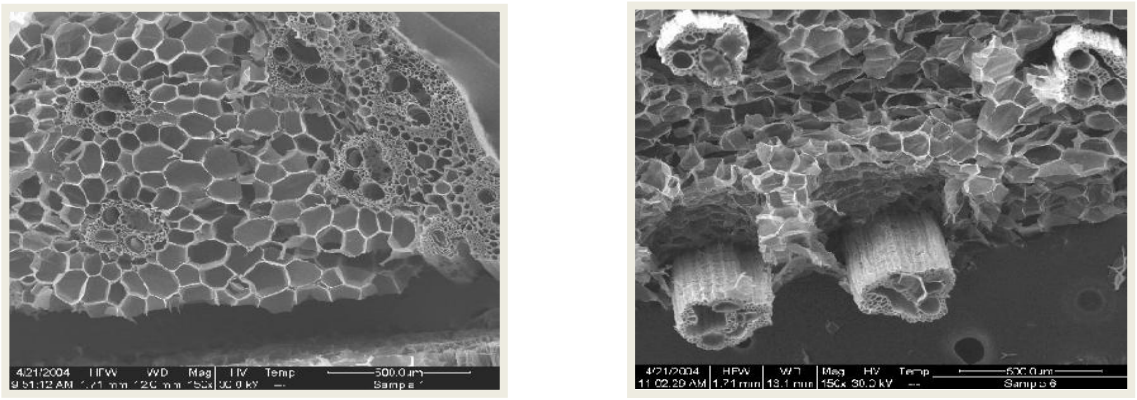

**Figure 4:** SEM images of plant cell wall chemically (a) and enzymatically (b) hydrolyzed (Source: NREL)

The final step is the fermentation of sugars obtained from previous step, which is typically performed by the yeast *Saccharomyces cerevisiae*. Current process is optimized for 6-carbon atoms sugars fermentation since most of yeasts can not ferment 5-carbon atoms sugars. Economically competitive ethanol production from lignocellulosic materials requires the efficient use of both the hexose and pentose monosaccharides. Thus, research is aimed at exploring new engineered yeasts able to ferment 5-carbon sugars.

Two main routes can be followed for ethanol production, such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (ssf).

In SHF the bioconversion of lignocellulose takes place in two separate reactors, thus separating the saccharification and the fermentation processes, whilst in ssf enzymatic hydrolysis and fermentation are carried out together, using a unique reactor. Taking place in the same reactor, this kind of process has a number of advantages such as the reduction of costs and the increase of hydrolysis rate; however, the ideal pH or temperature conditions for the saccharification step may differ from those for the fermentation step [6].

On the other side, Consolidated BioProcessing (CBP) can provide an important contribution to reduce ethanol production costs, moving from cellulosic feedstock to fuel ethanol tanks. Biological conversion is consolidated into a single process step where a single engineered microorganism is able to directly convert cellulose into ethanol using its own enzymatic machinery (see paragraph 1.5.3).

## 1.4 Enzymes involved in lignocelluloses conversion

The highest yields of fermentable sugars from cellulose portion are achieved through enzymatic hydrolysis, which involves three types of enzymes belonging to glycosyl hydrolases class, endo-glucanases, cello-biohydrolases and β–glucosidases. They act in a cooperative manner producing monomers of glucose. Exploiting hemicellulose portion of lignocellulosic biomass for fermentable sugars recovery is needed to increase saccharification yield. Complete enzymatic hydrolysis of both cellulose and hemicellulose requires a cocktail of enzymes finely designed, consisting not only of cellulases but also of xylanases, arabinofuranosidases, mannanases and all the enzymes acting on the highly heterogeneous structure of this macromolecule (Table 3). Other proteins, like expansin and swollenin have been recently shown to contribute to lignocellulose degradation, even if their roles have not been already clarified.

According to Carbohydrate-Active EnZYmes (CAZY) [12] and Fungal Oxidative Lignin Enzymes (FOLy) [13] databases, lignocellulose-degrading enzymes belong to Glycoside Hydrolases (GH), Polysaccharide Lyases (PL), Carbohydrate Esterases (CE), Lignin Oxidases (LO), and Lignin Degrading Auxiliary enzymes (LDA) families.

Focusing the attention on the GHs, they are a wide spread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. Glycoside hydrolysis can occur *via* overall retention or overall inversion of the anomeric configuration (Figure 4) [12]. Glycoside hydrolysis with inversion of the anomeric configuration is achieved through a single-displacement step, which involves oxocarbenium ion-like transition states**.** The reaction takes place with general acid and general base assistance from two amino acid side chains, normally glutamic or aspartic acids.

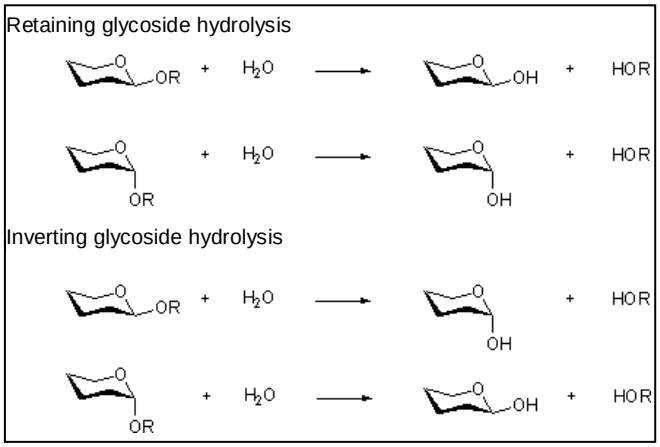


**Figure 4:** GHs mechanism of reaction

Retention is characterized by a double-displacement mechanism which involves a covalent glycosyl-enzyme intermediate and the formation of an oxocarbenium ion-like transition state in each step. Also in this case, reaction is mediated by an acid/base amino acid and a nucleophilic one, typically glutamate or aspartate. In the first step (glycosylation step), one residue plays the role of nucleophile, attacking the anomeric centre to displace the aglycon and form a “glycosyl enzyme” intermediate. At the same time, the other residue works as an acid catalyst by protonating the glycosidic oxygen, as the bond cleaves. In the second step (deglycosylation step), the intermediate is hydrolyzed by water, with the other residue now acting as a base catalyst which de-protonates the water molecule [14].

**Table 3:** Enzymes involved in the degradation of lignocellulose components

| Component | Enzyme |
|---|---|
| **Lignin** | Laccase, Manganese peroxidase, Lignin peroxidase |
| **Pectin** | Pectin methyl esterase, Pectate lyase, Polygalacturonase, Rhamnogalacturonan lyase |
| **Hemicellulose** | Endo-xylanase, Acetyl xylan esterase, β-xylosidase, Endomannanase, β-mannosidase,α-galactosidase, α-L-arabinofuranosidase, α- glucuronidase, Ferulic acid esterase, p-coumaric acid esterase |
| **Cellulose** | Cellobiohydrolase, Endoglucanase, β-glucosidase |

- **Cellulases**

Cellulases are involved in the hydrolytic scission of the β(1→4) glucosidic bond in cellulose, leading to the formation of glucose and short cellodextrins.
Cellulases can be grouped in three types: endoglucanases (EG) (EC 3.2.1.4), which hydrolyse internal β-1,4-D glucosidic linkages randomly in the cellulose chain; cellobiohydrolases (CBH, also known as exoglucanases) (EC 3.2.1.91), which progress along the cellulose line and cleave off cellobiose units from the ends; *β*- glucosidases (BG) (EC 3.2.1.21), which hydrolyse cellobiose to glucose. Despite the homopolysaccharide nature of cellulose, all the three cellulases are needed, acting synergistically to complete the conversion of cellulose into simple glucose monomers.
To facilitate sufficient contact and orientation between the catalytic domain and the substrate, CBHs and EGs have a cellulose-binding domain (CBD, or CBM (carbohydrate-binding module)), connected to the catalytic domain by a glycosylated flexible linker. CBD influences cellulase ability to dock with and degrade crystalline cellulose. CBDs have little impact on the activity of cellulases on a soluble substrate (such as carboxymethyl cellulose (CMC)), but removal of the CBD from the enzyme significantly affect the hydrolysis of crystalline cellulose. For instance, CBM anchors and directs the enzymes to targeted carbohydrate substrates and in some cases even disrupts crystalline cellulose microfibrils assisting cellulase reaction [15].
*Trichoderma reesei* has been so far recognized as the main producer of such enzymes, particularly producing two CBHs, five EGs and two BGs with an average enzymatic hydrolysis yield attested in the range 75-85% [16].

- **Xylanases**

endo-β-Xylanases (EXs) and β-Xylosidases are involved in the conversion of xylan into xylose monomers. Particularly, endo-β-Xylanases carry out the hydrolysis of the β(1→4) linked D-xylopyranosyl polysaccharides with different O-substitutions constituting the hemicelluloses.
EXs have a catalytic core belonging to GH8, 10, 11, 30 or 43 families. Moreover, they possess one or more CBMs or other domains.
β-xylosidases (BX, EC 3.2.1.37) hydrolyze the xylobiose or other xylooligosaccharides resulting from the action of EXs. They belong to GH3, 30, 39, 43, 52 and 54 families and can possess α-L-arabinofuranosidase activity, too.

- **Accessory enzymes**

Due to the high complexity of hemicelluloses, several accessory enzyme are needed for its complete conversion into simple sugars (xylose, mannose, arabinose). Acetyl xylan esterase (AXE, EC 3.1.1.72), feruloyl esterase (FAE, EC 3.1.1.73) and glucuronoyl esterase (GE, EC 3.1.1.-) are involved in the removal of ester substituents in xylan. Removal of arabinose substituent is carried out by α-L-arabinofuranosidases (AF, EC 3.2.1.55), a group of enzymes whose catalytic cores belong to GH3, 43, 51, 54, and 62 families according to CaZy database. They are able to hydrolyze terminal non reducing α-L-1,2-, α-L-1,3-, and α-L-1,5-arabinofuranosyl residues. It is possible to distinguish three different classes of arabinofuranosidases: α-L-arabinofuranosidases type A, acting on short oligosaccharides, type B α-L-arabinofuranosidases, able of hydrolyzing side-chain arabinose residues from polymeric substrates, and type C, specific for arabinoxylans and not able to hydrolyze the synthetic substrate p-nitrophenyl α-L-arabinofuranoside (pNPA), differently from the latter types. Enzymes from the three classes have been found in culture supernatant of various fungi. Degradation of β(1→3), (1→4), or (1→6) glucans can be carried out by β-glucanases (EC 3.2.1.-), a group of endo- or exo-acting glycosidic bond type-specific or promiscuous enzymes, belonging to GH3, 5, 12, 16, 17, 55, 64, and 81 families. Mannanases (EC 3.2.1.78) act in the degradation of (galacto)(gluco)mannans, β(1→4)-D-mannosyl or manno/glucopyranosyl polymers with variable α(1→6) D-Gal side chains. Mannanases are distribuited among GH5, 26, and 113 families.

### 1.4.1 Factors affecting lignocellulose hydrolysis

The heterogeneous nature of lignocellulose and the multiplicity of enzymes make it difficult to fully understand the interactions between enzyme and lignocellulose. One of the main factors affecting initial hydrolysis rate is the nature of the substrate, particularly the content of cellulose and hemicellulose. High substrate concentration generally causes hydrolysis rate decrease, problems in mixing and mass transfer.
The enzymatic hydrolysis of lignocellulose is also affected by hydrolysis temperature, time, pH, enzyme loading, product concentration, and biomass structural features.
Structural features are the most complicated factors, being correlated to the nature of the pretreatment. It is generally recognized that accessible surface area and lignin content play significant roles in influencing the rate and extent of biomass hydrolysis.
Lignin content, acetyl content, and cellulose crystallinity are key factors that determine biomass digestibility.
Moreover, it is well known that cellulolytic enzymes are inhibited by hydrolysis end-products such as cellobiose, glucose, or both.

Several methods have been developed to reduce the inhibition, such as the use of high dosage of enzymes, the addition of β-glucosidases and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (ssf). Inhibition can be also ascribed to the end products of pre-treatment, such as phenol compounds derived from lignin removal [17] and to the increase of solid material concentration in lignocellulose biomass [18, 19]. In general, the maximum hydrolysis efficiency of lignocelluloses can be observed when the substrate concentration is 5%, with a ration solid:liquid of 1:20 [19, 20].
A dosage in the range 5-35 Filter Paper Units (FPU) $g^{-1}$ of cellulose is generally used, providing a high glucose yield (300 – 600 mg $g^{-1}$ of pretreated biomass) in a reasonable time of 48-72 h [20].
The effect of surfactants on lignocellulose hydrolysis has been largely investigated, showing the positive effects of surfactants which can prevent denaturation of the enzymes and increase enzyme accessibility by disrupting the surface structure, enhancing enzyme-substrate interactions, and preventing non-productive adsorption of enzymes. Particularly, surfactants such as Tween 20/80 or polyethylene glycol, have been shown to increase the surface area available for cellulase hydrolysis of lignocelluloses.

### 1.5 Strategies to improve second generation bioethanol production

Currently, lignocellulosic ethanol production is economically uncompetitive with first-generation ethanol production, with the cost proving to be 250–300% greater, as reported by Stephen et al. (2012) [3]. The key barrier of the current technologies to produce cost-effective ethanol from lignocellulose is the high cost of ligno-cellulolytic enzymes needed to hydrolyze the (hemi)cellulose into monosaccharides [21]. In fact, the cost of hydrolytic enzymes, is currently generally reported to represent 15 – 20 % of the costs of the whole process.
As predicted by CGEE in 2006 (Centro de Gestao e Estudos Estrategicos), the world demand for cellulases and amylases was estimated as US$ 295 million and US$ 374 million, respectively, for 2009, and their market is expected to reach $4.4 billion by 2015, with a compound annual growth rate (CAGR) of 6% over the 5-year forecast period [22]. Over the last few years, significant progress has been made in reducing lignocellulolytic enzymes cost, with Novozymes claiming a 30-fold reduction in enzyme cost between 2001 and 2005 from $1.32 per liter of ethanol ($L^{-1}$) to between $0.026 and $0.048 $L^{-1}$ at the bench scale, this figure being later revised to $0.132 $L^{-1}$ in 2010 to represent industrial costs [4]. Cellulase enzyme cost contribution to the total production cost of lignocellulosic ethanol can be approximated by the equation:

$$E = \frac{P * L * C}{A * Y} * 1000$$

where *E* is the price of enzyme in $ $L^{-1}$ ethanol, *P* is the price of protein in $ $kg^{-1}$, *L* is the enzyme loading in FPU $g^{-1}$ cellulose, *C* is the cellulose content of the biomass (%), *A* is the enzyme activity in FPU $g^{-1}$ protein, and *Y* is the ethanol yield in L bone dry ton $(bdt)^{-1}$ [4]. Reduction of (hemi)cellulases production costs is strongly required to increase competitiveness of second generation bioethanol process of production. With this aim, different strategies can be adopted: i) isolation and identification of new microorganisms producing high levels of (hemi)cellulase activity; ii) production of hypercellulolytic mutants of organisms suitable for cellulase production; iii) genetic

modification to develop new enzymes with high specific activity and/or high temperature resistance; iv) use of cheap substrates for enzymes production.

### 1.5.1 Novel biocatalysts with improved catalytic properties

A variety of microorganisms, including bacteria, actynomycetes and fungi, possess the ability to degrade the cellulosic biomass into fermentable sugars. Microorganisms isolated from natural habitats can constitute the source of new enzymes with improved catalytic characteristics for lignocellulosic biomass conversion into fermentable sugars. Particularly, research is looking for (hemi)cellulases with higher efficiencies, increased stability at elevated temperatures and at extreme pH values, and higher tolerance to end-products inhibition

On the other side, protein engineering of enzymes already studied, through both rational design and direct evolution, is currently the best strategy to develop new biocatalysts which can well answer to the existing market demand [23, 24, 25].

Traditional random mutagenesis techniques, such as ultraviolet (UV) irradiation or chemical (e.g. N-methyl-N0-nitro-N-nitrosoguanidine) treatment, are useful and effective approaches for the development of strains with increased cellulase production. Such experiments have been so far successfully performed for preparation of new *T. reesei* strains with improved expression levels of cellulases as reviewed in Amore et al. [26].

### 1.5.2 Solid state fermentation (SSF)

Submerged fermentation (Smf) has been so far reported as the main process for production of lignocellulolytic enzymes, among those with industrial applications. More that 75% of the industrial enzymes are produced using SmF, one of the major reasons being that SmF supports the utilization of genetically modified organisms [27, 28]. In order to reduce cellulolytic/hemicellulytic enzymes costs of production, SSF holds economical, environmental and energetically advantages and it has been demonstrated to be the most suitable system for fungal enzymes production. SSF represents the growth of microorganisms in the absence or near-absence of free water [29], thus closely resembling the natural way of life of microorganisms. Differently from SmF, in SSF processes microbial growth and products formation occur at or near the surface of the solid substrate particles having low moisture contents (Figure 5). Thus, it is crucial to provide an optimized water content, and control the water activity ($a_w$) of the fermenting substrate. Water has also a strong impact on the physic-chemical properties of the solids and this, in turn, affects the overall process productivity. SSF shows a lot of advantages such as low energy consumption, simplicity of aeration because of the porosity of the materials, low risk of contamination.

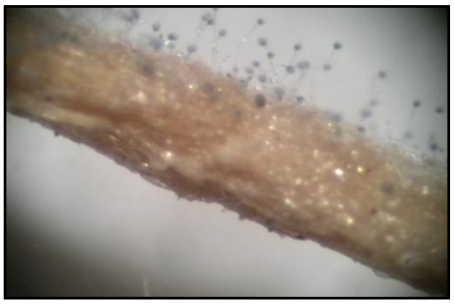

**Figure 5:** *Aspergillus fumigatus* on sugarcane bagasse

On the other side, the design itself of SSF process often results in a complicate downstream process of the enzymes of interest, due to the presence of particulates and to the complex composition of the substrates.

Agro-industrial residues are generally considered the best substrates for SSF processes, and a number of such substrates have been employed for the cultivation of microorganisms to produce enzymes [30]. Since biotechnological applications require large amounts of low cost enzymes, one of the most appropriate approaches

for this purpose is to utilize as substrate lignocellulosic wastes, some of which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis. Large quantities of agro-industrial wastes are generated all over the world and the environmental pollution problems associated with conventional disposal methods (land filling, incineration, feeding for animals) have been an impulse to the search for alternative, environment-friendly methods of handling biowastes (Couto, 2008) [31], such as their microbial transformation.
Particularly, agriculture and food farming residues, besides paper, green and organic fractions of municipal waste represent the most abundant lignocellulosic wastes to be used for second generation bioethanol production in the Mediterranean area [32].

### 1.5.3 Consolidated Bio Processing

Consolidated BioProcessing (CBP) can provide an important contribution to reduce ethanol production costs and move from cellulosic feedstock to fuel ethanol tanks. As a matter of the fact, it would be desirable to combine cellulase production, enzymatic hydrolysis and fermentation using an unique microorganism properly engineered to perform all these steps in a unique reactor. There are two main routes to perform CBP: category I CBP is based on the use of a cellulase producing microorganism, mainly fungi like *Trichoderma, Aspergillus*, *Rhizopus* and *Fusarium* engineered to ferment sugars with high titers of conversion, while category II CBP uses ethanologenic microorganisms, both yeasts (*Trichoderma, Aspergillus*, *Rhizopus* and *Fusarium*) and bacteria (*Escherichia coli* and *Zymomonas mobilis*) engineered to be cellulolytic.
The following paper **"Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production" (Paper I)** reviews recent efforts aimed at developing category I CBP process, which involved genetic manipulation of fungi naturally able to produce huge amount of glycosyl hydrolytic enzymes.

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# Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production

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ABSTRACT

Consolidated BioProcessing (CBP) can provide an important contribution to reducing ethanol production costs and moving from cellulosic feedstock to fuel ethanol tanks. Several efforts have so far been focused mainly on CBP category II engineering an ethanologen yeast or bacterium to be cellulolytic, but the limited ability of the category II CBP system for producing enzymes for lignocellulose degradation remains a challenge. As an alternative, category I CBP, aimed at engineering a cellulase producer to be ethanologenic, could be pursued, but it is still in its infancy. Some cellulolytic thermophilic bacteria have been described as potential candidates for category I CBP. However, only fungi naturally produce the needed titers of cellulases required for the complete saccharification of pretreated lignocellulose. In this review, potential of cellulolytic fungi as candidates for category I CBP is discussed.



**Contents**



*Abbreviations:* aCGH, array comparative genomic hybridization; ADH, alchol dehydrogenase; ARS, autonomous replicating sequence; ATMT, *Agrobacterium tumefaciens*-Mediated Transformation; AXE, acetyl xylan esterase; BG, Brewer's spent grain; CAE, p-coumaroyl esterase; CAZy, Carbohydrate-Active EnZymes; CBD, carbohydrate binding domain; CBH, cellobiohydrolase; CBM, carbohydrate binding module; CBP, Consolidate BioProcessing; CC, corn cobs; CD, carbohydrate domain; CE, carbohydrate esterase; CMC, carboxymethylcellulose; EG, endoglucanase; EMP, Embden-Meyerhof-Parnas; F6P, fructose-6-phosphate; FAE, feruloyl esterase; FDD, fungal dockerin domain; FPA, filter paper activity; G3P, glyceraldehyde-3-phosphate; GABA, gamma-aminobutyric acid; GH, glycoside hydrolase; *gpd*, glyceraldeide-3-phosphate dehydrogenase; GRAS, gGenerally Recognized as Safe; GT, glycosyl transferase; HSVtk, herpes simplex virus thymidie kinase; Lic, lichenase; MAN, mannanase; NR, nitrate reductase; NTG, N-nitrosoguanidine; Pdc, pyruvate decarboxylase; PEG, polyethylene glycol; PL, polysaccharide lyase; PPP, pentose phosphate pathway; PWS, pre-treated wheat straw; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, tricarboxylic acid; URS, upstream regulatory sequence; XYN, xylanase.

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## 1. Introduction

The extensive exploitation of fossil sources has been causing increasing concern both on security of their supply and alarm over greenhouse gas emission and global warming. Bioethanol has been recognized as a potential alternative to petroleum-derived transportation fuels, with several advantages, such as high octane number, low cetane number and high heat of vaporization [1]. However, the reduction of bioethanol cost needed for fossil fuel substitution mainly depends on the purchase price of feedstock and the cost of feedstock processing. As pointed out by Lynd et al. [2], even if cellulosic biomass is less expensive than corn and sugarcane, currently being the main bioethanol sources, the higher costs for its conversion make the near-term price of cellulosic ethanol higher than that of corn ethanol and even more than that of sugarcane ethanol. As a matter of fact, cellulosic bioethanol is not produced at competitive level yet, due to the high cost of processing. Nevertheless, in the long term, incorporating advanced technological improvements [3], the projected selling price of cellulosic ethanol is estimated less than the purchase cost of the other feedstocks considered [2]. Cellulosic biomass is the most promising feedstock for large scale-fuel production in the long term among the feedstock types reported in Table 1, because of its potential in low fuel-production price, large-scale production, and environmental benefits. Not less importantly, identification of lignocellulosic wastes as raw materials for effective large-scale bioethanol production remains an urgent priority in several countries [4].

Among the main routes to advance cellulosic ethanol [3], CBP [5] holds tremendous potential to reduce ethanol production costs. There are two main pathways to produce CBP strains: category I CBP aims at engineering a cellulase producer to make it ethanologenic, while category II CBP intends to engineer an ethanologen to be cellulolytic. Several efforts and studies have so far been focused mainly on CBP category II [6–8]. The primary requirements for the category II CBP strategy include functional production and secretion of a variety of exoglucanases and endoglucanases, growth on lignocellulose as sole carbon resource, and assimilation and fermentation of all sugars derived from lignocellulose. The bacteria *Zymomonas mobilis*, *Escherichia coli* and *Klebsiella oxytoca*, as well as the yeasts *Saccharomyces cerevisiae*, *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehatae* have been modified as candidates for category II CBP [6,7]. The limited ability of the category II CBP system to produce enzymes in sufficient quantity and quality for lignocellulose degradation remains a challenge. It was initially expected that this problem could be overcome by introduction of heterologous genes for various cellulolytic enzymes. However, there has been limited progress in producing some of these enzymes in active form and in sufficient quantities.

As an alternative, category I CBP can be pursued. The cellulolytic thermophilic bacteria *Geobacillus thermoglucosidasius*, *Thermoanerobacteriumsaccharolyticum* and *Thermoanerobacter mathranii* have been described as potential candidates for category I CBP [9]. However, only fungi naturally produce the needed titers of cellulases required for the complete saccharification of pretreated lignocellulose (30–50 mg enzyme per g of crystalline cellulose). The mesophilic fungus *Trichoderma reesei* is reported to be able to produce more than 100 g of cellulases per liter of culture broth [10], while the most productive cellulolytic bacteria produce only a few grams per liter. The ability to produce and secrete enzymatic complexes, such as the CBH I from *T. reesei*, requires a robust secretion system. Bacteria not having such systems are difficult or not viable to be engineered to produce cellulolytic enzymes in sufficient quantities for the biorefinery.

Some filamentous fungi belonging to the genera *Neurospora*, *Aspergillus*, *Trichoderma*, *Monilia*, *Rhizopus*, *Paecilomyces* and *Fusarium* have been reported to hold the ability to directly ferment cellulose to ethanol [11–13]. This conversion ability is thought to depend on two metabolic routes: one route involves production of cellulases to degrade cellulose to soluble sugars under aerobic conditions; the other produces ethanol and other byproducts, such as acetic acid, under anaerobic conditions. It was reported in 2002 that an organism isolated from cow dung suggested to be *T. harzianum* was capable of producing ethanol from cellulose [13].

In this review, the potential of fungi as I category CBP organisms is analyzed. The ability of these microorganims to produce cellulose degrading enzymes, availability of tools for their genetic manipulation and the state of art of their application to direct conversion of cellulose into ethanol together with the main bottlenecks and perspectives are described.

## 2. *T. reesei* as CBP organism

### 2.1. Cellulase production by T. reesei

Extensive research has been carried out to improve efficiency of cellulase cocktail from *T. reesei*, previously named *T. viride* QM6a, in order to decrease production costs of cellulosic bioethanol [14]. The cellulolytic machinery of *T. reesei* is one of the most widely studied and it includes seven cellulases and two β-glucosidases so far characterized [15], while *T. reesei* genome (http://genome.jgi-psf.org/Trire2/Trire2.home.html) contains ten cellulase and sixteen hemicellulase genes [16]. The components of the *T. reesei* cellulolytic system nowadays identified are reported in Table 2, according to CAZy (classification system of carbohydrate active enzymes) annotation [17].

Random mutagenesis of *T. reesei* has been performed to enhance its cellulase production and the cellulase hyperproducer strains QM9123, QM9414, CL847, NG14 and Rut-C30, all originating from the natural isolate QM6a and secreting up to 100 g/l of cellulases, have been selected and widely characterized.

Several efforts have been focused on elucidation of the genetic events responsible for cellulase production improvement in *T. reesei* mutant strains. Seidl et al. [18] showed that Rut-C30 lacks an 85 kb genomic fragment including 29 genes encoding transcription factors, enzymes of the primary metabolism and transport proteins. This loss is already present in the ancestor of Rut-C30 – NG14 –, and is not linked to the *cre1* locus. On the other hand, the deletion of the

**Table 1**
Comparison of feedstock categories with respect to several social objectives. Ratings: (•••••) excellent; (••••) very good; (•••) good; (••) fair; (•) poor.

| | (A) Large-scale production | | (B) Rural economic development | | (C) Petroleum displacement | | (D) Fossil fuel displacement/GHG reduction | | (E) Soil fertility and agricultural ecology | (F) Low-cost fuels (feedstock and conversion) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Feedstock type | Per unit | Total | Now | Future | Per unit | Total | Per unit | Total | | Now | Future |
| Cellulosic | •••• | •••• | •• | ••••• | ••••• | ••••• | ••••• | ••••• | ••••• | •• | ••••• |
| Starch-rich | •••• | •• | •••• | ••• | ••••• | ••• | ••• | •• | •• | ••• | ••• |
| Sugar-rich | ••• | •• | •••• | ••• | ••••• | ••• | ••••• | •••• | •••• | ••• | ••• |

Adapted from Lynd et al. [3] with the author's permission.

**Table 2**
Components of the *T. reesei* cellulolytic system.

| Enzyme | Family | No. residues | Size (Da) | Special domain | Accession number |
|---|---|---|---|---|---|
| Abf1 | GH54 | 500 | 51,115 | CBM42 | Q92455 |
| Aes1 | CE16 | 348 | 39,158 | – | A7J2C6 |
| Agl2 | GH36 | 746 | 82,079 | – | Q92457 |
| Glr1 | GH67 | 847 | 93,424 | – | Q99024 |
| Dpm1 | GT2 | 243 | 26,995 | – | Q9HGE2 |
| Pmt1 | GT39 | 773 | 88,706 | – | Q870E9 |
| Axe1 | – | 302 | 30,754 | CBM1 | Q99034 |
| Man5A | GH5 | 437 | 47,053 | CBM1 | Q99036 |
| Cel61A | GH61 | 344 | 35,511 | CBM1 | O14405 |
| Swo1 | – | 493 | 51,524 | CBM1 | Q9P8D0 |
| Bxl1 | GH3 | 797 | 87,191 | – | Q92458 |
| Cbh2 | GH6 | 471 | 49,641 | CBD | D3YNY1 |
| Cel1b | GH1 | 484 | 55,064 | – | Q7Z9M2 |
| Cel1A | GH1 | 466 | 52,241 | – | O93785 |
| Xyn2 | GH11 | 196 | 21,525 | – | B2CZF9 |
| Cel12a | GH12 | 234 | 25,159 | | O00095 |
| Agl3 | GH27 | 444 | 48,516 | – | Q92456 |
| Bga1 | GH35 | 1023 | 111,369 | – | Q70SY0 |
| Cel45A | GH45 | 242 | 24,411 | CBM1 | P43317 |
| Mds1 | GH47 | 523 | 56,206 | – | Q9P8T8 |
| Cel61b | GH61 | 249 | 26,828 | – | Q7Z9M7 |
| Abf2 | GH62 | 322 | 34,777 | – | Q7Z9N0 |
| Glr1 | GH67 | 847 | 93,424 | – | Q99024 |
| Cel74A | GH74 | 838 | 87,133 | CBM1 | Q7Z9M8 |
| Cel3B | GH3 | 874 | 93,948 | – | Q7Z9M5 |
| Cel3C | GH3 | 833 | 90,718 | – | Q7Z9M4 |
| Cel3D | GH3 | 700 | 77,090 | – | Q7Z9M1 |
| Cel3E | GH3 | 765 | 83,004 | – | Q7Z9M0 |
| Cel3A | GH3 | 744 | 78,433 | – | Q12715 |
| Cel5A | GH5 | 418 | 44,227 | CBM1 | P07982 |
| Cel5B | GH5 | 438 | 46,855 | – | Q7Z9M6 |
| Cel7B | GH7 | 459 | 48,208 | CBM1 | P07981 |
| Cel7A | GH7 | 513 | 54,073 | CBM1 | P62694 |

*cre1* locus, mediating glucose repression, and a frameshift mutation in the glucosidase II alpha subunit gene are specific of Rut-C30. Le Crom et al. [19] discovered that in Rut-C30, in addition to the 29 genes deleted during the generation of NG14, the truncation of *cre1* gene and the frameshift in glucosidase II, nearly 45% of the genes mutated encode transcription factors, components of nuclear import, mRNA metabolism, protein secretion, and vacuolar sorting. The knowledge of mutations in the hyperproducer *T. reesei* strains was widened by Vitikainen et al. [20], reporting an aCGH analysis of the high-producing strains QM9123, QM9414, NG14 and Rut-C30. These authors showed that the 85 kb deletion is not responsible for the high ability of cellulase producing in Rut-C30.

Elucidation of regulatory mechanisms at the transcription [21] and the signal transduction levels [22] have contributed to enhance the efficiency of the cellulolytic system from *T. reesei*. Metabolic engineering allowed advancing knowledge on these processes and provided relevant tools for improvement of cellulase production [23]. Other approaches, such as enzyme engineering [24] and directed evolution [25] also contributed to the optimization of cellulase production.

Hence, *T. reesei* represents a fundamental tool to make economically feasible production of second generation bioethanol.

### 2.2. Tools for genetic manipulation of T. reesei

A wide range of genetic tools have been developed for *T. reesei*. Different transformation strategies such as protoplasting based transformation [26], ATMT [27] and biolistic transformation [28] were shown successful for *T. reesei*. Different selection markers, such as hygromycin [29] and benomyl [30,31] resistance, the *Aspergillus nidulans amdS* gene, conferring ability to grow on acetamide as sole nitrogen source [32] and the auxotrophic markers *pyr4* [26] and *hxk1* [33] are available allowing construction of multiple mutants, that takes advantage of a *T. reesei* strain with non-homologous endjoining pathway [34]. Moreover, a sexual cycle was recently discovered in *T. reesei* [35] further increasing its industrial potential.

### 2.3. Potential of T. reesei as I category CBP organism

Potential of *T. reesei* as I category CBP organism was analyzed by Xu et al. [36]. These authors reported that *T. reesei* can produce ethanol from cellulose, through cellulase production and cellulose degradation during the initial aerobic growth phase, followed by fermentation of the resulting sugars to ethanol

when anaerobic growth conditions are established. They also reported that *T. reesei* is able to survive under anaerobic conditions up to 13 days. These observations are consistent with the fact that all the genes necessary for conversion of cellulosic sugars into ethanol are present in *T. reesei* genome (http://genome.jgi-psf.org/Trire2/Trire2.home.html).

*T. reesei* shows several advantages as CBP organism, including (i) the availability of cellulase hyperproducer strains showing known mutagenic events and genomic sequences [18–20]; (ii) the extensive knowledge of its physiology, its cellulolytic machinery and mechanisms involved in regulation of cellulase production [22,23,37]; (iii) the availability of a wide range of tools for its genetic manipulation [10,16]; (iv) the presence of the metabolic pathways needed to utilize all the lignocellulose sugars for production of ethanol.

However, some bottlenecks remain to be overcome to improve the feasibility of developing a category I CBP organism based on *T. reesei*. First of all, the ethanol yield and productivity are low [36], due to the low level of expression of the relevant genes involved in ethanol fermentation or to the low activity of the enzymes encoded by these genes. The low ethanol tolerance of *T. reesei* represents another main drawback to be overcome: ethanol stops glycolysis, thus inhibiting cell growth. Moreover, the obligate aerobe nature of *T. reesei*, mainly due to the fact that the genes encoding enzymes crucial for glycolysis are repressed in the absence of oxygen [38], hinders its growth without oxygen.

Hence, development of a *T. reesei* strain able to grow in the absence of oxygen and improvement of its ethanol production and tolerance are the main challenges to be faced to develop an ethalonogenic *T. reesei* strain. Improvement of cellulase hyperproducer *T. reesei* strains for their ability to grow in the absence of oxygen can be addressed by traditional methods of random mutagenesis, screening for the strains able to grow in the absence of oxygen, selection of the strain growing in the absence of oxygen for the longest times. This strain could be then engineered by introducing heterologous genes, such as *S. cerevisiae* PDC and ADH, to enhance its ethanol production. Ethanol tolerance of this strain could be finally improved, by further engineering it with modified versions of the genes responsible for ethanol tolerance, following elucidation of metabolic pathways involved in ethanol production and tolerance and identification of genes responsible for ethanol tolerance, achieved through differential proteomic and transcriptomic analyses in the presence and in the absence of growing ethanol concentrations.

## 3. *Aspergillus spp.* as CBP organisms

### 3.1. Cellulase production by Aspergillus spp.

Several manuscripts have so far reported the potential of *Aspergillus spp.* in (hemi)cellulases production at industrial scale. *Aspergillus* species are the major agents of (hemi)cellulose decomposition and thus possess the capability to produce a broad range of (hemi)cellulolytic enzymes [39]. A number of studies on *Aspergilli*, mainly *A. nidulans*, *A. niger* and *A. oryzae*, have identified enzymatic activities such as cellulases, xylanases, hemicellulases and pectinases, acting against a range of polymers found in the plant cell wall. These embrace both enzymes cleaving the polymer backbone and accessory proteins acting on side chains or cleavage products. Genomes of *A. nidulans*, *A. niger* and *A. oryzae* (http://www.aspgd.org/) contain genes encoding a wide range of carbohydrate active enzymes (Table 3).

Carbohydrate active enzymes produced by *A. terreus* and *A. fumigatus* are reported in Table 4. *A. terreus* cellulase system has been largely characterized and it was shown that the fungus has the ability to produce cellulase growing in both liquid and solid state systems [40,41]. *A. fumigatus* secretes several glycosidases [42,43]. There have been relatively few studies in which extracellular hydrolases from *A. fumigatus* have been characterized, even if a huge number of reports have demonstrated that environmental isolates of *A. fumigatus* are highly cellulolytic. It appears that *A. fumigatus* contains genes encoding more cellulose-binding domains (fungal type) than either *A. oryzae* or *A. nidulans*. There are several other glycosyl hydrolases without obvious CBDs as well as three putative cutinases lacking a CBD in *A. fumigatus* [44]. To increase cellulase production levels, mutagenesis experiments have also been carried out on *Aspergillus spp.* For instance, when the strain *Aspergillus* MAM-F23 was exposed to different doses of gamma irradiation, eleven mutants were produced among which one showed enhanced productivity in cellulolytic-revealed for FPA and hydrolysis of CMC and Avicel- and hemicellulolytic (xylanase, pectinase) activities [45].

### 3.2. Tools for genetic manipulation of Aspergillus spp.

Many efforts have been carried out to improve genetic manipulation of *Aspergilli*, due to their extensive use in several industrial sectors. A particular interest has been focused on the GRAS strains *A. niger* and *A. oryzae*, largely studied to adapt them as hosts for recombinant protein expression [46]. The most used method for transformation of *Aspergillus spp.* (mainly *A. niger*, *A. oryzae*, *A. sojae*, and *A. terreus*) is the chemical treatment of protoplasts produced by enzymatic digestion of the fungal cell wall [47]. Versatile selection systems including antibiotic resistance markers (*hph*, *ble*, *oliC3*), auxotrophic markers (*pyrG*, *pyrE*, *argB*, *adeA*, *adeB*, *niaD*, *trpC*, *sC*) and nutritional markers (*amdS*, *ptrA*), in combination with integrative and autonomously replicating vectors, have been so far developed, offering nowadays high flexibility for genetic manipulation of industrial *Aspergillus* strains [46,48]. As recently reviewed by Meyer et al. [48], strong advancement in research on *Aspergillus* spp. transformation has been accomplished in the last decade, resulting in (i) efficient genetic transformation systems, (ii) high-throughput gene targeting tools, (iii) expression systems for high level and controlled protein production and (iv) live-imaging techniques for cell biological studies.

### 3.3. Potential of Aspergillus spp. as I category CBP organisms

Most of *Aspergilli* have been shown to be able to produce ethanol from glucose. *A. terreus* was shown to be able to ferment glucose, several other hexoses, pentoses and disaccharides to ethanol [49]. With D-glucose as soluble substrate, the ethanol production was maximum in 5 days, exhibiting a yield of 2.46% (w/v) ethanol (96.5% theoretical yield). D-fructose and D-mannose were fermented giving high ethanol yields of 2.16% (w/v) (85% conversion) in 5 days and 1.98% (w/v) (78% conversion) in 6 days, respectively. With D-maltose as carbon source, 1.5% (w/v) ethanol, corresponding to 59% theoretical yield, was produced in 6 days. Lower levels (0.98% (w/v)) were obtained with D-galactose, giving 38% conversion in 6 days. The pentose sugar, D-xylose was poorly fermented by *A. terreus*, yielding only 0.36% (w/v) (14% conversion) ethanol in 6 days. An ethanol yield of 0.51% (w/v) (50% conversion) was obtained with D-arabinose in 5 days, indicating that D-arabinose was fermented more efficiently than D-xylose. The fermentation of disaccharides, D-sucrose and D-cellobiose by *A. terreus* resulted in yields of 2.13% (w/v) ethanol in 6 days and 2.37% (w/v) ethanol (88% conversion) in 5 days, respectively.

The main limitations of a cellulose CBP based on *Aspergillus spp.* consist in the very low ethanol yields that these

microorganisms exhibit in comparison with *S. cerevisiae*, and formation of the by-product lactate. Masuo et al. [50] performed global gene expression analysis of *A. nidulans*, revealing that hypoxia causes changes in the expression of genes involved in the initial glycolytic and related pathways for catabolizing glucose as a carbon source. Particularly, they found that *A. nidulans* oxidizes glucose and produces ethanol under hypoxic conditions, but the microorganism produces also lactate in these conditions.

As far as *Aspergillus spp*. genetic engineering experiments aimed at improvement of ethanol production yield are concerned, very encouraging results were achieved with *A. nidulans* expressing the *Z. mobilis pdc* gene fused to the *A. nidulans gpd* promoter [12].

In order to control natural products such as ethanol, Roze et al. [51] have applied a volatile profiling analysis for gaining rapid access to information on intracellular metabolism in the fungus *A. parasiticus*. They showed that the global regulator *veA* affected the volatile profile, negatively regulating catabolism of branched chain amino acids and synthesis of ethanol at the transcriptional level. In fact, disruption of this factor by mutation of the studied strain, resulted in 3–4 fold higher levels of ethanol.

**Table 3**
Sequences annotated as CAZymes in *A. nidulans*, *A. niger*, and *A. oryzae* genomes (http://www.aspgd.org/).

| GH fam | No. Seq | GT fam | No. Sequ | PL fam | No. Seq | CE fam | No. Seq | CBM fam | No. Seq |
|---|---|---|---|---|---|---|---|---|---|
| ***Aspergillus nidulans* FGSC A4 annotated CAZymes** | | | | | | | | | |
| 1 | 3 | 1 | 9 | 1 | 8 | 1 | 3 | 1 | 7 |
| 2 | 10 | 2 | 12 | 3 | 5 | 3 | 6 | 14 | 1 |
| 3 | 20 | 3 | 1 | 4 | 4 | 4 | 8 | 18 | 17 |
| 5 | 15 | 4 | 7 | 9 | 1 | 5 | 4 | 20 | 4 |
| 6 | 2 | 5 | 2 | 11 | 1 | 8 | 3 | 21 | 1 |
| 7 | 3 | 8 | 5 | 20 | 2 | 9 | 1 | 24 | 2 |
| 10 | 3 | 15 | 3 | | | 12 | 2 | 35 | 2 |
| 11 | 2 | 20 | 3 | | | 16 | 3 | 42 | 1 |
| 12 | 1 | 21 | 1 | | | NC | 3 | 43 | 3 |
| 13 | 13 | 22 | 4 | | | | | 48 | 1 |
| 15 | 2 | 24 | 1 | | | | | 50 | 2 |
| 16 | 13 | 25 | 4 | | | | | 63 | 1 |
| 17 | 5 | 31 | 5 | | | | | | |
| 18 | 19 | 32 | 7 | | | | | | |
| 20 | 2 | 33 | 1 | | | | | | |
| 24 | 1 | 34 | 3 | | | | | | |
| 25 | 3 | 35 | 1 | | | | | | |
| 26 | 3 | 39 | 3 | | | | | | |
| 27 | 3 | 41 | 1 | | | | | | |
| 28 | 9 | 48 | 1 | | | | | | |
| 31 | 10 | 50 | 1 | | | | | | |
| 32 | 2 | 57 | 2 | | | | | | |
| 35 | 4 | 58 | 1 | | | | | | |
| 36 | 4 | 59 | 1 | | | | | | |
| 37 | 1 | 62 | 3 | | | | | | |
| 38 | 1 | 66 | 1 | | | | | | |
| 39 | 1 | 69 | 3 | | | | | | |
| 43 | 15 | 71 | 2 | | | | | | |
| 45 | 1 | 90 | 2 | | | | | | |
| 47 | 7 | | | | | | | | |
| 51 | 2 | | | | | | | | |
| 53 | 1 | | | | | | | | |
| 54 | 1 | | | | | | | | |
| 55 | 6 | | | | | | | | |
| 61 | 9 | | | | | | | | |
| 62 | 2 | | | | | | | | |
| 63 | 1 | | | | | | | | |
| 65 | 1 | | | | | | | | |
| 67 | 1 | | | | | | | | |
| 71 | 5 | | | | | | | | |
| 72 | 5 | | | | | | | | |
| 74 | 2 | | | | | | | | |
| 75 | 2 | | | | | | | | |
| 76 | 7 | | | | | | | | |
| 78 | 8 | | | | | | | | |
| 79 | 1 | | | | | | | | |
| 81 | 1 | | | | | | | | |
| 88 | 2 | | | | | | | | |
| 92 | 5 | | | | | | | | |
| 93 | 2 | | | | | | | | |
| 95 | 3 | | | | | | | | |
| 105 | 3 | | | | | | | | |
| 114 | 2 | | | | | | | | |
| 115 | 1 | | | | | | | | |
| 125 | 1 | | | | | | | | |
| NC | 4 | | | | | | | | |

Table 3 (*Continued*)

| GH fam | No. Seq | GT fam | No. Sequ | PL fam | No. Seq | CE fam | No. Seq | CBM fam | No. Seq |
|---|---|---|---|---|---|---|---|---|---|
| ***Aspergillus niger* CBS 513.88 annotated CAZymes** | | | | | | | | | |
| 1 | 3 | 1 | 12 | 1 | 6 | 1 | 3 | 1 | 8 |
| 2 | 6 | 2 | 18 | 4 | 2 | 3 | 1 | 13 | 1 |
| 3 | 17 | 3 | 1 | | | 4 | 6 | 14 | 1 |
| 5 | 10 | 4 | 8 | | | 5 | 5 | 18 | 13 |
| 6 | 2 | 5 | 5 | | | 8 | 3 | 20 | 1 |
| 7 | 2 | 8 | 5 | | | 9 | 1 | 21 | 1 |
| 10 | 1 | 15 | 3 | | | 12 | 2 | 24 | 6 |
| 11 | 4 | 20 | 6 | | | 16 | 2 | 35 | 2 |
| 12 | 4 | 21 | 1 | | | NC | 2 | 42 | 1 |
| 13 | 18 | 22 | 3 | | | | | 43 | 4 |
| 15 | 2 | 24 | 1 | | | | | 48 | 3 |
| 16 | 13 | 25 | 5 | | | | | 50 | 2 |
| 17 | 5 | 31 | 6 | | | | | 63 | 1 |
| 18 | 14 | 32 | 11 | | | | | | |
| 20 | 3 | 33 | 1 | | | | | | |
| 26 | 1 | 34 | 3 | | | | | | |
| 27 | 5 | 35 | 1 | | | | | | |
| 28 | 21 | 39 | 3 | | | | | | |
| 29 | 1 | 41 | 1 | | | | | | |
| 30 | 1 | 48 | 1 | | | | | | |
| 31 | 7 | 50 | 1 | | | | | | |
| 32 | 6 | 57 | 2 | | | | | | |
| 33 | 1 | 58 | 1 | | | | | | |
| 35 | 5 | 59 | 1 | | | | | | |
| 36 | 2 | 62 | 3 | | | | | | |
| 37 | 1 | 66 | 1 | | | | | | |
| 38 | 1 | 69 | 6 | | | | | | |
| 43 | 10 | 71 | 4 | | | | | | |
| 47 | 5 | 76 | 1 | | | | | | |
| 51 | 4 | 90 | 3 | | | | | | |
| 53 | 2 | NC | 4 | | | | | | |
| 54 | 1 | | | | | | | | |
| 55 | 3 | | | | | | | | |
| 61 | 7 | | | | | | | | |
| 62 | 1 | | | | | | | | |
| 63 | 1 | | | | | | | | |
| 65 | 1 | | | | | | | | |
| 67 | 1 | | | | | | | | |
| 71 | 7 | | | | | | | | |
| 72 | 7 | | | | | | | | |
| 74 | 1 | | | | | | | | |
| 75 | 2 | | | | | | | | |
| 76 | 11 | | | | | | | | |
| 78 | 8 | | | | | | | | |
| 79 | 3 | | | | | | | | |
| 81 | 1 | | | | | | | | |
| 88 | 1 | | | | | | | | |
| 92 | 5 | | | | | | | | |
| 95 | 2 | | | | | | | | |
| 105 | 2 | | | | | | | | |
| 114 | 2 | | | | | | | | |
| 125 | 1 | | | | | | | | |
| NC | 7 | | | | | | | | |

## 4. *Fusarium spp.* as CBP organism

### 4.1. Cellulase production by Fusarium spp.

*F. oxysporum* displays strong cellulose degradation ability and it has been intensively studied for its cellulolytic system (Table 5), particularly in connection with its phytopathological role. Many manuscripts report that *Fusaria* are potential strains for cellulase production at industrial scale, since, differently from the commonly used sources of cellulases, *Aspergillus* and *Trichiderma* spp., *Fusaria* produce cellulases functioning in a broad range of temperature and pH. Research is recently focusing on characterization of new *Fusaria* strains isolated from soil or directly from infected vegetables (e.g. infected tomato) in order to optimize cellulase production [52]. Several soil borne *Fusaria* species, such as *F. tabacinum*, *F. solani*, *F. sporotrichoides*, *F. moniliform*, *F. camptocoras* and *F. oxysporum*, were recently screened for cellulase activity production and both liquid and submerged fermentations have been reported as suitable systems for high level cellulase yield by *Fusaria*, using CMC and wheat straw as substrates, respectively [53]. Solid state fermentation is a good system for high yield cellulase production by *Fusarium spp.*, as demonstrated by Qin et al. [54] who studied a novel thermostable cellulase producing fungus, *F. chlamydosporum* HML 0278, shown to produce the three major cellulase components in the presence of sugar cane bagasse and wheat bran as carbon source. These cellulolytic enzymes exhibit great stability within the pH range 4–10 and at temperatures below 70 °C, promoting their potential use in industrial bioconversion. Different isolates of *F. graminearum* were shown able to produce *in vitro* several cellulolytic and hemicellulolytic enzymes, whose roles in degradation of the main components of plant cell wall have been demonstrated [55].

Table 3 (*Continued*)

| GH fam | No. Seq | GT fam | No. Sequ | PL fam | No. Seq | CE fam | No. Seq | CBM fam | No. Seq |
|---|---|---|---|---|---|---|---|---|---|
| ***Aspergillus oryzae* RIB40 annotated CAZymes** | | | | | | | | | |
| 1 | 3 | 1 | 9 | 1 | 12 | 1 | 5 | 1 | 3 |
| 2 | 7 | 2 | 18 | 3 | 3 | 3 | 3 | 13 | 1 |
| 3 | 23 | 3 | 1 | 4 | 4 | 4 | 4 | 14 | 1 |
| 5 | 14 | 4 | 9 | 7 | 1 | 5 | 5 | 18 | 5 |
| 6 | 1 | 5 | 3 | 9 | 1 | 8 | 5 | 19 | 1 |
| 7 | 3 | 8 | 4 | 20 | 2 | 9 | 1 | 20 | 1 |
| 10 | 4 | 15 | 3 | | | 12 | 4 | 21 | 1 |
| 11 | 4 | 20 | 6 | | | 16 | 3 | 24 | 6 |
| 12 | 4 | 21 | 1 | | | | | 32 | 2 |
| 13 | 17 | 22 | 4 | | | | | 35 | 1 |
| 15 | 3 | 24 | 1 | | | | | 42 | 1 |
| 16 | 13 | 25 | 5 | | | | | 43 | 5 |
| 17 | 5 | 31 | 11 | | | | | 48 | 2 |
| 18 | 18 | 32 | 8 | | | | | 50 | 3 |
| 20 | 3 | 33 | 1 | | | | | 63 | 1 |
| 24 | 2 | 34 | 2 | | | | | NC | 1 |
| 25 | 1 | 35 | 1 | | | | | | |
| 26 | 1 | 39 | 3 | | | | | | |
| 27 | 3 | 41 | 1 | | | | | | |
| 28 | 21 | 48 | 1 | | | | | | |
| 31 | 10 | 50 | 1 | | | | | | |
| 32 | 4 | 57 | 2 | | | | | | |
| 33 | 1 | 58 | 1 | | | | | | |
| 35 | 7 | 59 | 1 | | | | | | |
| 36 | 3 | 62 | 3 | | | | | | |
| 37 | 1 | 66 | 1 | | | | | | |
| 38 | 1 | 69 | 4 | | | | | | |
| 43 | 20 | 71 | 5 | | | | | | |
| 47 | 5 | 76 | 2 | | | | | | |
| 51 | 3 | 90 | 4 | | | | | | |
| 53 | 1 | NC | 3 | | | | | | |
| 54 | 1 | | | | | | | | |
| 55 | 4 | | | | | | | | |
| 61 | 8 | | | | | | | | |
| 62 | 2 | | | | | | | | |
| 63 | 1 | | | | | | | | |
| 65 | 1 | | | | | | | | |
| 67 | 1 | | | | | | | | |
| 71 | 8 | | | | | | | | |
| 72 | 7 | | | | | | | | |
| 75 | 4 | | | | | | | | |
| 76 | 11 | | | | | | | | |
| 78 | 9 | | | | | | | | |
| 79 | 6 | | | | | | | | |
| 81 | 1 | | | | | | | | |
| 88 | 3 | | | | | | | | |
| 89 | 1 | | | | | | | | |
| 92 | 6 | | | | | | | | |
| 93 | 3 | | | | | | | | |
| 95 | 3 | | | | | | | | |
| 105 | 4 | | | | | | | | |
| 114 | 1 | | | | | | | | |
| 115 | 4 | | | | | | | | |
| 125 | 1 | | | | | | | | |
| NC | 9 | | | | | | | | |

Xiros et al. [56] have investigated the effect of several factors (e.g. enzyme loading, initial substrate concentration, temperature, pH, surfactant addition and catabolite repression) on the hydrolysis reaction by the crude enzyme extract from *F. oxysporum*. Its cellulolytic system, produced under submerged fermentation, was proven capable of efficiently hydrolysing hydrothermally pretreated wheat straw and it was demonstrated that hydrolysis rate was enhanced by removal of glucose which was so confirmed to be a catabolite repressor for *Fusarium sp*. cellulases. More recently, factors affecting cellulose and hemicellulose hydrolysis of alkali treated brewers spent grain by *F. oxysporum* enzyme extract were also evaluated [57]. Satisfactory cellulose conversion could be achieved by increasing the enzyme dosage in order to overcome the end-product inhibition, while the complexity of hemicellulose structure imposes the presence of specific enzyme activities in the enzyme mixture.

### 4.2. Tools for genetic manipulation of Fusarium spp.

Most of studies on genetic manipulation of *Fusarium* spp. have been so far carried out for *F. oxysporum* related to its phytopathological action. The first homologous transformation system of *F. oxysporum* was developed by Diolez et al. [58], based on cloning of the *F. oxysporum* gene *nia*, encoding NR. Transformation frequencies up to 600 transformants per μg of DNA were achieved, and events of gene replacements, single-copy homologous integrations and integrations at non-homologous sites were observed. Gene replacement events were observed at high frequency (in about 50% of the transformants), offering new applications for the transformation system in *F. oxysporum*.

A highly efficient transformation system, including protoplasting and PEG-mediated DNA transformation of protoplasts, was reported for *F. oxysporum* by Gareia-Pedrajas and Roncero [59],

**Table 4**
Components of the *A. terreus* and *A. fumigatusAf293* cellulolytic systems.

| Enzyme | Family | No. residues | Size (Da) | Special domain | Accession number |
|---|---|---|---|---|---|
| ***Aspergillus terreus*** | | | | | |
| Endo-β-1,4-glucanase I | GH7 | 471 | 49,328 | CBM1 | E5Q901 |
| Cellobiohydrolase | GH7 | 540 | – | CBM1 | AAW68437 |
| Endoglucanase | – | 404 | – | CBM1 | AAW68436 |
| Gla1 | GH15 | – | – | CBM20 | – |
| BglA | GH3 | 861 | 93,333 | – | BAE65953 |
| Endoglucanase | GH5 | 404 | 43,026 | | Q5G1M1 |
| XlnC | GH10 | 326 | 35,346 | | Q4JHP5 |
| α-L-arabinofuranosidase A (peptide fragment) | GH54 | – | – | – | – |
| α-L-arabinofuranosidase B1/B2 (peptide fragment) | GH54 | – | – | – | – |
| Orf18 (probable fragment) | GH115 | – | – | – | Q9Y7E2 |
| ***Aspergillus fumigatus*Af293** | | | | | |
| AglA | – | 532 | – | CBM13 | EAL91233 |
| AfuA | GH3 | 769 | – | – | EAL91070 |
| AfuA | GH3 | 873 | – | | EAL88289 |
| ManF (Subsp. IMI 385708) | GH5 | 438 | 47,327 | – | Q4WBS1 |
| XlnC | GH10 | 325 | 35,221 | – | Q0H904 |
| AGS3 | GH13 | – | – | – | EAL90874 |
| AFUA | GH16 | – | – | – | ACQ08899 |
| AglA | GH27 | – | – | – | EAL91233 |
| Afu8g06890 | GH28 | – | – | – | EAL85450 |
| Neg1 | GH30 | – | – | – | XP747510 |
| KDNase | GH33 | – | – | – | EAL89414 |
| LacA | Gh35 | 996 | 109,266 | – | Q6MY72 |
| AfAms1 (Subsp. YJ-407) | GH38 | 1088 | 123,748 | – | Q5I5K3 |
| AFUA_6G00770 | GH43 | – | – | – | EAL84189 |
| Man70 (Subsp. YJ-407) | GH47 | 503 | 55,446 | | Q6PWQ1 |
| Mannosidase I | GH47 | 591 | 67,318 | – | Q5I5K2 |
| ExgO | GH55 | 947 | 100,622 | – | Q6MY43 |
| AfA5C5.025 | GH61 | 373 | 38,383 | – | Q6MYM8 |
| Cwh41 (Subsp. YJ-407) | GH63 | 822 | 93,129 | – | Q6S9W4 |
| Gel1 | GH72 | – | – | – | – |
| Gel2 | GH72 | – | – | – | – |
| Gel3 | GH72 | – | – | – | – |
| Gel4 | GH72 | – | – | – | – |
| Gel5 | GH72 | – | – | – | – |
| Gel6 | GH72 | – | – | – | – |
| Gel7 | GH72 | – | – | – | – |
| Csn | GH75 | – | – | – | EAL84291 |
| Engl1 | GH81 | 727 | 78,928 | – | Q9UVV0 |

based on the complementation of a NR mutant with the homologous *nitI* gene and on the presence of ARS and telomeric sequences in the vector.

Mullins et al. [60] described application of ATMT to *F. oxysporum* through the construction of novel binary vectors, bringing the bacterial hygromycin B phosphotransferase gene (hph) under the control of the *Aspergillus nidulans* trpC promoter as a selectable marker, and selection of the conditions of ATMT affecting the transformation efficiency and the copy number of inserted T-DNA in *F. oxysporum*.

Khang et al. [61] reported a method of gene replacement in *F. oxysporum*, based on ATMT with a mutant allele of the target gene flanked by the *HSVtk* gene as a conditional negative selection marker against ectopic transformants. The *HSVtk* gene product converts 5-fluoro-2′-deoxyuridine to a compound toxic to diverse fungi. Because ectopic transformants express *HSVtk*, while gene replacement mutants lack *HSVtk*, growing transformants on a medium amended with 5-fluoro-2′-deoxyuridine facilitates the identification of targeted mutants by counter-selecting against ectopic transformants.

### 4.3. Potential of F. oxysporum as I category CBP organism

More than twenty years ago it was reported for the first time that *F. oxysporum* is able to directly convert biomass to ethanol [62,63]. *F. oxysporum* produces a broad range of cellulases and xylanases [64–68]. Therefore, it is not necessary to perform a separate enzymatic hydrolysis of the lignocellulosic raw material if this fungus is used for bioethanol production.

Panagiotou et al. [69] have examined the growth, substrate consumption, product and by-product formation of *F. oxysporum* in a minimal glucose-based medium in aerobic, anaerobic and oxygen-limited batch cultivations. Under anaerobic conditions, they found the highest ethanol yield (1.66 mol ethanol/mol of glucose corresponding to 80% of the theoretical maximum yield), due to a high glycolytic flux, characterized by high efflux of G3P and F6P from the PPP to the EMP pathway. The TCA cycle was primarily active under aerobic cultivation, while the presence of high levels of GABA under anaerobic conditions suggested a functional GABA bypass and a possible block in the TCA cycle in these conditions.

Xiros and Christakopoulos [70] evaluated the potential *F. oxysporum* for consolidated bioconversion of BG and ethanol production. An ethanol yield of 109 g ethanol per kg of dry BG was obtained with alkali-pretreated BG under microaerobic conditions (0.01 vvm), corresponding to 60% of the theoretical yield based on total glucose and xylose content of BG. Comparing fermentations carried out by using sugar mixtures simulating BG's carbohydrates content, alkali-pretreated and untreated BG, it was shown that BG hydrolysis is the bottleneck of the bioconversion process.

The most important bottlenecks of using *F. oxysporum* in I category CBP of lignocellulose are the slow conversion of cellulose and formation of significant amounts of acetic acid as a by-product.

**Table 5**
Components of the *F. oxysporum* cellulolytic system.

| Enzyme | Family | No. residues | Size (Da) | Special domain | Accession number |
|---|---|---|---|---|---|
| Cell7A | GH7 | 514 | 54,704 | CBM1 | P46238 |
| CelF | GH10 | 385 | 41,225 | CMBM1 | P46239 |
| Endoglucanase B | GH6 | 462 | 49,208 | CBM1 | P46236 |
| Putative endoglucanase type K | GH45 | 376 | 39,236 | CBM1 | P45699 |
| Xyl3 | GH10 | 384 | 41,217 | CBM1 | O59937 |
| FoAf1 | GH43 | 449 | 49,441 | CBM35 | B6F260 |
| Foap2 | GH27 | 540 | 59,459 | CMB35 | B7XH21 |
| Foap1 | GH27 | 549 | 58,429 | CMB35 | B7XH22 |
| Foaf2 | GH54 | 499 | 51,659 | CMB42 | B6F261 |
| AbfB (subsp. dianthi) | GH54 | 499 | 51,647 | CMB42 | Q86ZN9 |
| Gas1 | GH72 | 539 | 58,286 | CBM43 | Q2KN79 |
| Lip1 | CE5 | 230 | 23,372 | – | A6N6J6 |
| Fogal1 | GH5 | 420 | 47,283 | – | A0ZSY6 |
| Eg1 | GH7 | 429 | 46,445 | – | P46237 |
| FoTomIck | GH10 | 335 | 37,120 | – | Q8TGC1 |
| Tom1 (subsp. lycopersici) | GH10 | 335 | 37,138 | – | O93976 |
| Xyl2 (subsp. lycopersici 42–87) | GH10 | 328 | 35,781 | | O59938 |
| Xyl4 | GH11 | 231 | 25,638 | – | Q9C1R1 |
| Xyl5 | GH11 | 295 | 30,858 | – | Q9C1R2 |
| Fogp1 | GH27 | 409 | 44,795 | – | E2S091 |
| Pg1 (subsp. dianthi) | GH28 | 370 | 38,325 | – | Q14U03 |
| Pgx4 (subsp. dianthi) | GH28 | 464 | 51,671 | – | Q14TV4 |
| Pg5 (subsp. dianthi) | GH28 | 360 | 37,208 | – | Q14TR5 |
| Pgx1 (subsp. dianthi) | GH28 | 455 | 49,957 | – | Q14TM1 |
| Ara1 | GH93 | 379 | 41,411 | – | Q2A0P2 |
| Chs2 (subsp. lycopersici) | GT2 | 1041 | 117,488 | – | Q5YCX0 |
| Chs3 (subsp. lycopersici) | GT2 | 978 | 110,365 | – | Q5YCW9 |
| Chs1 (subsp. lycopersici) | GT2 | 901 | 102,271 | – | Q5YCX1 |
| ChsVb (subsp. lycopersici) | GT2 | 1780 | 198,287 | – | A7L5W4 |
| ChsV | GT2 | 1863 | 207,193 | – | Q873Z8 |
| Fks1 | GT48 | 1785 | 203,391 | – | A7U5Z3 |
| Pl1 | PL3 | 240 | 24,858 | – | C6KF40 |

In order to increase ethanol productivity by lignocellulose CBP with *F. oxysporum*, Anasontzis et al. [71] performed the homologous overexpression of an endo-xylanase, followed by screening of transformants for their high extracellular xylanase activities under normally repressing conditions (glucose as sole carbon source). When ethanol yield in CBP systems with CC or wheat bran as carbon source was evaluated for the transformants in comparison to the wild type, transformants were shown to produce approximately 60% more ethanol.

Efforts to increase ethanol production yield in *F. oxysporum* have been also carried out by expression of yeast transaldolase (*tal*) genes [72,73]. *F. oxysporum* strains carrying *tal* genes from *S. cerevisiae* [72] and *P. stipitis* [73],displayed an ethanol yield increase of 28.83% and 11.71% on xylose media compared to the parental strain, respectively.

## 5. *Rhizopus spp.* as CBP organisms

### 5.1. Cellulase production by Rhizopus spp.

To the best of our knowledge, relatively few studies have been so far reported on the production of cellulases by *Rhizopus spp.* and very few manuscripts reported purification and characterization of their cellulases. In 2002, Murashima et al. [74] have purified and characterized two new endoglucanases from *R. oryzae*, shown to have maximum activity in a pH range of around 5–6 and at temperature value of 55 °C. Similar results were reported by Takii et al. [75] for a β-glucosidase from the same specie. Carbohydrate active enzymes produced by *R. oryzae* and *R. stolonifer* are reported in Table 6. Search for carbohydrate active enzymes according to CAZy annotation in *R. oryzae* (genome

**Table 6**
Components of the *Rhizopus* cellulolytic system.

| Enzyme | Family | No. residues | Size Da | Special domain | Accession number |
|---|---|---|---|---|---|
| ***Rhizopus oryzae*** | | | | | |
| Rce1 | GH45 | 338 | 34,749 | CBM1 | Q8J1L2 |
| Rce2 | GH45 | 360 | 37,427 | CBM1 | Q8J1L1 |
| Rce3 | GH45 | 366 | 38,036 | CBM1 | Q8J1L0 |
| RaGa | GH15 | 579 | 62,110 | CBM21 | Q09GR5 |
| amyA | GH15 | 604 | 65,198 | CBM21 | B7XC04 |
| amyA | GH15 | 604 | 65,142 | CBM21 | Q2VC81 |
| Glucoamylase | GH15 | 579 | 62,152 | CBM21 | Q7Z7X9 |
| cda | CE4 | 446 | 49,178 | – | Q67G19 |
| Amy | GH13 | 462 | 50,896 | – | E2G4G0 |
| 1,3(4)-Beta-glucanase | GH16 | 315 | 34,059 | – | A1KXF8 |
| Pg | GH28 | 383 | 39,705 | – | Q5W9U0 |
| ***Rhizopus stolonifer*** | | | | | |
| CDA | CE4 | 447 | 46,942 | – | Q32XH4 |
| EG1 | GH18 | 219 | 24,195 | – | D1GCA0 |
| Chitin synthase | GT2 | 190 | 21,219 | – | Q02072 |

website: http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/MultiHome.html) showed, in contrast to other filamentous fungi, a low number of GHs and a high number of GTs and carbohydrate CEs, thus revealing the ability to better use easily digestible sugars rather than complex plant cell wall polysaccharides [76]. Studies on cellulase production by *R. oryzae* have been recently carried out by growing it on agro wastes, such as dried flower, sweet lime peel, dried grass, water hyacinth, potato peel, reaching yields of cellulase activity in the range of 50–900 U/mL after 72 h [77,78]. The strain *R. stolonifer* var. reflexus TP-02 isolated from ecological forests on Huangshan Mountain was found to produce huge amounts of cellulase activity, thus leading to the study of several genes encoding the endoglucanases EGX, EG27, EG45 and, more recently, EG1 [79].

### 5.2. Potential of Rhizopus spp. as I category CBP organisms

*R. oryzae* has been generally reported able to produce ethanol even if together with significant amounts of lactic acid as by-product. One of the first studies on ethanol fermentation by *Rhizopus* sp. was performed by Fujio et al. [80] on *R. koji* grown on uncooked cassava starch, and the maximum productivity of ethanol was of 2.3 g ethanol/L broth h, around the 50% compared to the productivity of a yeast system grown on glucose.

Three strains of *R. oryzae* were examinated for ethanol production by growing them in the presence of different carbon sources, glucose and xylose being the best substrates for growth and ethanol production [81]. Large amounts of lactic acid were produced as by-product in the presence of glucose, while xylitol by-production was observed in xylose medium.

Lactate production in *R. oryzae* cultivations was investigated also by Büyükkileci et al. [82], demonstrating that increase of spore concentration in the inoculum improves biomass and ethanol production and decreases lactate production.

*Rhizopus sp.* (strain W-08) has been studied for direct conversion of raw corn flour to ethanol, setting up a fed batch process of simultaneous saccharification and fermentation. Ethanol concentration of 21% (v/v) was obtained after 48 h, achieving a conversion efficiency of raw corn flour to ethanol of 94.5% of the theoretical ethanol yield [83], thus confirming that fungal mycelia enhance yeast ethanol productivity and tolerance.

Production of lactic acid as by-product was reported also by Abedinifar et al. [84] and Thongchul et al. [85]. The latter authors showed that high-concentration sugar solutions can be produced from cassava pulp treated with enzymes or acids and that cassava pulp hydrolysate could be directly used as a carbon source for *R. oryzae*, to produce cell biomass, lactic acid and ethanol.

A *R. stolonifer* strain was shown to have high ethanol productivity in comparison to *S. cerevisiae* and it was used for production of traditional rice wine by Song et al. [86].

Hence, the main limitation of use of *Rhizopus* as a I CBP organism for ethanol production is formation of lactate as by-product. This drawback can be faced by interfering with lactic acid metabolism through suppression/silencing of lactate dehydrogenase gene. As a matter of fact, RNA silencing of lactate dehydrogenase gene in *R. oryzae* provided a 85.7% (g/g) decrease in lactic acid production and an increase of 15.4% (g/g) in ethanol yield as compared with wild type *R. oryzae* [87].

## 6. Other fungi

Exploring biodiversity can contribute to moving from cellulosic feedstock to ethanol fuel tanks [88]. Besides *T. reesei*, several and diverse fungi can be found as natural colonizers of cellulosic materials. Among these, anaerobic (Table 7) and thermophilic (Table 8) fungi deserve particular attention as producers of carbohydrate active enzymes and are below described. However, use of these fungi for I category CBP requires many efforts to improve knowledge of their metabolic pathways and to develop genetic tools for enzyme/organism enhancement by genetic engineering. Another limitation of the anaerobic fungi is that they are very sensitive to oxygen and temperature and generally do not survive in conditions other than those found in the intestinal tracts of animals [89].

### 6.1. Anaerobic fungi

Anaerobic fungi are present in the gastrointestinal tract of herbivorous animals and play an active role in the plant fiber degradation by producing a wide array of hydrolytic enzymes in the rumen. Seventeen different anaerobic fungi belonging to the five genera *Caecomyces*, *Neocallimastix*, *Piromyces*, *Anaeromyces* and *Orpinomyces* have been so far reported [90–92]. Anaerobic fungi are able to hydrolyze plant materials, such as grass and straw, by producing plant degrading enzymes, such as cellulases, β-glucosidases, xylanases, β-glucanases/lichenases and mannanases [93], thus releasing sugars and other compounds resulting from sugars fermentation such as formate, acetate, ethanol, lactate, $CO_2$, and $H_2$ [93,94]. An extensive description of (hemi)cellulolytic enzymes of anaerobic fungi has been reported by Ljungdahl [95]; the most important features of these enzymatic systems are below reported.

#### 6.1.1. Cellulolytic enzyme systems of anaerobic fungi

Anaerobic fungi are able to efficiently hydrolyze cellulose, hemicelluloses and other plant materials producing various degrading enzymes (Table 7) [93,95], most of which are associated with cellulosomes, even if free enzymes are also present. Cellulosomes are extracellular multienzyme complexes containing 20 or more different carbohydrate hydrolytic enzymes bound together by non-catalytic scaffolding proteins [96]. Besides the catalytic sites, the enzymes within cellulosomal complexes exhibit modules named *dockerins* for binding to cohesin modules of scaffolding proteins [96,97]. CBMs are also present in polypeptides of cellulosomes. No scaffolding polypeptide has been so far isolated from a cellulosome of an anaerobic fungus, and there is limited knowledge on cohesins in fungal scaffolding proteins. On the contrary, there is more detailed knowledge on fungal FDDs or docking domains, whose amino acid sequences are very different from those of bacterial dockerins [98,99]. Most enzymes in cellulosomes of anaerobic fungi contain two copies of FDD, each consisting of about 40 highly conserved amino acids, linked by a short novel linker sequence. FDDs are cysteine-rich domains and the cysteines are important for the structure of FDDs, as demonstrated for the N-terminal FDD of Cel45A from *Piromyces equi*, for which the three-dimensional structure has been solved [98]: reduction of the disulfides caused the loss of the structure. Three amino acids conserved in all FDDs – tyrosine (Y8), tryptophan (W35), and aspartic acid (D23) – are essential for docking to the cellulosomes [99].

The cellulosome from *Neocallimastix frontalis* [100], consisting of at least six different polypeptides with very high activity against cotton fiber, is the first cellulosome-type complex isolated from an anaerobic fungus.

The cellulase/hemicellulase system of the fungus *Orpinomyces* PC-2, extensively described by Ljungdahl [95], consists of 17 cellulase/hemicellulase enzymes, including ten cellulases – six of GH family 6 (CelA, CelC, CelD, CelF, CelH and CelI) and four of family 5 (CelB, CelE, CelG, and CelJ) –; one β-glucosidase (BglA) of GH family 1; five enzymes involved in hemicellulose hydrolysis, including xylanase XynA GH11, lichenase or β-glucanase LicA GH16, mannanase ManA GH5, acetyl xylan esterase AxeA, and feruloyl esterase FaeA (Table 7). All the cellulases, except CelF, contain two copies of

**Table 7**
Components of the cellulolytic system in anaerobic fungi.

| Enzyme | Family | No. residues | Size (Da) | Special domain | Accession number |
|---|---|---|---|---|---|
| ***Orpinomyces sp.* strain PC-2a** | | | | | |
| CelA | GH6 | 459 | 50,560 | FDD3 | U63837 |
| CelB | GH5 | 471 | 53,103 | FDD | U57818 |
| CelC | GH6 | 449 | 49,390 | FDD | U63838 |
| CelD | GH6 | 455 | 50,279 | FDD | AAC090661 |
| CelE | GH5 | 477 | 53,635 | FDD | U97153 |
| CelF | GH6 | 432 | 46,736 | CBD | U97154 |
| CelG1 | GH5 | 193 | 21,150. | FDD | U97155 |
| CelH | GH6 | 491 | 53,956 | FDD | AAL01211.1 |
| CelI | GH6 | 490 | 54,051 | FDD | AAL01212.1 |
| BglA | GH5 | 229 | 24,907 | FDD&CBD | AF177207 |
| CelJ1 | GH1 | 663 | 75,228 | None | AF016864 |
| XynA | GH11 | 362 | 39,542 | FDD | U57819 |
| LicA | GH16 | 245 | 27,929 | None | U63813 |
| ManA | GH5 | 579 | 64,425 | CBD&FDD | AF177206 |
| AxeA | CE6 | 313 | 34,845 | None | AF001178 |
| FaeA | CE1 | 530 | 59,013 | None | AF164351 |
| CypB | – | –203 | 21,969 | None | U17900 |
| CelB | GH6 | 412 | 43,829 | CBM1 | Q6EH22 |
| CelA | GH6 | 510 | 53,951 | CBM1 | Q6EIY8 |
| Cel A | GH5 | 482 | 54,646 | CBD&CDD | Q01409 |
| CBH6 | GH6 | 431 | 46,020 | CBM1 | B0FEV4 |
| CelA | GH6 | 428 | 45,675 | CBM1 | Q12646 |
| Xyns20 | GH11 | 335 | 36,094 | CBM1 | A8TGA1 |
| XynB | GH10 | 860 | 88,052 | CBM1 | Q02290 |
| Xyns20e | GH11 | 671 | 72,469 | – | B8YG19 |
| BnaII | CE2 | 392 | 42,988 | – | O13496 |
| Estw1-6 | CE3 | 319 | 33,758 | – | Q6A4K5 |
| BnaI | CE6 | 393 | 42,759 | – | O13495 |
| CelB | GH5 | 473 | 53,070 | CBD&CDD | Q12647 |
| CelD | GH5 | 1232 | 140,617 | CBD&CDD | O59943 |
| Lic6 | GH16 | 245 | 27,805 | – | B0FEV5 |
| ***Piromyces sp.* and *Piromyces sp.* E2** | | | | | |
| ManB | *GH26* | 179 | 19,719 | *CBM35* | Q870B3 |
| ManA | *GH26* | 606 | 68,055 | *CBM35* | P55296 |
| ManB | *GH26* | 571 | 64,397 | *CBM35* | P55297 |
| ManC | *GH26* | 569 | 64,115 | *CBM35* | P55298 |
| FaeA | CE1 | 140 | 16,110 | CBB CDD | Q870B0 |
| bgl1A | GH1 | 664 | 75,801 | – | Q9C122 |
| Cel1D | GH1 | 110 | 11,983 | – | Q870B5 |
| Cel1C | GH1 | 665 | 76,089 | – | Q870B6 |
| Cel1B | GH1 | 540 | 62,476 | – | Q870B7 |
| Cel3a | GH3 | 867 | 93,632 | CBM&CDD | Q875K3 |
| Cel9a | GH9 | 778 | 86,034 | CBM&CDD | Q8NJX5 |
| Cel9a | GH9 | 771 | 85,186 | CBM&CDD | Q8NJX6 |
| XYNA | GH11 | 625 | 68,049 | CBM&CDD | Q12667 |
| Cel48a | GH48 | 753 | 83,489 | CBM&CDD | Q8J1E3 |

Data on *Orpinomyces sp.* strain PC-2a were adapted from Ljungdahl [95] with the author's permission.

FDD, indicating that these cellulases are cellulosome-associated, while CelF, that contains a CBD instead of the FDDs, is probably a free enzyme. The four family 5 cellulases differ from the family 6 cellulases since they have N-terminal instead of C-terminal CDs.

The sequence identity between CelE from *Orpinomyces* PC-2 and CelB from *N. patriciarum* is 67.9% [101]. Three family 5 cellulases (CelA, CelB2, and CelB29) have been characterized from *Orpinomyces joynii* [102,103]. The gene for the CelB29 enzyme from *O. joynii* shares 99% sequence identity with CelB from *Orpinomyces* PC-2.

Cellulosome-type complexes with endoglucanase, xylanase, mannanase, and β-glucosidase activities, containing at least 10 polypeptides have been found in *Piromyces* [104].

β-Glucosidases from the anaerobic fungi *N. frontalis* [105], *Piromyces* E2 [106], and *Orpinomyces* [107] have been characterized. β-Glucosidases BglA of *Orpinomyces* and Cel1A of *Piromyces*, belonging to GH family 1, are not associated with cellulosomes as indicated by lack of FDDs. The addition of the recombinant BglA to cellulase cocktail of *T. reesei* improved avicel saccharification allowing its complete hydrolysis into glucose [108]. Another β-glucosidase, Cel3A, responsible for the formation of glucose from cellobiose by the cellulosomal complex during cellulose hydrolysis, has been isolated from *Piromyces sp.* strain E2, differing from the above mentioned family 1 β-glucosidases for its cellulosome-associated nature [106]. On the other hand, no family 3 β-glucosidase is present in *Orpinomyces* PC-2 cellulosome.

### *6.1.2. Hemicellulases and associated enzymes from anaerobic fungi*

Anaerobic fungi produce the enzymes needed for degrading several types of hemicelluloses, including accessory enzymes such as xylan esterase, feruloyl- and *p*-coumaroyl esterases, and α-(4-O-methyl)-glucoronidase.

Two feruloyl esterases (FAE-I and FAE-II), and one *p*-coumaroyl esterase (CAE) have been purified from *Neocallimastix* strain MC-2, and their synergetistic action with commercial xylanases from *T. viride* was demonstrated [109]. The gene *faeA* (GenBank accession no. AF164351), coding for a feruloyl esterase from *Orpinomyces* PC-2 has been isolated and sequenced.

Several xylanases have been described in anaerobic fungi. Three xylanases – XYLA, XylB, and XynC – have been characterized from *N. patriciarum* [110–112]. XYLA and XylC have CDs belonging to GH

**Table 8**
Components of the cellulolytic system in thermophilic fungi.

| Enzyme | Family | No. residues | Size (Da) | Special domain | Accession number |
|---|---|---|---|---|---|
| ***Talaromyces emersonii*** | | | | | |
| CbhII | GH6 | 459 | 48,562 | CBM1 | Q8NIB5 |
| Axe | CE1 | 300 | – | – | ADX07526 |
| Bg1 | GH1 | 489 | 55,811 | – | Q8X214 |
| Beta-glucosidase | GH3 | 857 | 92,386 | – | Q8TGI8 |
| Bxl1 | GH3 | 796 | 86,784 | | Q8X212 |
| Aven | GH3 | 793 | 85,775 | – | Q8J261 |
| Eg1 | GH5 | 334 | 36,395 | – | Q8WZD7 |
| Cbh1 | GH7 | 455 | 48,737 | – | Q8TFL9 |
| Xylanase | GH10 | 408 | – | – | CAD34597.1 |
| GA | GH25 | 618 | 65,429 | CBM20 | Q9C1V4 |
| Gal1 | GH27 | 452 | 49,352 | – | A7XZT2 |
| BGal1 | GH35 | 1008 | 109,848 | – | Q8X213 |
| Acid trehalase | GH65 | 1066 | 116,509 | | Q6V7X7 |
| AGlu | GH67 | 837 | 91,902 | – | Q8X211 |
| ***Thermoascus auranticus*** | | | | | |
| Bgl1 | GH3 | 861 | 93,477 | – | Q0ZUL0 |
| Blg2 | GH3 | 866 | 93,313 | – | A9QUC3 |
| Eg1 | GH5 | 335 | 36,932 | | Q8TG26 |
| Cel7A | GH7 | 457 | 48,832 | | A7WNU2 |
| Cbh1 | GH7 | 457 | 48,775 | – | Q8TG37 |
| XynA | GH10 | 329 | 35,686 | – | P23360 |
| Chit1 | GH18 | 399 | 44,012 | | A6YJX1 |
| GH61A | GH61 | 250 | – | – | – |

family 11, and they are in the cellulosome, while XylB has a family 10 CD and a domain related to a CBM of family 1. *N. frontalis* has also several xylanases belonging to either GH family 10 or family 11 [113,114]. XYN3 shows a FDD at the C terminus, indicating its cellulosome-associated nature [115].

An essential enzyme for complete xylan degradation is acetyl xylan esterase, which removes the acetyl group from arabinoxylan thus avoiding that the xylan hydrolysis by xylanases is hindered by acetylation of the xylose unities. The gene *axeA*, coding for an acetyl xylan esterase, has been isolated from *Orpinomyces* PC-2 [116], and it shares 56% amino acid sequence identity with the acetyl xylan esterase BnaA from *N. patriciarum* [117]. *N. patriciarum* has two additional acetyl xylan esterases, BnaB and BnaC, not showing homology with BnaA [117]. A great increase of the rate of acetylated xylan hydrolysis was observed combining recombinant BnaA and XynA from *N. patriciarum* and AxeA and XynA from *Orpinomyces* [116,118].

The gene encoding the mannanase Man A has been isolated from *Orpinomyces* PR-2 [119] and genes coding for three mannanases – MANA, MANB, and MANC – have been isolated from *P. equi* [120]. It is possible that the CBM of ManA is involved in the binding of the fungal cellulosome to lignocellulose. A novel CBM-containing protein, NCP1, has been found associated with the cellulosome of *P. equi* [121].

Finally, the gene *licA*, encoding a protein homologous with β-glucanases from mesophilic, thermophilic, and ruminal anaerobic bacteria has been isolated from *Orpinomyces* PC-2 [122]. The encoded protein, LicA, exhibits 1,3-1,4-β-D-glucan 4-glucano hydrolase (β-glucanase; lichenase) activity, and it does not exhibit a FDD and it is not connected with the cellulosome. LicA has been expressed in *E. coli*, and a major part of the enzyme is found in the extracellular supernatant. Recombinant LicA from the *E. coli* extracellular supernatant hydrolyzes lichenin and barley β-glucan, while it does not hydrolyze laminarin, arboxymethylcellulose, pustulan, or xylan.

### 6.2. Thermophilic fungi

Although mesophilic engineered organisms or enzymes have so far been the preferred choices for the production of biofuels, mainly due to deep knowledge of metabolic pathways in these organisms as well as established genetic tools for engineering them, in the last few years, alternative approaches have being focused on thermophilic organisms and their enzymes, due to their robustness and versatility [123]. An ethanologenic process based on thermophiles displays several advantages over a mesophilic process such as: (i) ability of thermophiles to ferment not only the pentose and hexose sugar fraction of biomass but also hydrolysate materials and, in some cases, structurally complex polycarbohydrates, such as cellulose; (ii) the remarkable tolerance of thermophiles to tolerate fluctuations in pH, temperature and environmental change; (iii) easier downstream ethanol recovery due to the use of high temperatures, allowing ethanol removal and recovery by applying only a mild vacuum, which will facilitate continuous distillation or 'stripping' of ethanol as opposed to conventional distillation; (iv) lower chance of microbial contamination at the higher adopted temperatures; (v) reduction of gas solubility at higher temperatures, having the double advantage of helping to maintain the near anaerobic environment-needed for the fermentative process, and minimizing the growth of obligatory aerobic contaminants; (vi) possibility to reduce energy input, which is required to cool mesophilic fermentations between the pre-treatment of feedstock and the post-fermentative distillation process, thus improving process economics. Looking for thermophilic cellulolytic fungi, combining high cellulolytic abilities of fungi with the advantages of thermophilic ethanologenesis, could therefore represent another important strategy to develop improved bio-systems for ethanol production.

Some filamentous fungi produce cellulases that retain relatively high cellulose-degrading activity at elevated temperatures, particularly those from the species *Talaromyces emersonii* [124–126], *Thermoascus aurantiacus* [127–129], *Chaetomium thermophilum* [130], *Myceliophthora thermophila* [131], *Thielavia terrestris* and *Corynascus thermophilus* [132].

#### 6.2.1. T. emersonii

Three cellobiohydrolases (EC 3.2.1.91) from *T. emersonii*, CBH IA, CBH IB and CBH II, have been characterized by Tuohy et al. [126]. These enzymes are single subunit glycoproteins, showing remarkable thermostability and catalysing the hydrolysis of microcrystalline cellulose but not of a soluble cellulose derivative (CMC) and barley 1,3;1,4-L-D-glucan. The gene (*cbh2*) encoding CBH II

was isolated and sequenced, and regulation of its transcription and expression was analyzed [133]. The analysis of deduced amino acid sequence revealed that CBH2 has a modular structure consisting of a fungal type CBM separated from a catalytic domain by a proline/serine/threonine rich linker. The protein is homologous to family 6A fungal cellobiohydrolases, comprising enzymes responsible for hydrolysis of β-1,4 glycosidic bonds with inversion of configuration of the anomeric carbon. High sequence identity (67%) between the catalytic domain of Cel 6A from *T. reesei* and the *T. emersonii cbh2* gene product allowed predicting structure of the *T. emersonii* catalytic domain as a variant of the classical TIM α/β fold. The X-ray structure of native CBHIB from *T. emersonii*, PDB 1Q9H, was solved to 2.4 Å by molecular replacement [124], showing that it is a glycoprotein consisting of a large domain with a β-sandwich structure, representing the characteristic fold of GH7 family. The long cellulose-binding tunnel and the catalytic residues of GH7 Cel7A from *T. reesei* are conserved in 1Q9H. Deletions and other changes in loop regions are responsible for differences in the binding and catalytic properties of *T. emersonii* 1Q9H.

Three thermostable endo-β-D-glucanases (EG V–VII: EG V, 22.9 kDa; EG VI, 26.9 kDa; EG VII, 33.8 kDa) exhibiting maximum activity against mixed-link 1,3;1,4-β-D-glucans have been characterized from *T. emersonii* [134]. Time-course hydrolysis studies of 1,4-β-D-glucan (CMC), 1,3;1,4-β-D-glucan from barley (BBG) and lichenan confirmed the endo-acting nature of EG V–VII and verified preference for 1,3;1,4-β-D-glucan substrates. Both EG VI and EG VII enzymes also exhibit activity against 1,3-β-glucan (laminaran), in contrast to EG V.

UV-mutant strains of *T. emersonii* (TC2, TC5) displaying enhanced activity against mixed linkage cereal β-glucans were developed [135].

The gene encoding a thermostable β-glucosidase (*cel3a*) was isolated from *T. emersonii* and expressed in *T. reesei* [136]. Cel3a belongs to GH family 3, showing approximately 56 and 67% identity with Cel3b (GenBank AAP57755) from *T. reesei*, and a β-glucosidase from *A. niger* (GenBank CAB75696), respectively. Cel3a is thermostable with an optimum temperature of 71.5 °C and was a specific β-glucosidase with no β-galactosidase side activity. Cel3a was also active against natural cellooligosacharides releasing glucose. It displayed transferase activity producing mainly cellobiose from glucose and cellotetrose from cellobiose.

Cloning of two β-glucosidase genes (*bg1* and *aven1*) from *T. emersonii* having very different biological functions was reported by Collins et al. [137]. The *bg1* gene, encoding a putative intracellular β-glucosidase, shows significant similarity to other fungal glucosidases of GH family 1, known to be involved in cellulose degradation. Solka floc, methyl-xylose, gentiobiose, beech wood xylan, and lactose induced expression of *bg1*, whereas glucose repressed expression. A second β-glucosidase gene isolated from *T. emersonii*, *aven1*, encodes a putative avenacinase, that deglycosylates the anti-fungal saponin, avenacin, rendering it less toxic to the fungus. This gene displays homology with other fungal saponin-hydrolysing enzymes and β-glucosidases of GH 3 family.

O'Connell et al. [138] reported the purification and characterization of a further glucan hydrolase from *T. emersonii*, an exoacting β-1,3-glucanase, with preference for β-1,3-linkages. The purified exo-β-1,3-glucanase degrades laminaran releasing glucose as the sole product of hydrolysis. This enzyme displays great thermostability, and '*de novo*' sequence analysis suggests that this exo-β-1,3-glucanase belongs to GH family 5. Fungal exo-β-1,3-glucanases assigned to GH5 are associated with sporulation, ascospore thermoresistance and cell wall modification in the source organisms.

The gene coding for β-xylosidase, *bxl1*, from *T. emersonii* has been cloned [139], and the deduced amino acid sequence exhibits homology with *A. niger*, *A. nidulans*, *A. oryzae*, and *T. reesei* β-xylosidase gene products, and with some β-glucosidases, all of which belong to GH family 3. The *bxl1* gene was shown to be induced by xylan and methyl-β-D-xylopyranoside, and also by D-xylose except at high concentrations. The presence of six CreA binding sites in the *bxl1* promoter suggested that the observed repression by D-glucose may be mediated by this catabolite repressor.

Waters et al. [140] reported production of a cocktail of novel extracellular hydrolyzing thermozymes from *T. emersonii* on low-cost carbon inducers, such as tea leaves, wheat bran, wheat flour, sorghum and glucose, and its characterization in substrate hydrolysis.

#### 6.2.2. *T. aurantiacus*

*T. aurantiacus* is known to produce thermostable cellulases, including β-glucosidases [127,141,142].

An extracellular GH family 5 endoglucanase from *T. aurantiacus* was purified and characterized by Parry et al. [128]. The crystal structure of this 35 kDa thermostable endoglucanase was determined [143]. The active site contains eight critical residues, conserved in family 5. In addition, aromatic residues that line the substrate-binding cleft and that are possibly involved in substrate-binding were identified. A number of residues seem to be conserved among members of the subtype, including a disulphide bridge between Cys212 and Cys249.

Genes for a thermostable endo-β-1,4-glucanase and a thermostable cellobiohydrolase from *T. aurantiacus* were cloned [144,145]. A β-glucosidase gene from *T. aurantiacus* was also cloned based on the amino acid similarities of GH family 3 [146]. Hong et al. [147] reported purification of a thermostable β-glucosidase (BGLI) from *T. aurantiacus*, and cloning of the gene (*bgl1*) encoding this enzyme and its expression in yeast *Pichia pastoris*. The deduced amino acid sequence encoded by *bgl1* showed high similarity with the sequence of GH family 3.

Novel GH-7 family cellobiohydrolases from the thermophilic fungi *Acremonium thermophilum*, *T. aurantiacus* and *Chaetomium thermophilum* were cloned, expressed and characterized in comparison to cellobiohydrolases from *T. reesei* [148]. All these acidic cellobiohydrolases were more thermostable (by 4–10 °C) and more active (two- to fourfold) in hydrolysis of microcrystalline cellulose (Avicel) at 45 °C than *T. reesei* Cel7A. The *C. thermophilum* Cel7A showed the highest specific activity and temperature optimum when measured on soluble substrates. The most effective enzyme for Avicel hydrolysis at 70 °C, however, was the 2-module version of the *T. aurantiacus* Cel7A, which was also relatively weakly inhibited by cellobiose.

To develop functional enzymes in cellulose hydrolysis at or above 70 °C the cellobiohydrolase (CBHI/Cel7A) of *T. aurantiacus* was cloned and expressed in *T. reesei* Rut-C30 under the strong *cbh1* promoter [149].

## 7. Concluding remarks

CBP is the main route to reducing the cost of cellulosic ethanol. The limited ability of bacteria of producing enzymes in sufficient quantity and quality for lignocellulose degradation makes fungi alternative and better candidates for CBP. Cellulolytic fungi produce a large repertoire of saccharolytic enzymes to digest lignocellulose efficiently, assimilate all lignocellulosic sugars, and convert these sugars to ethanol, showing that they naturally possess all pathways for conversion of lignocellulose to bioethanol. For the development of fungi as CBP organisms, the remaining challenges to be met are their low ethanol yields, mainly due to by-product formation, and slow rates of fermentation.

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### 1.5.4 The Biorefinery Concept

The use of all the components of lignocellulose biomass to produce a large spectrum of biobased products and implement the biorefinery concept, is another challenge for further improving competitiveness of second generation bioethanol production.

Biorefinery, defined as the “co-production of fermentable sugars and bio-products from lignocellulose” [1], integrates biomass conversion processes and equipments to the production of fuels, power, and chemicals from the biomass itself, with an analogous concept to today petroleum refineries which produce multiple fuels and products from petroleum.

By producing multiple products, a biorefinery can exploit all the biomass components and the intermediates coming out from the different steps of lignocellulose conversion. For instance cellulose can be converted in glucose, for production not only of ethanol, but also xylitol, mannytol, sorbytol, lactic acid; hemicellulose can be hydrolysed in pentoses, useful in ethanol and xylose/xylitol production, whereas lignin can be a source of energy, as power and heat, besides being a source of phenols and other aromatic chemicals, and products like resins and fuel additives. The main challenges of research is thus aimed at finding the optimum methods to exploit all the chemistry and the energy embedded in the biomass, ensuring costs minimization and cost competitive end products.

The active role played by the US D.O.E. (Dept. of Energy), has made the USA a leading player in the emerging biorefineries. The largest example of these new technologies is corn oil extraction from the feedstock used for ethanol production. Corn oil can be used for the production of a wide spread of bio-based products such as biodiesel, feed, and building blocks for a variety of industrial application.

In Brazil, the highly developed sugar cane and ethanol industry is now attracting investments in bio-based plastics, such as PVC (Polyvinyl chloride) among those obtained from the ethylene.

As far as Europe is concerned, despite public police incentives, few efforts have been focused on the development of the biorefinery concept, mainly due to the fragmented nature of the European Research&Development (R&D) and insufficient resources for large demonstration plants. However, results achieved by the Biorefinery Euroview team [33] show that among the main European agro-industrial sites, nine can be classified as biorefineries, eight as future biorefineries, and four as R&D centers, pilot or demonstration plants on biorefinery subject, suggesting a new tendency which takes in to account the strong positive impact of the biorefinery on the economy and the environment.

## 1.6 Aims of the thesis

The overall aim of this project is the development of improved biocatalysts - cellulases, hemicellulases (xylanases) and accessory enzymes (e.g. arabinofuranosidases, pectinases, mannanases) - for conversion of lignocellulosic biomass into fermntable sugars for bioethanol production. As far as (hemi)cellulases are concerned, the strategy of this work was the isolation and selection of new microorganisms, bacteria and fungi, from three different habitats: industrial based compost, Western Ghat (India) and a "maâsra" (Morocco). The main outlines concerning this task are following reported:

**1)** Isolation and selection of new (hemi)cellulolytic microorganisms.
**2)** Morphological characterization of the selected microorganisms.
**3)** Optimization of (hemi)cellulolytic enzymes production by the selected microorganisms.
**4)** Identification and characterization of the (hemi)cellulolytic enzymes produced by the selected microorganisms.
**5)** Set up of recombinant expression systems of the gene(s) coding for the enzyme(s) of interest.

On the other side, the fungus *Pleurotus ostreatus* so far mainly studied for its laccase machinery, has been explored as a source of accessory enzymes for hemicelluloses conversion. In particular, it has been shown able to produce high level of arabinofuranosidase activity when grown on tomato processing waste.
Concerning this task, following goals have been fixed in this work:

**6)** Identification of the arabinofuranosidase (named PoAbf) produced during *Pleurotus ostreatus* growth on tomato waste by proteomic analyses.
***7)*** Gene and cDNA synthesis of PoAbf, in order to set up recombinant expression systems and characterize the recombinant enzyme.
**8)** Study of the catalytic/structural properties of the recombinant PoAbf (rPoAbf) and design and preparation of rPoAbf site-directed mutants, to study the effect of glycosylation on enzyme stability (rPoAbf S160G) and to elucidate the reaction mechanism of the enzyme (rPoAbf E364G and rPoAbf E471G).

The operative goals previously described aim at the general object of **selecting the best biocatalysts** among those developed, to be adopted in the hydrolysis of lignocellulose, as a component of minimal enzymatic cocktails finely designed.

*Chapter 2*

# *Isolation and characterization of new (hemi)cellulolytic microorganisms and their enzymes*

## 2.1 Introduction

Both aerobic and anaerobic microorganisms are involved in the degradation of plant cell walls. Aerobic bacteria are generally found in soil, water, on plant materials, in humus, animal feces and leaf litter. The rumen of various ruminants and the gut of termites are the major source of anaerobic bacteria involved in degradation of plant materials, being implicated in the host organism nutrition. Aerobic fungi play a key role in the degradation of plant materials, producing a wide spread of enzymes naturally evolved for lignocelluloses conversion. The genus *Trichoderma,* comprising almost 100 species, has been the one mostly studied so far, with *T. reesei* being recongnized as the major producer of cellulase activity. These species can be easily found in decomposing wood and plants, in the soil and on agricultural wastes. Anaerobic fungi acting in lignocelluloses conversion, such as *Anaeromyces, Caecomyces, Cyllamyces, Neocallimastix, Orpinomyces and Piromyces,* can be found in intestinal tract of large herbivorous animals. Table 4 adapted from Tamaru et al. [35], lists the main species of cellulolytic bacteria and fungi and the natural habitats from which they have been isolated, whilst the following paper **"Cellulolytic *Bacillus* strains from natural habitats for bioethanol production- A review" (Paper II)** reviews the research so far performed on cellulolytic *Bacillus* spp. strains isolation.

**Table 4:** Cellulolytic bacteria and fungi and their natural habitats (adapted from Tamaru et al. 2010 [35])

| **Aerobic bacteria** | | **Anaerobic bacteria** | |
|---|---|---|---|
| **Specie** | **Habitat(s)** | **Specie** | **Habitat(s)** |
| ***Brevibacterium*** | compost | ***Acetivibrio*** | *Sludge* |
| ***Cellulomonas*** | Soil, leaf litter, Forest humus soils, Sugar cane field | ***Clostridium*** | *Soil, Wood digester, Rotting grass, Wood chips, Pig intestine, compost* |
| ***Cellvibrio*** | Bovine faeces, soil | ***Ruminococcus*** | *Rumen* |
| ***Paenibacillus*** | Compost | ***Spirochaeta*** | *Hot spring* |
| ***Pseudomonas*** | Soil, sludge | ***Thermotoga*** | *Hot spring* |
| ***Streptomyces*** | Soil | | |
| ***Sorangium*** | Soil | | |
| ***Acidothermus*** | Hot spring | | |
| ***Talaromyces*** | Compost | | |
| **Aerobic bacteria** | | **Anaerobic bacteria** | |
| **Habitat(s)** | **Specie** | **Habitat(s)** | **Specie** |
| ***Aspergillus*** | Soil, rotten wood | ***Anaeromyces*** | Rumen |
| ***Agaricus*** | Mushroom compost | ***Caecomyces*** | Rumen |
| ***Penicillium*** | Soil, rotten wood | ***Cyllamyces*** | Rumen |
| ***Phanerochaete*** | compost | ***Neocallimastix*** | Rumen |
| ***Trichocladium*** | Soil | ***Orpinomyces*** | Rumen |
| ***Trichoderma*** | Soil, rotting canvas | ***Piromyces*** | Rumen |
| ***Humicola*** | Soil, compost | | |

Thus, this chapter deals with the results concerning the isolation and selection of new microorganisms, and their (hemi)cellulolytic enzymes identification and characterization. In the following section 2-I, **paper III**, **"Industrial waste based compost as a source of novel cellulolytic strains and enzymes"** and **paper IV**, **"Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces* sp. G12 isolated from compost"** describe results achieved starting from the isolation of new cellulolytic microorganisms from industrial waste based compost, whilst section 2-II deals with the work carried out at IRD-IMEP (Marseille, France) and NIIST-CSIR (Trivandrum, India) on isolation and selection of thermophilic fungi and bacteria, respectively.

***Accepted for publication in Chimica OGGI / Chemistry TODAY*** **(Paper II)**

**Cellulolytic *Bacillus* strains from natural habitats - A review**

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**ABSTRACT**

Fossil fuel reserves depletion, global warming, costly and problematic waste recycling and population growth greatly induce to find renewable energy sources. Second generation bioethanol produced from lignocellulosic materials exhibits great potential as liquid biofuel to substitute gasoline.
Production costs of enzymes involved in cellulose hydrolysis into fermentable sugars, represent the main obstacle to achieve competitive production of cellulosic ethanol. Cheaper and more efficient biocatalysts for the saccharification step are, therefore, required for making the whole process more competitive. The biodiversity of natural niches has been so far exploited for the isolation of new cellulolytic microorganisms whose enzymes are naturally evolved for an efficient conversion of lignocelluloses into fermentable sugars.
This review discusses advances in isolation of bacteria, namely *Bacillus* spp., from several natural habitats and their ability to produce cellulase activity.

**1. INTRODUCTION**

Second generation ethanol, produced from lignocellulosic materials, such as agricultural wastes, plant wastes and other non-food wastes, offers a more attractive greenhouse gas emissions profile than other biofuels, with a 86 % reduction of greenhouse gas emissions in comparison to gasoline [1]. Moreover, use of lignocelluloses favors overcoming the so called *fuel versus food* conflict rising from the use of agricultural products (first generation bioethanol) normally addressed to human diet. The main challenges to improve the whole process associated with the conversion of cellulosic biomass to ethanol include increasing polysaccharides hydrolysis yield, decreasing loading of hydrolytic enzymes, optimizing or eliminating the pretreatment and developing a consolidated bioprocessing where the enzyme production, the hydrolysis step and fermentation of sugars into ethanol occur in a single process [2, 3].
An enormous range of different habitats such as soil, compost piles, decaying plant materials, rumens, sewage sludge, invertebrates like termites (gut), forest waste piles, wood processing plants, leaf litter, animal faces, paper mills and water, especially from hot springs have been so far investigated as sources of both aerobic and anaerobic bacteria producing several different enzymes of industrial interest. The aerobic microorganisms, especially fungi, play a major role in the degradation of plant materials and they are found on decomposing wood and plants, in the soil and on agricultural wastes [2]. However, the isolation of bacteria as a source of novel enzymes for cellulose hydrolysis is now strongly investigated for the several advantages they exhibit in comparison to fungi. The high growth rates of bacteria, the possibility to easily engineering them, and last but not least the higher probability to isolate them from environmental niches that naturally drive them to the production of enzymes resistant to environmental stresses are some their main advantages.
This review focuses on the isolation of *Bacillus* strains, that have been so far recognized as good producers of cellulase enzymes, from compost, soil and other sources explored for their natural biodiversity (Table 1).

**2. COMPOST AS A SOURCE OF NEW CELLULOLYTIC *Bacillus* STRAINS**

Composting process mainly depends on the combined and sequential action of specific taxonomic and functional microbial groups which produce a series of hydrolytic enzymes that contribute to the bioconversion of the organic materials. During composting numerous types of microorganisms are involved in degradation of wastes and their diversity is influenced by biomass type. Since the organic substances used for this process are mostly derived from plant material, composting of this recalcitrant biomaterial is more likely to be led by a microbial community with high lignocellulolytic activity. Therefore, in the last decades, composting has been used as source of novel microorganisms and enzymes to be applied to the conversion of lignocellulose to biofuels [4, 5]. Although fungi are the main cellulase-producing microorganisms and most cellulases currently used in industry are of fungal origin, bacteria and

actinomycetes have also been reported to produce cellulase [6, 7] and play specific roles in the biodegradation of organic materials during composting.
Cellulolytic bacteria have been isolated from a wide diversity of composts. Bacteria species such as *Pseudomonas*, *Klebsiella* and *Bacillus* have been isolated from different compost environments. Strom [8] reported that bacteria that are the dominant in the thermophilic phase of the process include *Bacillus* since the 87% of the randomly selected colonies during this phase belong to this genus. Similarly, Raut et al. [9] observed an increasing in bacterial biomass during rapid composting due to dominance of actinomycetes and thermophilic bacteria, especially *Bacillus* spp. formed during the thermophilic phase. In particular, *B. subtilis*, *B. licheniformis* and *B. circulans* are reported as typical bacteria of the thermophilic phase of composting process [10].
Eida and co-workers [11] isolated cellulolytic bacteria using Dubos mineral salt agar containing azurine crosslinked (AZCL) HE-cellulose. Bacterial identification was performed based on the sequence analysis of 16S rRNA genes, and cellulase, xylanase, β-glucanase, mannanase, and protease activities were characterized using insoluble AZCL-linked substrates. Twenty-one isolates were obtained belonging to the genera *Streptomyces*, *Microbispora*, *Paenibacillus*, and *Cohnella*, all showing cellulase, xylanase, β-glucanase, and mannanase activities. Based on enzyme activities expressed as the ratio of hydrolysis zone diameter to colony diameter, it was suggested that some *Paenibacillus* strains (SDCB10, SDCB11) contribute to efficient cellulolytic and hemicellulolytic processes during composting, clarifying the role of these bacteria in the biodegradation of organic wastes.
In their study, Abu-Taleb and co-workers [12] isolated from compost the strain *Bacillus amyloliquefaciens* C23 that showed a high ability to produce cellulase enzymes. Since cellulase yield depends on a complex relationship involving a variety of factors such as inoculum size, pH value, temperature, presence of inducers, medium additives, aeration and growth time, these authors investigated and optimized the nutritional and environmental parameters for improving cellulase production by this cellulolytic bacterial strain.
Recently, 90 bacteria were isolated from raw composting materials obtained from vegetable processing industry wastes using carboxymethylcellulose (CMC) as substrate [13]. The bacteria producing the highest cellulolytic activity levels were identified by morphological, physiological, biochemical and molecular analyses as *Bacillus licheniformis* strain 1, *Bacillus subtilis* subsp. *subtilis* strain B7B, *Bacillus subtilis* subsp. *spizizenii* strain 6, and *Bacillus amyloliquefaciens* strain B31C. The strain *B. amyloliquefaciens* B31C was shown to produce the highest cellulase activity levels in comparison to the other isolated *Bacillus* strains. The cellulase purified from this strain showed a significant thermo-resistance and a range of optimum temperatures from 50 to 70°C, resulting an interesting candidate as biocatalyst in lignocelluloses conversion for second generation bioethanol production.
Thermophilic microorganisms from compost, identified as *Bacillus* strains by 16S rDNA sequence analysis, were isolated by Mayende et al. [14] using CMC as substrate. Authors screened the isolates for cellulase and polyphenol oxidase activity and performed temperature optimum and temperature stability studies on the enzymes. Their results showed that thermophilic microorganisms isolated from a composting environment are able to produce thermophilic cellulases which show activity at temperature up to 80° C.
Cellulolytic *Bacillus* strains were also isolated by Kim and co-workers [15] from different environments.Three *Bacillus* strains were selected for their highest cellulase activity and in particular, the strain C5-16, isolated from compost and identified as *Bacillus subtilis*, produced different enzymatic activities, such as CMCase, avicelase, β-glucosidase, and xylanase.

## 3. SOIL AS A SOURCE OF NEW CELLULOLYTIC *Bacillus* STRAINS

Among the different microorganisms inhabiting in the soil, bacteria are the most abundant and predominant organisms, Bacilli being the most numerous, followed by Cocci and Spirilla.
As proposed in the Bergey's Manual of Systematic Bacteriology, most of the soil bacteria can be taxonomically divided in the three orders, *Pseudomonadales*, *Eubacteriales* and *Actinomycetales*.
Soil bacteria can also be classified on the basis of their physiological activity and the way in which they obtain carbon, nitrogen, energy and other nutrient requirements (autotrophs or heterotrophs).

Soil bacteria are able to degrade all the components of lignocelluloses. Particularly, the most abundant bacteria of the genus *Bacillus* spp. are involved in cellulose, hemicelluloses and proteins degradation, producing a wide spectra of enzymes used for these reactions.
A lot of cellulolytic bacteria belonging to *Bacillus* genus have been so far isolated from soil and characterized for the production of enzymes needed in lignocelluloses to ethanol process.
In 1984, Robson and Chambliss [16] reported isolation of a cellulolytic *Bacillus* strain, DLG from soil near Lake Mendota (Wisconsin), representing one of the first examples of *Bacillus* sp. isolation from soil.
Heck et al. [17] demonstrated the potential of some *Bacillus* strains isolated from Amazonian environment for production of cellulases. The isolates were proved to be excellent biological systems, showing some advantage over filamentous fungi, such as faster growth and lower probability of contaminations.
*Bacillus alcalophilus* S39 was isolated from soil and it distinguished as a potential cellulase producer [12]. Studying the effect of growth parameters on the enzyme production levels, it was shown for this strain that glucose represents the best carbon source for biomass accumulation, whilst CMC was the best substrate for CMCase, Filter paper (FP) and β-glucosidases production. As far as nitrogen source is concerned, ammonium sulphate and peptone were shown to be the most suitable nitrogen source for cells growth and cellulase activity production, respectively, whilst pH 7 was optimum for enzyme production. The production of cellulase by the new isolate was demonstrated to be influenced even by physical parameters such as temperature and shaker rate, 150-200 rpm and 30°C being the best values, respectively.
*B. amyloliquefaciens* DL-3 was isolated from soil as a bacterium able to hydrolyze rice hull, a major cellulosic waste-material in Korea, consisting of lignin, cellulose and hemicelluloses [18]. Even in this case, cellulase activity production was optimized by changing both carbon and nitrogen source, demonstrating that rice hull is a better carbon source than glucose, fructose, sucrose and maltose. Recently, Kim et al. [15] collected a total of 176 samples from soil, compost, and animal waste slurry on Jeju Island, South Korea. The isolates were screened for cellulolytic activity production and three clones showing relatively higher cellulolytic activity and broader pH optimum were selected. The selected isolated from the soil was designated as *Bacillus subtilis* S52-2 and characterized from both morphological and physiological points of view. It was shown able to produce CMCase, avicelase, beta-glucosidase and even xylanase activities.
Afzal et al. [19] defined the best parameters for cellulase production by a *Bacillus* strain closely related to *Bacillus cereus*, and named as *Bacillus cereus* MRLB1.
Total population of cellulose degrading bacteria was studied in a landfill ecosystem as a part of microbial diversity study [20]. Samples were obtained from 3 and 5 feet depth of a local landfill being operated for past 10 years. Among many isolates, 22 bacterial strains were selected based on their capability to decompose CMC. All isolates were Gram positive, endospore forming and alkalophilic bacteria with optimum growth pH 9–10. They were grouped based on the phenotypic and chemotaxonomic characters and analysis of 16S rRNA gene indicated that these strains belong to different species of the genus *Bacillus*. Maximum CMCase activity of 4.8 U/ml at 50°C was obtained by strain *Bacillus* LFC15.
In their study, Vijayaraghavan et al. [21] isolated five cellulase-secreting bacterial strains from a paddy field located in Nagercoil, Kanyakumari (southwestern region of India). Among the five bacteria, the strain showing the highest cellulolytic activity on the CMC agar plate, was identified as *Bacillus* sp., based on morphological and biochemical characteristics, according to Bergey's Manual of Systematic Bacteriology. Effect of pH on cellulase activity production was studied, showing that cellulase production is higher at pH 6.5, similarly to most of the *Bacillus* strains.
*Bacillus subtilis* SJ01 was isolated from soil samples in Grahamstown, South Africa and studied for CMCase, avicelase and xylanase activity production. The enzymes have been purified and analysed by zymography, showing their ability to hydrolyze natural substrates like birchwood xylan, as a multi-enzyme complex [22]. Similarly, Otajevwo et al. [23] selected two *Bacillus* strains, *Bacillus subtilis* and *Bacillus circulans* from a collection of microorganisms isolated from soil situated in three different locations of Benin City, Nigeria. Both the isolates produce the highest cellulolytic activities level at around 35 °C, *Bacillus subtilis* giving an optimal yield of residual fermentable sugars at pH 5.

Soil samples from agricultural fields, the premises of paper, cotton and wool industries and rich in rotting rice straw have also been showed a good source for cellulase-producing bacteria [24, 25,26].
Soil samples from an hot spring, Hammam pharoan, in Sinai desert (in the east of Egypt, at the red sea beach), have been enriched with cellulose with the aim of isolating thermophilic cellullase producing bacteria [27]. Isolation of cellulolytic microorganisms from soil samples enriched by incubation with cellulose for about 4 weeks and incubated at 70°C, resulted in 3 different microorganisms showing high similarity with *Anoxybacillus flavithermus*, *Geobacillus thermodenitrificans* and *Geobacillus stearothermophilus*.

## 4. OTHER HABITATS FOR NEW CELLULOLYTIC *Bacillus* STRAINS

Several other sources have been exploited for the isolation of cellulolytic bacteria belonging to *Bacillus* genus. Besides soil, decaying plants materials and compost piles, bacteria occur in the anaerobic rumen of various ruminants and the gut of termites, where they hydrolyze plant materials for the host organism's nutrition. The aerobic bacteria are also usually found in water, on plant materials, in humus, in animal faeces, in sugar cane fields and in leaf litter.
Several manuscripts show the potential of cow dung as a good source for bacteria involved in several physiological roles. Cow-dung is a mixture of dung and urine, generally in the ratio of 3:1 containing crude fibre, crude protein, cellulose, hemicellulose and several minerals such as N, K, S, traces of P, Fe, Co, Mg, P, Cl, Mn, etc. [28]. As reported by Swain and Ray [29], *Bacillus subtilis* strains isolated from cow-dung have several beneficial attributes, including biocontrol, plant growth promotion, sulphur oxidation, phosphorus solubilization and production of industrially important enzymes amylase and cellulase (1.5–1.8 mg of reducing sugar $24h^{-1}$ $ml^{-1}$). Cow dung has been showed an interesting source of cellulolytic bacteria even by Bai et al. [30] and Deka et al. [31] who both isolated *Bacillus subtilis* strains.
Microbial system found in the gut of organisms thriving on cellulosic biomasses is one of the major source for cellulolytic enzymes. In fact, invertebrates like termites (Isopteran), bookworm (Lepidoptera), snails have a syntrophic symbiotic microflora in their guts responsible for cellulosic feed digestion.
Sarkar and Upadhyay [32] isolated a novel *Bacillus thermoalcaliphus* from a termite mound, producing a cellulase that is stable at elevated temperatures under alkaline conditions.
In their work, Huang et al. [33] isolated 270 aerobic and facultative anaerobic bacteria from the gut of *Holotrichia parallela larvae*. A *B. licheniformis* SVD1 strain was isolated from a biosulphidogenic bioreactor and studied for the presence of multy-enzyme complexes acting on several substrates such as cellulose, birchwood xylan and bagasse [34].
Bacterial cultures have been isolated also from the flour mill effluents around Tirupati, India and among the isolates two *Bacillus* strains, FME 1 and FME 2, were identified. Both the isolates were shown able to produce CMCase and FP activities on cellulose powder, whatman filter paper and a lignocellulosic substrate, namely rice husk [35].
Liang et al. [36] used, as the original bacterial source, lyophilized samples from a pilot plant performing an aerobic and thermophilic process to degrade swine waste from a swine finishing building (Southern Illinois University). The new isolate *Brevibacillus* sp. was shown able to grow on different carbon sources, and, except in the presence of xylan and xylose, to release cellulases in the medium as revealed by the filter paper activity assay.
Litter of swampy forest in Pesisir Selatan and mountain forest in Lembah Anai Tanah Datar were shown suitable cellulolytic bacteria source. The isolates were cultivated in selective media to obtain bacteria from the genus *Bacillus*; thus six *Bacillus* strains from swampy forest and three *Bacillus* strains from mountain forest were obtained [37]. A *Bacillus subtilis* strain has been isolated from molasses obtained in Kom Ombo sugars factory. Factors affecting cellulase activity production have been studied, showing that the strain is able to use as carbon source for cellulase production a wide spectrum of substrates such as cellulose, filter paper or starch [38].
Maki et al. [39] used different lignocellulosic samples for isolation of cellulase-producing bacteria. Dry and aged waste products leftover from pulp and paper mill processing were obtained from the area of Red Rock (Ontario, Canada) whilst a sludge material produced from the kraft processing of fine paper was obtained from a paper mill in Thunder Bay (Ontario, Canada). Moreover, a commercial fertilizer called Efficient Microorganism Dust, was analyzed as bacteria source. Several of these isolates were analyzed for CMcellulase and Filter paper activities production, showing great potential for the application in lignocelluloses conversion.

Water samples are of also of great interest for isolation of biotechnologically useful microbes, especially when originated from the so called "hot spring". For instance, Acharya and Chaudhary [40], isolated and identified two new *Bacillus* strains, as *B. licheniformis* MVS1 and *Bacillus* sp. MVS3. Effects on cellulase activity production of several parameters were studied, showing that rice straw and wheat bran can be used as a cheap carbon source which stimulate the production of thermophilic cellulases.
*Bacillus* spp. CH43 and HR68 were isolated from hot springs in Chiredzi (43°C, pH 8.5) and Chimanimani (55°C, pH 8.5–9), respectively (Zimbabwe), and their cellulases have been purified and characterized by Mawadza et al. [41].

## 5. CONCLUSIONS AND PERSPECTIVES

Second generation bioethanol represents a good alternative to the fossil fuels and moreover, its production can be achieved in frame of a biorefinery where the traditional chemical processes can be easily substituted with bioprocesses for the production of a wide spread of industrial green products.
Cellulases are needed in the hydrolysis step involved in the second generation ethanol for cellulose conversion into fermentable sugars but cost of their production are still high, thus efforts to improve the lignocellulose to ethanol conversion process are needed. For instance, research aims at cellulases having better catalytic performances thus requiring lower enzymes' loading and reducing time of reaction in the lignocelluloses hydrolysis step. Habitats as soil, compost, decaying plant material, rumens, sewage sludge, invertebrates gut, forest waste piles, wood processing plants, leaf litter, animal faces, paper mills and water have been successfully explored as a source of novel cellulolytic microorganisms. The ability of the most of these microbes to produce cellulolytic enzymes resistant to the recalcitrant conditions typically founded in the process of second generation bioethanol production could help improving competitiveness of the whole process.

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**Table 1**- Various *Bacillus* sp. isolated from natural habitats and tested for cellulase activity

| Microorganism | Source | Enzymatic activity | References |
|---|---|---|---|
| *Paenibacillus* strains (SDCB10, SDCB11) | Compost | Cellulase/ hemicellulase | [11] |
| *Bacillus amyloliquefaciens* C23 | Compost | Cellulase | [12] |
| *Bacillus amyloliquefaciens* B31C | Compost from vegetable processing of industry wastes. | Cellulase | [13]. |
| *Bacillus* strains | Compost | Cellulase | [14] |
| *Bacillus* spp. | Compost | CMCase, avicelase, β-glucosidase, xylanase | [15] |
| *Bacillus* strain DLG | Soil near Lake Mendota (Wisconsin. | Endoglucanase and exoglucanase | [16] |
| *Bacillus* strains | Amazonian environment | Cellulase | [17] |
| *Bacillus alcalophilus* S39 | Soil | CMCase, filter paper activity, β-glucosidases | [12] |
| *Bacillus amyloliquefaciens* DL-3 | Soil | CMCase | [18] |
| *Bacillus subtilis* S52-2 | Soil | CMCase, avicelase, β-glucosidase, xylanase | [19] |
| *Bacillus cereus* MRLB1 | Soil | Cellulase | [20] |
| *Bacillus* LFC15 | Landfill ecosystem | CMCase | [21] |
| *Bacillus* sp. | Nagercoil, Kanyakumari (southwestern region of India) | CMCase | [22] |
| *Bacillus subtilis* SJ01 | Soil samples in Grahamstown, South Africa | CMCase, avicelase and xylanase | [23] |
| *Bacillus subtilis* and *Bacillus circulans* | Benin city, nigeria. | Cellulase | [24] |
| *Anoxybacillus flavithermus*, *Geobacillus thermodenitrificans*, *Geobacillus stearothermophilus* | Hot spring, Hammam pharoan, in Sinai desert. | Cellulase | [28] |
| *Bacillus subtilis* | Cow dung | CMCase | [30] |
| *Bacillus subtilis* | Cow dung | Cellulase | [31] |
| *Bacillus subtilis* | Cow dung | CMCase | [32] |
| *Bacillus thermoalcaliphus* | Termite mound | Cellulase | [33] |
| *Bacillus licheniformis* SVD1 | Biosulphidogenic bioreactor | Multy-enzyme complexes | [35] |

| | | | |
|---|---|---|---|
| *Bacillus* FME1strain<br>*Bacillus* FME2 strain | Flour mill effluents around Tirupati, India | CMCase and FPactivity | [36] |
| *Brevibacillus* sp. | Degraded swine waste performing with an aerobic and thermophilic process (Southern Illinois University) | Cellulase | [37] |
| *Bacillus* strains<br>*Bacillus* strains | Litter of swampy forest in Pesisir Selatan<br><br>Mountain forest in Lembah Anai Tanah Datar | Exoglucanase, endoglucanase | [38] |
| *Bacillus subtilis* | Molasses obtained in Kom Ombo sugars factory | Cellulase | [39] |
| Cellulase-producing bacteria | Dry and aged waste products leftover from pulp and paper mill processing (Ontario, Canada); sludge material from the kraft processing of fine paper (Ontario, Canada); commercial fertilizer | Cellulase | [40] |
| *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3 | Water samples | Cellulase | [41] |
| *Bacillus* spp. CH43 | Hot springs in Chiredzi (43°C, ph 8.5), Zimbabwe | Cellulase | [42] |
| *Bacillus* spp. HR68 | Chimanimani (55°C, pH 8.5–9), Zimbabwe | Cellulase | [42] |

## 2.2 Results

### *Section 2-I*

### 2.2.1 Industrial waste based compost as a source of novel cellulolytic strains and enzymes (Paper III)

FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

# Industrial waste based compost as a source of novel cellulolytic strains and enzymes

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## Abstract

Ninety bacteria isolated from raw composting materials were screened for their cellulolytic activity on solid medium containing carboxymethylcellulose. The bacteria producing the highest cellulolytic activity levels were identified by 16S rRNA sequencing as *Bacillus licheniformis* strain 1, *Bacillus subtilis* subsp. *subtilis* strain B7B, *Bacillus subtilis* subsp. *spizizenii* strain 6, and *Bacillus amyloliquefaciens* strain B31C. Cellulase activity production by the most productive strain *B. amyloliquefaciens* B31C was optimized in liquid culture varying the carbon source. Comparison of growth curves of *B. amyloliquefaciens* B31C at temperatures from 28 to 47 °C indicated its thermotolerant nature. Moreover, analysis of time courses of cellulase activity production in this thermal range showed that increase of temperature from 28 to 37 °C causes an increase of cellulase activity levels. Investigating the enzymes responsible for cellulase activity produced by *B. amyloliquefaciens* B31C by proteomic analyses, an endoglucanase was identified. It was shown that the purified enzyme catalyzes carboxymethylcellulose's hydrolysis following Michaelis–Menten kinetics with a $K_M$ of 9.95 mg $ml^{-1}$ and a $v_{max}$ of 284 μM $min^{-1}$. It shows a retention of 90% of its activity for at least 144 h of incubation at 40 °C and exhibits a range of optimum temperatures from 50 to 70 °C.

## Introduction

Second generation bioethanol produced from lignocelluloses represents one of the best alternatives to the fossil fuels. Despite its many advantages, cellulosic bioethanol is not industrially produced at competitive level yet mainly due to the high cost of cellulolytic enzymes (Lynd *et al.*, 2008), and new more efficient and low cost cellulolytic enzymes should be developed.

Filamentous fungi are the major source of cellulases and hemicellulases (Baldrian & Valásková, 2008), but the production costs of these enzymes are very high. Bacteria, which have high growth rate as compared to fungi have good potential to be used in cellulase production. Various bacterial strains have the ability to produce cellulase complexes aerobically as well as anaerobically. However, the application of bacteria to cellulase production is not widely applied, yet.

Isolation of cellulolytic bacteria from soil (Elberson *et al.*, 2000; Heck *et al.*, 2002; An *et al.*, 2005; Sangkharak *et al.*, 2011; Kim *et al.*, 2012), water (Heck *et al.*, 2002), compost (Kang *et al.*, 2007; Eida *et al.*, 2012; Kim *et al.*, 2012), invertebrates (Gupta *et al.*, 2012), flour mill effluents (Kumar *et al.*, 2009), decaying vegetables (Sakthivel *et al.*, 2010) and animal waste slurry (Kim *et al.*, 2012) have been so far reported.

The aim of this study was to isolate a new bacterium as a source of thermostable cellulases in order to identify and characterize its cellulolytic enzyme(s). Identification and characterization of the endoglucanase produced by the most productive selected strain *Bacillus amyloliquefaciens* B31C, isolated from raw composting materials, was reported.

## Materials and methods

### Cellulolytic bacteria isolation

Cellulolytic microorganisms were isolated from mature compost obtained from agro-industrial wastes collected in region Campania (Italy) and consisting of pomace with kernel (65%), liquid sewage sludge (22%) from industrial processing of vegetable (potatoes and carrotoes) and borland molasses (13%). Representative samples of 1 kg were taken from the external (right and left side of the pile, about 5–10 cm of depth) and internal central part (at about 40 cm of depth) of biomass. Microbial isolates were obtained in solid media following the method described by Hankin & Anagnostakis (1977) with some modifications. Initial compost suspensions were prepared by the addition of 20 g (w/v) of compost samples to 180 mL of quarter strength Ringer's solution (Oxoid, Ltd, Oxford, UK) in 250 mL Erlenmeyer flasks. After shaking, suitable dilutions were made in the same solution and were used to inoculate solid media of growth composed by 5 g $L^{-1}$ carboxymethylcellulose (CMC; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1 g $L^{-1}$ $(NH_4)NO_3$, 1 g $L^{-1}$ yeast extract, 50 mL $L^{-1}$ standard salt solution, 1 mL $L^{-1}$ trace elements solution, 0.02% Remazol Brilliant Blue R (Moore *et al.*, 1979), 10 g $L^{-1}$ bacteriological agar, at pH 7.0. After incubation at 28 °C for 7 days, the plates were flooded with a Remazol Brilliant Blue R solution to put better in evidence the presence of clear haloes around the cellulolytic colonies. Single colonies were picked and checked for purity by repetitive streaking on CMC solid medium.

### Screening on solid and liquid media

Solid media composition, used for the screening of microbial isolates, was the same described above without Remazol Brilliant Blue R. The plates were incubated at 28 °C for 4 days. Afterwards, the strains were assayed for their ability to degrade CMC by incubation with 0.1% Congo red solution for 30 min followed by washing with 5 M NaCl (Kluepfel, 1988). All the strains with a clear halo around the colonies were chosen as positive. A comparison of the cellulase production was then carried out by agar spot method. After adjusting the turbidity of tested bacterial suspensions by comparison with McFarland Turbidity Standard at the value 0.5 (corresponding to about $1.5 \times 10^8$ CFU $mL^{-1}$), in 25 mL of Ringer solution (Sigma-Aldrich), cells were spotted on agar medium in triplicate. Spots were incubated at 28 °C for 4 days and stained with 0.1% Congo red. Experiments were performed in duplicate.

The liquid medium adopted for analysis of cellulase production levels contained 1% CMC, 0.7% yeast extract, 4 g $L^{-1}$ $KH_2PO_4$, 4 g $L^{-1}$ $Na_2HPO_4$, 0.2 g $L^{-1}$ $MgSO_4.7H_2O$, 0.001 g $L^{-1}$ $CaCl_2.2H_2O$, 0.004 g $L^{-1}$ $FeSO_4.7H_2O$ (Abou-Taleb *et al.*, 2009). When indicated, CMC was replaced with an equivalent amount of glucose or cellobiose (AppliChem, Germany).

### Phenotypic characterization of microbial isolates

Morphological analysis of colony of each bacterial strain was carried out observing shape (regular/irregular/rhizoid/punctiform/filamentous), edge (entire/undulate), surface (dry/viscid/powdery), elevation (flat/raised) and colour of colony.

The presence of the enzyme catalase was detected evaluating the immediate appearance of effervescence after dissolving a single colony in 3% hydrogen peroxide (Hanker & Rabin, 1975).

The presence of the enzyme cytochrome oxidase was detected with commercial Oxidase strips following manufacturer's instructions (Oxoid Ltd).

Gram-positive and gram-negative microorganisms were distinguished by means of KOH test as described by Halebian *et al.* (1981).

The cellular morphology was studied with the optic microscope Eclipse E200 (Nikon).

### Inoculum preparation and fermentation process

The bacterial strains were pre-inoculated dissolving a single colony in 3 mL of liquid medium having the composition described in the paragraph 'Screening on solid and liquid media' of section 'Materials and methods' and incubated over night at 28 °C. Fermentation was carried out in 250 mL plugged Erlenmeyer flasks, each containing 20 mL of medium and inoculated with volumes of preinoculum corresponding to 0.1 OD. Fermentations were incubated at 28 °C on rotary shaker at 225 rpm. When indicated, a higher temperature in the range 28–47 °C was adopted. From time to time, samples of liquid cultures were withdrawn and used for measurement of optical density ($OD_{600\ nm}$) and extracellular cellulase activity as below reported. The results of these analytical determinations reported in the figure and table correspond to mean values of the three replicates with a standard deviation lower than 10%.

### CMCase assay on solid medium

A preliminary analysis of levels of cellulase production in liquid medium was performed observing CMC hydrolysis on solid medium. Culture supernatants from different growth times (4–9, 14, 15, 17, 20 and 24 h) were assayed

on solid CMC medium (Lynd *et al.*, 2008) by 0.1% Congo red staining after 1 h incubation at 50 °C.

### Azo-CMCase assay

*endo*-1,4-β-Glucanase activity produced in liquid or submerged culture was assayed by using Azo-CMC (Megazyme, Ireland) as substrate, following supplier's instructions.

### Intracellular protein extraction

Intracellular crude protein extract was obtained by using a French press (Constant System, UK). Pellets obtained after 7 and 15 h of incubation were resuspended in Na phosphate 50 mM pH6.5, before applying a pressure of around 2.5 kbar.

### 16S rRNA gene partial sequence

Total genomic DNA of selected strains was extracted and purified using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) according to the supplier's recommendations. Two synthetic oligonucleotide primers at the 5′ and 3′ end of the 16S rDNA gene, described by Weisburg *et al.* (1991), fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′; *Escherichia coli* positions 8–17 and 1540–1524, respectively), were used to amplify the 16S rRNA gene for all the bacteria, using previously reported conditions (Blaiotta *et al.*, 2002). The PCR amplification fragment was sequenced by Primm srl (Milan, Italy). The sequences were analyzed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S. A., Olivet Cedex, France) and compared to the GenBank nucleotide data library using the Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov), in order to determine their closest phylogenetic relatives.

The partial 16S rDNA gene sequences of the isolates 1, B7B, 6 and B31C have been submitted to EMBL, and the accession numbers are HE590856, HE590857, HE590858 and HE585988, respectively.

### Strain deposition in culture collection

The characterized strain B31C used in this study was deposited in the publicly accessible culture collection DSMZ, and it was assigned to the collection number DSM 25043 *Bacillus* sp. B31C.

### Determination of protein concentration

Protein concentration of crude enzyme preparation was determined by Bradford method using Biorad reactive (München, Germany) following the procedure suggested by the supplier. Bovine serum albumin was used to set up the standard curve.

### Enzyme identification

Proteins secreted by *Bacillus* sp. B31C were precipitated from the cultures by the addition of ammonium sulphate up to 80% saturation, after removing cells by centrifugation. Precipitated proteins were brought in 20 mM Tris–HCl pH 7, by dialysis through ultrafiltration devices with a cut-off of 10 kDa (Millipore S.p.A., Vimodrone, Italy).

Semi-denaturing gel electrophoresis was carried out loading non-denatured and not-reduced samples on a SDS polyacrylamide gel (Laemmli, 1970). Proteins showing cellulolytic activity were visualized following a modified version of the method reported by Beguin (1983). After electrophoresis, the gel was soaked in 20 mM Tris–HCl pH 7 and gently shaken to remove SDS and allow renaturation of the proteins in the gel. The gel was then laid on the top of a thin sheet of 1.5% agar containing 1% CMC. After 1 h incubation at 40 °C, zones of CMC hydrolysis were revealed by staining the agar replica with 0.1% congo red.

Protein identification by mass spectrometry was performed on slices of interest from the non-denaturing PAGE as previously described (Lettera *et al.*, 2010).

### Enzyme purification

Proteins secreted by *Bacillus* sp. B31C precipitated with 80% ammonium sulphate and brought in 20 mM Tris–HCl pH 7 as above described, were loaded on HiTrap Phenyl FF high sub (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A (0.02 M Tris–HCl, 1.2 M (NH4) 2SO4, pH 7.5), and the proteins were eluted isocratically with buffer B (0.02 M Tris–HCl pH 7.5). Fractions containing activity were combined and concentrated on an Amicon PM-10 membrane and analyzed by SDS-PAGE.

### Enzyme characterization

#### Optimum temperature and temperature resistance

To determine the optimum temperature of the purified enzyme, the incubation (10 min) with the substrate Azo-CMC dissolved in 100 mM sodium acetate buffer pH 4.8 was performed at different temperatures. The thermo-resistance of CelB31C was studied by incubating the purified enzyme in 100 mM sodium acetate buffer pH 4.8, at 30–70 °C. The samples withdrawn were assayed for residual Azo-CMCase activity.

The results of the experiments reported in the text and figures correspond to mean values of the three replicates with a standard deviation lower than 10%.

### Determination of $v_{max}$ and $K_M$

For the experiments of enzyme kinetics characterization, cellulase activity was assayed in the total reaction mixture of 1 mL containing 0.5 mL of suitably diluted enzyme and 0.5 mL of 2% (w/v) CMC solution in 50 mM citrate buffer at pH 4.8. This mixture was incubated at 50 °C for 30 min. The release of reducing sugars was determined by the 3, 5-dinitrosalicylic acid method (Miller, 1959). One unit of cellulase activity was defined as the amount of enzyme that liberated 1 μmol reducing sugar per minute from substrate.

The values of Michaelis–Menten constants ($K_M$ and $v_{max}$) of purified CelB31C were identified by linear regression plots of Lineweaver and Burk. The enzyme was incubated at 50 °C with the substrates of different concentrations of CMC ranging from 0.5 to 50 mg $mL^{-1}$ in 50 mM citrate buffer at pH 4.8.

The results of the experiments reported in the text and figures correspond to mean values of the three replicates.

## Results and discussion

### Screening of cellulolytic microorganisms on solid medium

By screening of 90 microorganisms isolated from mature compost on CMC solid medium (Moore *et al.*, 1979; Kluepfel, 1988), 31 cellulolytic microorganisms showing the presence of a clear halo around the colonies were selected. A finer screening of these isolates was performed in normalized growth conditions (4 days at 28 °C), and 15 cellulolytic bacteria with a halo diameter from 6 to 17 mm were selected for further studies.

### Phenotypic characterization of the selected microorganisms

The 15 microorganisms selected on solid medium were characterized from a phenotypic point of view by analysis of colony and cell morphology, gram reaction, and the presence of catalase and oxidase activities. On the basis of these results, the analyzed microorganisms were grouped in five phenotypes (Table 1).

### Screening of cellulolytic microorganisms in liquid medium

A further screening of the 15 selected microorganisms was performed by cultivating them in liquid growth medium and assaying culture samples for cellulase production by plate method on CMC. The strains showing a significant halo intensity were chosen for the further analyses by quantitative CMCase assay with the substrate Azo-CMC. In the Table 2, the values of the maximum Azo-CMCase activity measured for each strain are reported. All the analyzed strains exhibited low activity levels in the earlier growth stages (starting from the fifth hour), and achieved a maximum AZO-CMCase level between the 14th and 24th hour, corresponding to the stationary growth phase.

### Molecular identification of selected cellulolytic microorganisms

The cellulolytic strains 1, B7B, 6 and B31C were identified by sequencing of 16S rRNA gene. 16S rRNA gene sequence analysis showed that the closest relative species (showing a 99% identity) for the strains 1, B7B, 6 and B31C are *Bacillus licheniformis*, *Bacillus subtilis* subsp. *subtilis*, *Bacillus subtilis* subsp. *spizizenii* and *B. amyloliquefaciens*, respectively.

*Bacillus* spp. have been widely reported as cellulolytic microorganisms (Robson & Chambliss, 1984; Fukumori *et al.*, 1985; Kawai *et al.*, 1988; Abou-Taleb *et al.*, 2009). *Bacillus subtilis*, *B. megaterium* and *B. amyoliquefaciens* from soil, *Bacillus pumilis* from rot biomass, *Paenibacillus polymyxa* and *Brevibacillus* sp. from compost are among the species studied for cellulase production (Lynd *et al.*, 2002; Lee *et al.*, 2008; Tamaru *et al.*, 2010).

**Table 1.** Phenotypes of the selected microorganisms; *net diameter measured from the edge of the colony to the outer edge of the halo, after 4 days of growth

| Phenotype | A | B | C | D | E |
|---|---|---|---|---|---|
| Strain | 1; 7; B23; B35 | 6; 8; 34; 38 | $14_{-5}$ A; B7B; B9A; B31B2; E4 | B31B1 | B31C |
| Colony morphology | Irregular, rhizoid flat, dry, yellow | Irregular, flat dry, yellow | Irregular, flat, viscid, yellow | Irregular, flat, transparent | Regular, flat, viscid, light yellow |
| Gram reaction | + | + | + | + | + |
| Catalase activity | + | + | + | + | + |
| Oxidase activity | + | + | + | + | + |
| Cell morphology | Rod-shaped endospore-forming bacteria | Rod-shaped endospore-forming bacteria | Rod-shaped endospore-forming bacteria | Rod-shaped endospore-forming bacteria | Rod-shaped no endospore-forming bacteria |
| Halo size* (mm) | 7.5–10.5 | 6–10 | 12–15 | 14.5 | 14.5 |

**Table 2.** Maximum value of Azo-CMCase activity measured for each strain and the corresponding time of production

| Phenotype | Strain | Maximum value of AZO-CMCase activity (U $mL^{-1}$) | Time (h) |
|---|---|---|---|
| A | 1 | 0.06 | 14–24 |
| B | 6 | 0.08 | 23 |
| | 34 | 0.06 | 20 |
| C | $14_5$A | 0.04 | 17 |
| | B31B2 | 0.04 | 9–24 |
| | B7 B | 0.07 | 14–24 |
| | B9 A | 0.08 | 17–24 |
| | E4 | 0.04 | 14 |
| D | B31B1 | 0.03 | 17 |
| E | B31C | 0.11 | 23 |

Among the manuscripts so far reported on characterization of native cellulases from *Bacillus* spp. (Robson & Chambliss, 1984; Fukumori *et al.*, 1985; Kawai *et al.*, 1988; Singh *et al.*, 2004; Kotchoni *et al.*, 2006; Lee *et al.*, 2008; Liang *et al.*, 2009; Afzal *et al.*, 2010; Annamalai *et al.*, 2011; Zhu *et al.*, 2011; Rawat & Tewari, 2012), only one regards a strain of *B. amyoliquefaciens* (Lee *et al.*, 2008).

## Optimization of culture conditions of *B. amyloliquefaciens* B31C for cellulase production

Cellulase production by the most productive strain *B. amyloliquefaciens* B31C was analyzed in liquid cultures varying the carbon source and growth temperature. The effect of carbon source was analyzed by replacing CMC with an equivalent amount of each one of the alternative tested carbon sources, glucose and cellobiose. Both the tested sugars stimulated *B. amyloliquefaciens* B31C growth, reaching a maximum of *c.* 4 OD $mL^{-1}$ vs. the *c.* 3 OD $mL^{-1}$ reached in CMC medium. The order of growth levels reached with the different sugars glucose > cellobiose > CMC is in agreement with previous results (Liang *et al.*, 2009) for *Brevibacillus* sp.

CMC was shown the most effective carbon source for cellulase production by *B. amyloliquefaciens* B31C, with a maximum value of 0.06 U $mL^{-1}$, that was 3- and 9- fold higher than maximum cellulase activity production in the presence of cellobiose and glucose, respectively. Similar results have been reported for *Bacillus* sp. (Paul & Varma, 1993; Abou-Taleb *et al.*, 2009). In *B. amyloliquefaciens* B31C, glucose causes a decrease of cellulase production suggesting its role of catabolite repressor. Cellobiose and glucose substrates have been shown to be inducers of cellulase production for some cellulolytic microbes (Robson & Chambliss, 1984; Paul & Varma, 1990), whilst glucose has been also reported as a catabolite repressor (Abou-Taleb *et al.*, 2009).

Effects of growth temperature on cellulase activity production of *B. amyloliquefaciens* B31C were also analyzed, culturing the microorganism at 28, 37 and 47 °C. Both the growth and the activity levels were enhanced by increasing temperature from 28 °C (Fig. 1). Growth rate and activity levels for the different temperatures follow the order 37 °C > 47 °C > 28 °C. Ray *et al.* (2007) reported that the minimum cellulase yield by *B. subtilis* and *Bacillus circulans* was observed when fermentation was carried out at 45 °C, while the maximum level was obtained at 40 °C. Immanuel *et al.* (2006) also reported a maximum endoglucanase activity in *Cellulomonas*, *Bacillus* and *Micrococcus* sp. at 40 °C.

Comparison of growth curves of *B. amyloliquefaciens* B31C at temperatures from 28 to 47 °C indicated its thermotolerant nature as expected as a consequence of its adaptation to adverse environmental conditions. Intracellular extracts from bacteria harvested after 7 and 15 h at 37 °C were also assayed for AZO-CMCase activity but no activity was detected.

## Identification of the cellulolytic enzymes produced by the selected *B. amyloliquefaciens* B31C

Proteins responsible for cellulase activity of the most productive strain *B. amyloliquefaciens* B31C were tentatively identified after a fractionation on a semi-denaturing SDS-PAGE where samples from the supernatant of the cell cultures after ammonium sulfate precipitation were loaded without any denaturing treatment. The resulting gel was laid over another gel containing CMC as substrate for cellulase activity detection (Fig. 2). Five protein bands, in correspondence to the area visualized for cellulase activity were excised from the SDS-PAGE and subjected to protein identification after *in situ* digestion and LC-MS/MS analysis of the peptide mixtures. Raw data were used to search protein databases with the MS/MS ion search program on a MASCOT server against the whole unreviewed set of protein entries (3201) that are present in the UniProtKB/TrEMBL database for *B. amyloliquefaciens* strain FZB42.

Interestingly, in one of the protein bands (band 4 in Fig. 2), three peptides matched corresponding peptides of the GH5 family endoglucanase BglC from *B. amyloliquefaciens* FZB42 (UniProt entry: A7Z597), covering 9% of its protein sequence.

Apparently, none of the other proteins identified in the excised bands seems to be responsible for the cellulase activity, although the presence of proteins in *B. amyloliquefaciens* B31C, other than the identified one, with cellulase activity, cannot be ruled out.

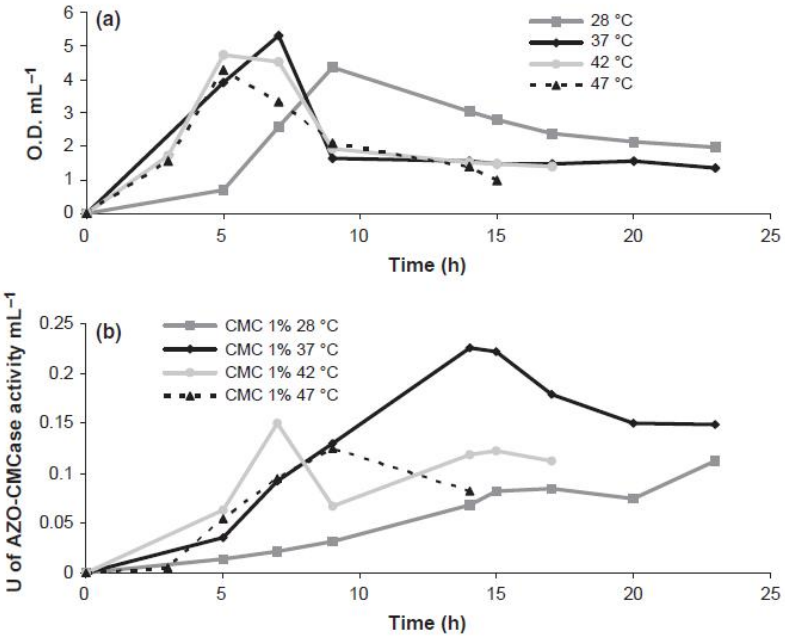


**Figure 1.** Time course of growth (a) and cellulase activity production (b) by *Bacillus amyloliquefaciens* strain B31C in the presence of 1% CMC at different temperatures.

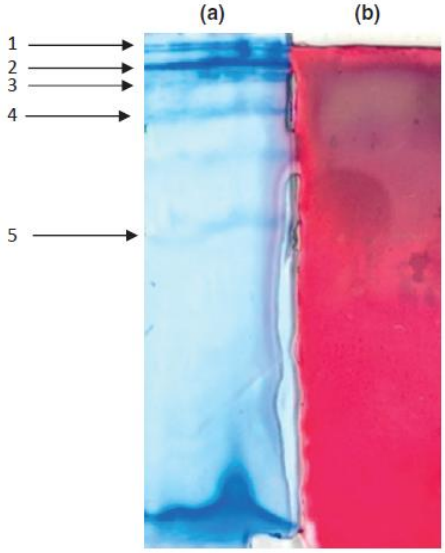


**Figure 2.** Analyses of supernatant of *Bacillus amyloliquefaciens* B31C strain by (a) SDS-PAGE without denaturating treatments stained with Blue Comassie and (b) PAGE containing CMC stained with Congo red for cellulase activity detection.

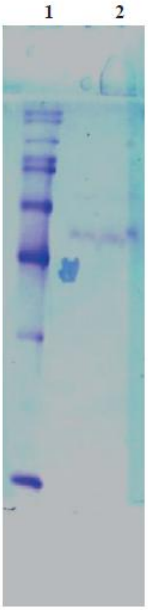


**Figure 3.** SDS-PAGE profiling of the purified CelB31C protein. Lane 1: protein molecular weight marker; lane 2: purified CelB31C.

## Characterization of the enzyme CelB31C

The enzyme CelB31C purified to apparent homogeneity (Fig. 3) was subjected to characterization. The estimated molecular weight deduced from SDS-PAGE was shown 55 000 Da. These results are close to those of Afzal *et al.* (2010) and Zhu *et al.* (2011) reporting *Bacillus* spp. purified CMCases with a molecular weight of 65 and 64 kDa, respectively and very different from those of Kotchoni *et al.* (2006), Singh *et al.* (2004) and Rawat & Tewari (2012) who have reported the molecular mass of purified CMCases from *Bacillus* spp. of around 185, 170 and 183 kDa, respectively.

A range of optimum temperatures from 50 to 70 °C was identified for CelB31C (Fig. 4a). An optimum temperature of 60 °C was reported for the other purified CMCases from *Bacillus* spp. (Singh *et al.*, 2004; Afzal *et al.*, 2010; Zhu *et al.*, 2011; Annamalai *et al.*, 2011; Rawat & Tewari, 2012).

The enzyme showed a higher thermoresistance (Fig. 4b) than the other cellulases so far characterized from *Bacillus* spp. (Singh *et al.*, 2004; Afzal *et al.*, 2010; Zhu *et al.*, 2011; Annamalai *et al.*, 2011; Rawat & Tewari, 2012) and from *B. amyloliquefaciens* DL-3 (Lee *et al.*, 2008).

CelB31C follows a Michaelis–Menten kinetic towards CMC: the $K_M$ for this substrate is 9.95 ± 1.4 mg $mL^{-1}$ similar to those reported by Rawat & Tewari (2012) and Afzal *et al.* (2010), and the $v_{max}$ 710.7 ± 3.5 mg $mL^{-1}$ $min^{-1}$ similar to that reported by Rawat & Tewari (2012) and 10-fold higher than that of the cellulase described by Afzal *et al.* (2010).

## Conclusions

The strain *B. amyloliquefaciens* B31C isolated from compost produces extracellular proteins exhibiting higher cellulase activity levels than the other analyzed strains. An endoglucanase matching with the endoglucanase BglC from *B. amyloliquefaciens* FZB42 (UniProt entry: A7Z597) was identified in *B. amyloliquefaciens* B31C. The cellulase purified from B31C, CelB31C, shows a significant thermoresistance and a range of optimum temperatures from 50

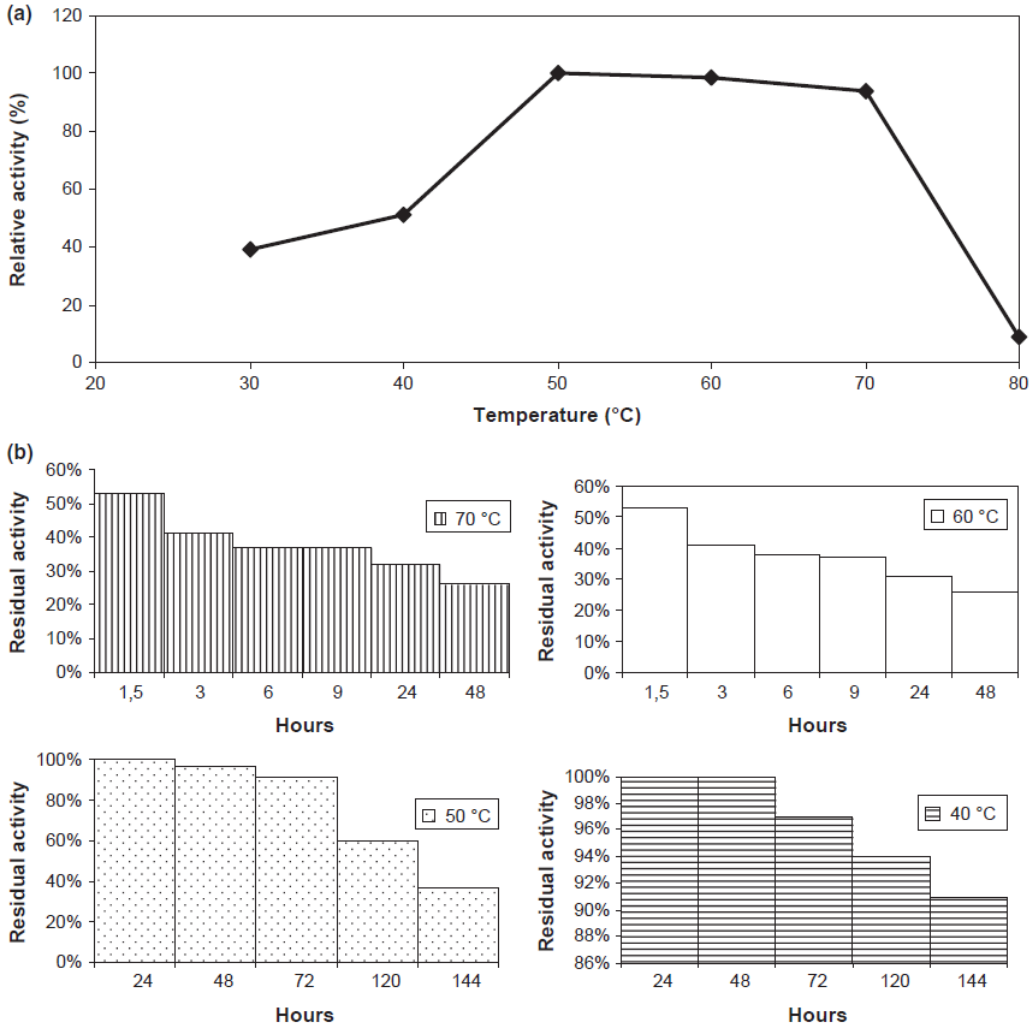


**Figure 4.** Influence of temperature on the enzyme activity and thermo-resistance of CelB31C. (a) Effect of temperature on the Azo-CMCase enzyme activity. The Azo-CMCase activity was measured at the temperatures ranging from 30 to 80 °C; (b) Thermo-resistance. Thermo-resistance was determined after incubating the enzyme at different temperatures ranging from 30 to 80 °C, at pH 6.2 for different intervals of time, and the residual enzyme activities were determined by the Azo-CMCase assay.

to 70 °C. Retention of 90% of activity for at least 144 h of incubation of the purified enzyme CelB31C at 40 °C and the observation that its activity was not affected by increasing the assay temperature up to 70 °C, highlight its potential for cellulose conversion. CelB31C could be therefore an interesting candidate as biocatalyst in pretreatment stage for second generation bioethanol production.

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## 2.2.2 Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces* sp. G12 isolated from compost (Paper IV)

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# Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces* sp. G12 isolated from compost

Antonella Amore[1], Olimpia Pepe[2], Valeria Ventorino[2], Leila Birolo[1,3], Chiara Giangrande[1] and Vincenza Faraco[1,3*]

**Abstract**

**Background:** The use of lignocellulosic materials for second generation ethanol production would give several advantages such as minimizing the conflict between land use for food and fuel production, providing less expensive raw materials than conventional agricultural feedstock, allowing lower greenhouse gas emissions than those of first generation ethanol. However, cellulosic biofuels are not produced at a competitive level yet, mainly because of the high production costs of the cellulolytic enzymes. Therefore, this study was aimed at discovering new cellulolytic microorganisms and enzymes.

**Results:** Different bacteria isolated from raw composting materials obtained from vegetable processing industry wastes were screened for their cellulolytic activity on solid medium containing carboxymethylcellulose. Four strains belonging to the actinomycetes group were selected on the basis of their phenotypic traits and cellulolytic activity on solid medium containing carboxymethylcellulose. The strain showing the highest cellulolytic activity was identified by 16S rRNA sequencing as belonging to *Streptomyces* genus and it was designated as *Streptomyces* sp. strain G12. Investigating the enzymes responsible for cellulase activity produced by *Streptomyces* G12 by proteomic analyses, two endoglucanases were identified. Gene coding for one of these enzymes, named CelStrep, was cloned and sequenced. Molecular analysis showed that the *celstrep* gene has an open reading frame encoding a protein of 379 amino acid residues, including a signal peptide of 37 amino acid residues. Comparison of deduced aminoacidic sequence to the other cellulases indicated that the enzyme CelStrep can be classified as a family 12 glycoside hydrolase. Heterologous recombinant expression of CelStrep was carried out in *Escherichia coli*, and the active recombinant enzyme was purified from culture supernatant and characterized. It catalyzes the hydrolysis of carboxymethylcellulose following a Michaelis–Menten kinetics with a $K_M$ of 9.13 mg/ml and a $v_{max}$ of 3469 μM $min^{-1}$. The enzyme exhibits a half life of around 24 h and 96 h at 60°C and 50°C, respectively and shows a retention of around 80% of activity after 96 h at 40°C.

**Conclusions:** In this manuscript, we describe the isolation of a new cellulolytic strain, *Streptomyces* sp. G12, from industrial waste based compost, the identification of the enzymes putatively responsible for its cellulolytic activity, the cloning and the recombinant expression of the gene coding for the *Streptomyces* sp. G12 cellulase CelStrep, that was characterized showing to exhibit a relevant thermoresistance increasing its potential for cellulose conversion.

**Keywords:** *Streptomyces*, Cellulases, Recombinant expression

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## Background

Demand for energy has more than doubled in the last 30 years in the Mediterranean where an under-exploitation of biomass for biofuel production takes place, biomass accounting for just 21% of the total renewable capacity. Adopting lignocellulosic residues as raw materials for effective large-scale production of bioethanol fuel in the Mediterranean is an urgent requirement if the region's growing energy demands are to be met and the climate change effects there alleviated [1]. Lignocellulosic biomass is an attractive material for production of a wide range of high added value products, such as fuel ethanol. Lignocellulose is the most abundant renewable resource on Earth, and it constitutes a large component of the wastes originating from municipal, agricultural, forestry and some industrial sources. The use of lignocellulosic materials would minimize the conflict between land use for food and feed production and energy and bioproducts feedstock production. This raw material is less expensive than conventional agricultural feedstock and can be produced with lower input of fertilizers, pesticides and energy [2].

However, cellulosic biofuels are not produced at a competitive level yet, due to the high cost of processing with the currently available technologies [3].

Conversion of cellulose into fermentable sugars for ethanol production is currently performed by enzymatic hydrolysis carried out by cellulases, produced by a wide variety of microorganisms, depolymerizing such a polymer and playing a major role in the recycling of biomass. Wild type and mutant strains of *Trichoderma* spp., have long been considered to be the most powerful producers of cellulases [4]. The production costs of these enzymes are very high and a huge amount of hydrolytic enzymes is required for hydrolysis on industrial scale.

Bacteria, showing higher growth rate than fungi have good potential to be used in cellulase production. Among bacteria, the actinomycetes *Thermomonospora fusca* [5-8], *Streptomyces thermodiastaticus* [5], *Thermomonospora curvata* [9-13], *Streptomyces viridosporus* and *S. setonii* [14] as well as other strains of *Streptomyces* sp. [15,16] and other actinomycetes [17] were reported as producers of cellulolytic activity.

This study was aimed at isolating a new cellulolytic bacterium and characterizing the enzymes responsible for its cellulolytic capabilities in order to catch better biocatalysts for second generation ethanol production. Identification of the enzymes putatively responsible for the cellulolytic activity of the strain *Streptomyces* sp. G12, the most active cellulase producer among the cellulolytic actinomycetes isolated from compost, is reported. Moreover, cloning and sequencing of the gene encoding one of the identified cellulases and its heterologous recombinant expression in *Escherichia coli* are described along with characterization of the recombinant enzyme.

## Methods

### Cellulolytic bacteria isolation

Cellulolytic microorganisms were isolated from mature compost obtained from agro-industrial wastes consisting of pomace with kernel (65%), liquid sewage sludge (22%) from industrial processing of vegetable (potatoes and carrotoes) and borland molasses (13%). Representative samples of 1 Kg were taken from the external (right and left side of the pile, about 5-10 cm of depth) and internal central part (at about 40 cm of depth) of biomass. Microbial isolates were obtained in solid media following the method described by Hankin and Anagnostic [18] with some modifications. Initial compost suspensions were prepared by the addition of 20 g (w/v) of compost samples to 180 ml of quarter strength Ringer's solution (Oxoid, Ltd., Oxford, UK) in 250 ml Erlenmeyer flasks. After shaking, suitable dilutions were made in the same solution and were used to inoculate solid media of growth composed by 5 g $L^{-1}$ carboxymethylcellulose (CMC) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1 g $L^{-1}$ $(NH_4)NO_3$, 1 g $L^{-1}$ yeast extract, 50 ml $L^{-1}$ standard salt solution, 1 ml $L^{-1}$ trace elements solution, 0.02% Remazol Brilliant Blue R [19], 10 g $L^{-1}$ bacteriological agar, at pH 7.0. After incubation at 28°C for 7 days, the plates were flooded with a Remazol Brilliant Blue R solution to put better in evidence the presence of clear haloes around the cellulolytic colonies. Single colonies were picked and checked for purity by repetitive streaking on CMC solid medium. All isolates were kept at 4°C on solid medium until analyses.

### Screening on solid and liquid media

Solid media composition, used for the screening of microbial isolates, was the same described above without Remazol Brilliant Blue R. The plates were incubated at 28°C for 4 days. Afterwards, the strains were assayed for their ability to degrade CMC by incubation with 0.1% Congo red solution for 30 minutes followed by washing with 5 M NaCl [20]. All the strains with a clear halo around the colonies were chosen as positive. A comparison of the cellulase production was then carried out by agar spot method. After adjusting the turbidity of tested bacterial suspensions by comparison with McFarland Turbidity Standard at the value 0.5 (corresponding to about $1.5 * 10^8$ $CFUmL^{-1}$), in 25 mL of Ringer solution (Sigma-Aldrich), cells were spotted on agar medium in triplicate. Spots were incubated at 28°C for 4 days, stained with 0.1% Congo red and activity halos dimensions were measured from the border of the colony to the outer edge of the halo. Experiments were performed in duplicate.

The liquid medium adopted for analysis of cellulase production levels contained 1% CMC, 0.25% $(NH_4)_2SO_4$, 0.05% yeast extract, 0.27% $KH_2PO_4$, 0.53% $Na_2HPO_4$, 0.02% NaCl, 0.02% $MgSO_4$-$7H_2O$, 0.005% $CaCl_2$ [5]. As far as *Streptomyces* strain G12 is concerned, other carbon sources were tested replacing CMC with an equivalent carbon amount of glucose, cellobiose, xylose or xylan (AppliChem, Germany), or using a combination of 0.6% CMC and 0.4% glucose or cellobiose. For this strain, submerged fermentation was also carried out using wheat straw (2% w/v) as carbon source and testing different concentrations of yeast extract (0.05%, 0.1%, 0.7%). The straw was reduced in small piece and sieved to have different dimension particles (< 0.8 mm, 0.8-2 mm, > 2 mm), moisturized with water (1:1 w/v) and autoclaved for 1 h at 110°C.

### Phenotypic characterization of microbial isolates

Morphological analysis of colony of each bacterial strain was carried out observing shape (regular/irregular/rhizoid/punctiform/filamentous), edge (entire/undulate), surface (dry/viscid/powdery), elevation (flat/raised) and colour of colony.

The presence of the enzyme cytochrome oxidase was detected with commercial Oxidase strips (Oxoid Ltd, England).

Gram-positive and gram-negative microorganisms were distinguished by mean of KOH test as described by Halebian et al. [21].

The cellular morphology was studied with the optic microscope Eclipse E200 (Nikon).

### Inoculum preparation and fermentation process

The bacterial strains were pre-inoculated dissolving a single colony in 10 mL of liquid medium having the composition described in the second paragraph of Methods section "Screening on solid and liquid media" and incubated over night at 28°C. Fermentation was carried out in 250 ml plugged Erlenmeyer flasks, each containing 50 mL of medium and inoculated with volumes of pre-inoculum corresponding to 0.8 O.D. After pre-inocula homogenization by ULTRA-TURRAX®, The inocula were incubated at 28° C on rotary shaker at 225 rpm for fifteen days. When indicated, a higher temperature in the range 28-47°C was adopted. From time to time, samples of liquid cultures were withdrawn and used for measurement of optical density ($O.D._{600nm}$) and extracellular cellulase activity. The results of these determinations reported in the figures and tables correspond to mean values of three independent experiments performed in three replicates.

### Intracellular protein extraction

Intracellular crude protein extract of *Streptomyces* G12 was obtained by using a French press (Constant System, UK). Pellets obtained after 6 days of growth in liquid culture were resuspended in sodium phosphate 50 mM pH 6.5, before applying a pressure of around 2.5 kbar.

### CMCase ctivity spot assay on solid medium

A preliminary analysis of levels of cellulase production in liquid medium was performed spotting 50 microliter of culture supernatants from different growth times (4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 14 h, 15 h, 17 h, 20 h and 24 h) on solid CMC medium. After 1 h incubation at 50°C, the CMC plates were flooded by 0.1% Congo red staining, as described in the second paragraph of Methods section "Screening on solid and liquid media".

### Azo-CMC assay

*endo*-1,4-ß-Glucanase activity produced in liquid or submerged culture was assayed by using Azo-CMC (Megazyme, Ireland) as substrate, following supplier instructions and determined by referring to a standard curve.

### Azo-avicelase assay

Avicelase activity was measured by using Azo-Avicel (Megazyme, Ireland) as substrate, following supplier instructions.

### 16S rRNA partial sequence

Total genomic DNA of selected strains was extracted and purified using InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA) according to the supplier's recommendations. Two synthetic oligonucleotide primers at the 5' and 3' end of the 16S rDNA, described by Kumar et al. [22] 9F GAGTTTGATCCTGGCTCAG and 1541R AAGGA GGTGATCCAACC were used to amplify the 16S rRNA gene. PCR was performed as previously reported [23]. The PCR amplification fragment was purified by agarose (1.5% wt/vol) gel electrophoresis using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequencing was ordered at Primm srl (Milan, Italy). The sequences were analysed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S. A., Olivet Cedex, F) and compared to the GenBank nucleotide data library using the Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov), in order to determine their closest phylogenetic relatives.

The partial 16S rDNA sequence of the isolate G12 has been submitted to EMBL, and the accession number is HE585989.

### Determination of protein concentration

Protein concentration of crude enzyme preparation was determined by Bradford method using Biorad reactive (München, Germany) following the procedure suggested by the supplier. Bovin serum albumin (BSA) was used to set up the standard curve.

## Enzyme identification

### *Protein fractionation*

Secreted proteins produced by *Streptomyces* sp. G12 were precipitated from the cultures corresponding at the maximum cellulase production by the addition of ammonium sulphate up to 80% saturation, after having removed cells by centrifugation. Precipitated proteins were recovered by centrifugation at 7500 rpm for 45 minutes at 4°C and brought in 50 mM $Na_2HPO_4$ pH 6.5, by dialysis through ultrafiltration devices with a cut-off of 10 kDa (Millipore S.p.A., Vimodrone Italy).

### *Zymogram analyses*

Semi-denaturing gel electrophoresis was carried out loading non-denatured and not-reduced samples on a SDS polyacrylamide gel, performed as described by Laemmli [24]. Proteins showing cellulolytic activity were visualized following a modified version of the assay reported by Béguin [25]. After electrophoresis, the gel was soaked in the same buffer used for dissolving proteins and gently shaken to remove SDS and rinature the proteins in the gel. The gel was then laid on the top of a thin sheet of 1.5% agar containing 1% CMC. After 1 h incubation at 40°C, zones of CMC hydrolysis were revealed by staining the agar replica with 0.1% of Congo red.

### *Protein identification by mass spectrometry*

Slices of interest from the semi-denaturing PAGE were cut and *in situ* digested after extensive destaining with 0.1 M $NH_4HCO_3$ pH 7.5 and acetonitrile, reduction of disulphide bonds for 45 minutes in 100 μl of 10 mM dithiothreitol, 0.1 M $NH_4HCO_3$, pH 7.5 and carboxyamidomethylation of thiols for 30 minutes in the dark by addition of 100 μl of 55 mM iodoacetamide dissolved in the same buffer. Enzymatic digestion was performed by adding to each slice 100 ng of proteomic-grade trypsin 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 50 μl of 10 mM $NH_4HCO_3$ pH 7.5 were added and incubated for 18 hours at 37°C. Peptides were extracted with 20 μl of 10 mM $NH_4HCO_3$, 1% formic acid, 50% acetonitrile at room temperature.

Peptide mixtures were filtered on 0.22 μm PVDF membrane (Millipore) and analysed by LC-MSMS on 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 is concentrated and washed in 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as the eluent. The sample is then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at a flow rate of 400 nl/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 is 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. MS/MS spectra were measured automatically when the MS signal is over the threshold of 50000 counts. Double and triple charged ions were preferably isolated and fragmented over single charged ions. Raw data from nanoLC–MS/MS analyses transformed in *mz.data* format and used to query nonredundant protein databases with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA). Additional search parameters were a peptide mass tolerance set at 10 ppm and a fragment mass tolerance of 0.6 Da, up to 3 allowed missed cleavages, carbamidomethylation of cysteines as fixed modification, oxidation of methionine, and cyclization of N-term Q to pyro-Glu as variable modifications. Only doubly and triply charge ions were considered. Ions score is -10 log(P), where P is the probability that the observed match is a random event. The threshold above which the individual ions score indicates identity or extensive homology ($p < 0.05$) can vary from search to search. In our searches, on average, individual ion scores >25 indicated identity or extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a nonprobabilistic basis for ranking protein hits (http://www.matrixscience.com/help/interpretation_help.html). Trypsin, dithiothreitol, iodoacetamide and $NH_4HCO_3$ were purchased from Sigma. Trifluoroacetic acid (TFA)-HPLC grade was from Carlo Erba (Milan, Italy). All other reagents and solvents were of the highest purity available from Baker.

## Gene isolation and sequencing

Chromosomal high-molecular weight DNA from *Streptomyces* sp. G12 was prepared as described by Raeder and Broda [26]. To synthesize gene coding for the enzyme CelStrep from *Streptomyces* sp. G12, Polymerase Chain Reaction (PCR) experiments were performed using *Streptomyces* sp. G12 genomic DNA as template and degenerate and specific oligonucleotide primers reported in Table 1: central region was amplified with the oligonucleotides 1 FW/1 REV, the 5' terminal region

**Table 1 Nucleotide sequences of oligonucleotides used to amplify *celstrep* gene**

| Primer | Nucleotide sequence | Annealing temperature (°C) |
|---|---|---|
| 1 FW | GCCACCGACTCSGGCTTC | 62 |
| 1 REV | CKGTTGAACCAGATCAT | 48-50 |
| 2 FW | ATGCCSCGSCTSCGSCACCAC | 74 |
| 2 REV | CGCCCCCACGGCGAACC | 62 |
| 3 FW | CGCCTCGTACGACATCTGG | 62 |
| 3 REV | SACAGTSGAGCASGCSGTSCC | 74 |

with the oligonucleotides 2 FW/2 REV, the 3' terminal region with the oligonucleotides 3 FW/3 REV and their sequencing by dideoxy chain termination method was performed by PRIMM Sequencing Service (Naples, Italy), using universal and specific oligonucleotide primers.

### Analysis of gene and protein sequences

Sequence of gene coding for the enzyme CelStrep from Streptomyces sp. G12, named *celstrep*, was deposited with the EMBL Data Library under accession number HE862416. Alignments of DNA and of deduced amino acid sequences were generated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Signal peptide prediction was achieved using SignalP V4.0 (http://www.cbs.dtu.dk/services/SignalP/). Potential N-glycosylation sites (Asn-XXX-Ser/Thr) were computed on the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/).

### Heterologous recombinant expression

Gene *celstrep* was cloned into the expression vector pET28a (Novagen, Inc.) by *Nde*I and *HindIII* restriction sites to generate celstrep-pET28a, and it was expressed in *E. coli* strain BL21 CodonPlus (DE3) RP (Novargen Ltd).

Cells were cultured with a rotary shaker at 37°C until 1 $OD_{600}$ and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h at 37°C.

After centrifugation, supernatant was assayed for Azo-CMCase activity and the cells were used to obtain intracellular crude protein extract by using a French press (Constant System, UK). Pellets of liquid cultures were resuspended in Na phosphate 50 mM pH6.5, before applying a pressure of around 2.5 kbar. After centrifugation, the soluble fraction was also adopted to assay cellulase activity.

### Recombinant enzyme purification

Proteins present in the culture supernatant of *E. coli* expressing *celstrep* at maximum production time were precipitated with ammonium sulphate up to 80% saturation and brought in 20 mM Tris–HCl pH 7. The proteins were then loaded on HiTrap Phenyl FF high sub (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A (0.02 M Tris–HCl, 1.2 M (NH4)2SO4, pH 7.5), and the proteins were eluted isocratically with buffer B (0.02 M Tris–HCl pH 7.5). Fractions containing activity were combined and concentrated on an Amicon PM-10 membrane and analyzed by SDS-PAGE.

### Recombinant enzyme characterization

#### *Optimum temperature and temperature resistance*

To determine the optimum temperature of the purified recombinant enzyme, the substrate of the activity assay (Azo-CMC) was dissolved in 100 mM sodium acetate buffer at pH 4.8 and the incubation (10 min) was performed at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C. The thermo-resistance of CelStrep was studied by incubating the purified enzyme preparation in 100 mM sodium acetate buffer pH 4.8, at 40°C, 50°C, 60°C, 70°C and 80°C. The samples withdrawn were assayed for residual Azo-CMCase activity.

The results of these experiments reported in the manuscript correspond to mean values of three independent experiments performed in three replicates.

#### *Determination of $v_{max}$ and KM*

For the experiments of enzyme kinetics characterization, cellulase activity was assayed in the total reaction mixture of 1 ml containing 0.5 ml of suitably diluted enzyme and 0.5 ml of 2% (w/v) CMC solution in 50 mM citrate buffer at pH 4.8. This mixture was incubated at 40°C for 30 min. The release of reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method [27]. One unit of cellulase activity was defined as the amount of enzyme that liberated 1 μmol reducing sugar per minute from substrate.

The values of Michaelis–Menten constants ($K_M$ and $v_{max}$) of purified recombinant cellulase were identified by linear regression plots of Lineweaver and Burk. The enzyme was incubated at 50°C with the substrates of different concentrations of CMC ranging from 0.5 to 50 mg/ml in 50 mM citrate buffer at pH 4.8.

The results of these experiments reported in the manuscript correspond to mean values of three independent experiments performed in three replicates.

## Results and discussion

### Screening and selection of cellulolytic microorganisms

#### *Screening and phenotypic characterization of cellulolytic microorganisms*

Ninety microorganisms isolated from mature compost obtained from agro-industrial wastes were screened for their cellulolytic activity on CMC solid medium, by Congo red staining. From this preliminary selection, 4 cellulolytic bacteria with a clear halo diameter from 6 to 17 mm around the colonies (data not shown) were selected.

The four microorganisms selected on solid medium were characterized from a phenotypic point of view by analysis of colony and cell morphology, gram reaction, and the oxidase activities. On the basis of these results, the analyzed microorganisms were grouped in two phenotypes. All the strains showed myceliar morphology,

**Table 2 Phenotype of the selected actinomycetes strains**

| PHENOTYPE | F | G |
|---|---|---|
| **Strain** | **$14_9$, $14_{13}$** | **$14_{12}$, G12** |
| **Colony morphology** | Grey regular mycelium with white margins | White-grey regular mycelium |
| **Cell morphology** | Spore-bearing hyphae | Spore-bearing hyphae |
| **Gram reaction** | + | + |
| **Oxidase activity** | + | + |
| **Halo size* (mm)** | 11 | 10 |

*net diameter measured from the edge of the colony to the outer edge of the halo, after 4 days of growth.

share positive gram reaction and presence of oxidase activities (Table 2).

### *Screening of cellulolytic microorganisms in liquid medium*

A further screening of the four selected microorganisms was performed by cultivating them in liquid medium and assaying culture supernatants for cellulase production by both plate method on CMC and AZO-CMCase activity assay. The qualitative plate medium assay showed that cellulase production by the actinomycete strain G12 starts from the 1st day with a maximum at the 6th day. AZO-CMCase activity assay confirmed G12 to be the most productive strain, with a production ~ 4-fold higher than the other tested strain, as shown in Figure 1.

### Molecular identification

The strain G12 was identified by sequencing of 16S rRNA gene and it was shown to belong to *Streptomyces* sp.

### Optimization of cellulolytic activity production by the selected *Streptomyces* G12 strain

The effect of carbon source on cellulase production by *Streptomyces* G12 strain was tested, using different concentrations (0.1%, 0.5%, and 1%) of CMC, substituting CMC with glucose, cellobiose, xylose or xylan, and also testing combinations of CMC and glucose or cellobiose. 1% CMC was proved to be the best condition for cellulase activity production, reaching a value of 0.0875 ± 0.025 $UmL^{-1}$ (at the 3rd day), that was 1.75-, 2.5-, 3-, 5- and 7-fold higher than that reached in the presence of 0.5% CMC, xylan, cellobiose, xylose and 0.1% CMC, respectively.

Then, the order of cellulase activity production was 1% CMC > 0.5% CMC > xylan > cellobiose > xylose > 0.1% CMC. As far as cellulase activity production by actinomycetes is concerned, very variable effects were reported for other strains. Cellobiose and glucose have been previously reported as good [6,7,28] or poor [16,29-31] inducers but they may also act as repressors of cellulase activity in some cases [6,32,33]. Xylose and xylan are often reported as non inducing cellulase activity [7,16,30], but in some cases they stimulate cellulase production [29,30,34].

The effect of nitrogen source concentration and nature was also investigated, but no positive effect was obtained either replacing yeast extract with 0.3 $gL^{-1}$ urea [33] or increasing yeast extract concentration by 2-fold [15,35]. When influence of temperature was analyzed, similar cellulase activity levels by *Streptomyces* G12 were achieved (Figure 2A). The highest cellulase production (0.1$UmL^{-1}$) in liquid medium was achieved at 28°C, in the presence of 1% CMC and 0.05% yeast extract, after 6 days of incubation (Figure 2A).

Because of the low endoglucanase activity levels obtained by growing *Streptomyces* sp. strain G12 on the tested soluble substrates, an inducing carbon source was searched among insoluble substrates, often reported as good

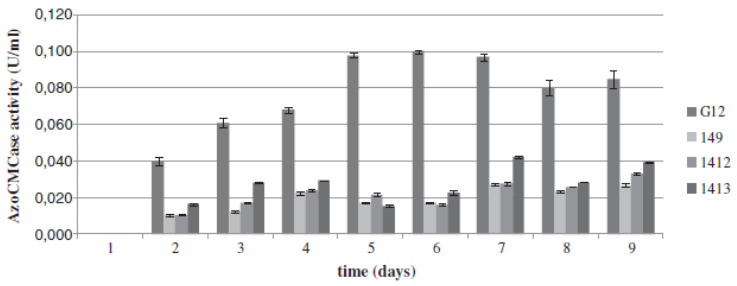


**Figure 1 Cellulase activity production of the selected actinomycete strains.** The results reported in this figure correspond to mean values of the three independent experiments performed in three replicates.

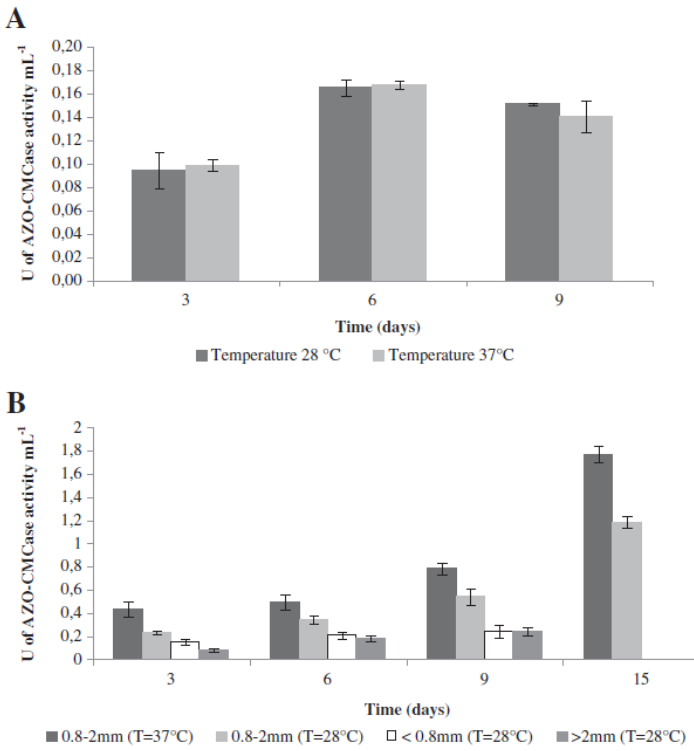


**Figure 2 Cellulase activity production levels achieved by *Streptomyces* G12 in: (A) liquid culture in the presence of 1% CMC at different temperatures; (B) submerged fermentation on wheat straw with different sizes (<0.8 mm; 0.8-2 mm; >2 mm) at 28°C and on wheat straw (0.8-2 mm particles) at different temperatures.** The results reported in this figure correspond to mean values of the three independent experiments performed in three replicates.

inducers of lignocellulosic activity [7,16,29,36]. Therefore, a submerged fermentation on wheat straw was set up. These fermentation conditions were shown to be the best ones among those explored, thus encouraging application of the isolated strain to waste upgrading by solid state fermentation. Comparing different wheat straw sizes (<0.8 mm; 0.8-2 mm; >2 mm), it was shown that particles in the range 0.8-2 mm give the highest production level (Figure 2B). When yeast extract concentration was increased up to 0.7% [15,28], no effect on cellulase activity production was revealed. Temperature strongly affects cellulase production in the presence of wheat straw as carbon source, and increasing from 28 to 37°C enhances cellulase activity by 1.5-fold (Figure 2B). Analysis of time course revealed that cellulase production continuously increases up to around 1.80 $UmL^{-1}$ at the 15th day of the growth.

### Cellulases identification

Proteins putatively responsible for cellulase activity were tentatively identified after a fractionation on a semi-denaturing SDS-PAGE where samples from the supernatant of the cell cultures were loaded without any denaturing treatment. The resulting gel was laid over another gel containing CMC as substrates for cellulase activity detection. Two activity positive bands were visualized, and in correspondence to these active bands, slices were excised from the SDS-PAGE and subjected to protein identification after *in situ* digestion and LC-MS/MS analysis of the peptide mixtures. Raw data were used to search non redundant NCBI database available on the net with no taxonomic restriction with the MS/MS ion search program on a MASCOT server as described above.

Several proteins could be confidently identified in different *Streptomyces* spp., two of which belong to the class of Carbohydrate active enzymes: 4 peptides matched to peptides present in the sequence of a GH6 family endoglucanase from *Streptomyces halstedii* (P33682), accounting for 19% of its protein sequence, and 8 peptides matched to peptides that are in the sequence of a GH12 family cellulase from *Streptomyces xylophagus* (D2D3J0), accounting for 41% of its protein sequence (Table 3).

### Sequences of the cellulase gene and its derived protein

The gene coding for the cellulase from *Streptomyces* G12 strain similar to GH12 family cellulase from

**Table 3 Results of protein identification in the SDS-PAGE in correspondence to the active bands including as significative only proteins identified with at least two peptides**

| Identified protein (Accession number) | Total score [a] | Matched sequence (individual ion score) | Sequence coverage (%) |
|---|---|---|---|
| Endoglucanase I (gi\|544459) | 342 | LPILVAYNIYNR (44) | 19% |
| | | AAAINASIANTPMAR (66) | |
| | | YGYTKPFWDTSR (50) | |
| | | QAPNTWVYMDAGNPR (92) | |
| beta-1,4-endoglucanase (gi\|224555766) | 418 | TEIMIWFNR (25) | 41% |
| | | VGPIQPIGSQVGTADVAGR (91) | |
| | | WGTSATQCVTATDSGFR (27) | |
| | | TDGVNRTEIMIWFNR (43) | |
| | | SYPSVFNGCHYTNCSPGTALPAR (33) | |
| | | WGTSATQCVTATDSGFRVTQADGSVPTDGAPK (37) | |
| | | LGFTLPSGQSVVHAWNASVAPSSGAVTATGPADSPR (49) | |
| | | INGISSAPSSISYGYVDNAVYNASYDIWLDPTPR (49) | |

[a] Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 48 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. (http://www.matrixscience.com/help/interpretation_help.html).

*Streptomyces xylophagus* (D2D3J0) was synthesized by PCR on *Streptomyces* G12's genomic DNA using as primers both degenerate oligonucleotides whose sequences were designed on the basis of sequences of peptides identified by proteomics or sequences of peptides in similar proteins and specific oligonucleotides whose sequences were designed on the basis of sequence of amplified central region (Table 1), to amplify three overlapped fragments.

A signal peptide sequence of 37 amino acids was singled out by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The mature protein is 342 amino acids in length and has a calculated molecular mass of 35369.67 Da.

Three potential N-glycosylation sites (Asn-X-Ser/Thr) were found in deduced amino acid sequence namely Asn154, Asn192 and Asn 293.

Similarity searches performed with Basic Local Alignment Search Tool (BLAST http://www.ebi.ac.uk/Tools/sss/wublast/) using the deduced amino acid sequence of CelStrep as the query revealed its belonging to family GH12 enzymes (http://www.cazy.org/GH12.html; [37]) extracted from the Carbohydrate-active enzymes database (http://www.cazy.org/, [38]). The best hits (99% and 93% identities) were with Beta-1,4-endoglucanase from *Streptomyces xylophagus* (EMBL Accession FJ441063) and eglS Cellulase from *Streptomyces rochei* [39] (EMBL Accession X73953), respectively, followed by enzymes from *Streptomyces ghanaensis* ATCC 14672 (EMBL Accession DS999641) and *Streptomyces griseoflavus* Tu4000 (EMBL Accession GG657758) displaying 84 and 83% identities respectively.

The family GH12 glycoside hydrolases have a catalytic mechanism with retention of configuration with two glutamates involved in catalysis, one acting as an acid/base and the other as a nucleophile [37].

Three-dimensional structures of family GH12 cellulases have been obtained for one enzyme from Archea *Pyrococcus furiosus* DSM 3638 (GenBank Accession AAD54602.1), five bacterial enzymes namely from *Bacillus licheniformis* ATCC 14580/DSM13 [40], from *Rhodothermus marinus* [41], from *Streptomyces lividans* 1326 [42], from *Streptomyces* sp. 11AG8 [43], from *Thermotoga maritima* MSB8 [44], and six fungal enzymes, namely from *Aspergillus aculeatus* F-50, *Aspergillus aculeatus* ATCC 16872 [45], *Aspergillus niger* CBS 120.49/N400 [46], *Humicola grisea* ATCC 22081 [47] *Hypocrea jecorina* QM9414 [48], *Hypocrea schweinitzii* ATCC 66965 [49]. The catalytic domain is a β-jelly roll catalytic domain.

Based on the sequence alignment, the potential catalytic glutamates of CelStrep are located at positions 156 (nucleophile) and 240 (acid/base).

### Recombinant expression system

Because of the difficulties to purify the native CelStrep enzyme from *Streptomyces* G12 due to the presence of another cellulase isoform with very similar chromatographic behavior (data not shown), recombinant expression system was set up to characterize the new cellulase. The enzyme CelStrep was expressed in *E. coli*. Synthesis of *celstrep* gene was performed by MrGene (http://mrgene.com). The full-length *celstrep* gene sequence including sequence coding for signal peptide was optimized for recombinant expression by adapting it to the *E. coli* codon usage.

The *celstrep* gene was expressed under the control of T7 RNA polymerase promoter in *E. coli* strain BL21 CodonPlus (DE3) RP. Protein expression was induced with 1 mM IPTG added to the cells grown until 1 $OD_{600}$, for 6 hours at 37°C. 1 $Uml^{-1}$ of Azo-CMCase activity was detected in the culture supernatant, and a similar activity level was revealed in the soluble fraction of intracellular proteins.

This achievement suggests the applicability of the developed *E. coli* system as a cell factory for extracellular production of other bacterial cellulases for their purification and characterization, also considering that not native cellulases are present in the bacterial host differently from the fungal host *Trichoderma reesei* that, on the contrary, is more appropriate for cellulase overproduction due to the very higher level of recombinant expression [50].

### Characterization of the recombinant enzyme CelStrep

The recombinant enzyme CelStrep was purified from culture supernatant of transformed *E. coli* strain BL21 to apparent homogeneity (Figure 3) and subjected to structural and functional characterization. The few manuscripts so far reported on characterization of cellulases from *Streptomyces* spp. mostly concern native enzymes [51,52]. Only one *Streptomyces* cellulase was so far produced by recombinant expression in *E. coli* [53].

The estimated molecular weight deduced from SDS-PAGE was shown to be around 37,000 Da, similar to that deduced from gene sequence of the mature protein. These results are close to those of Wittmann et al. [52], Irdani et al. [53] and Theberge et al. [51] reporting CMCases with a molecular weight of 36, 29 and 46 kDa, respectively.

The observed extracellular production of the mature active protein suggests that *celstrep* leader sequence could allow extracellular production of other recombinant proteins showing the potential of the developed *E. coli* system for extracellular production of bacterial cellulases and, possibly, other enzymes, for their characterization.

An optimum temperatures of 50°C was identified for CelStrep (Figure 4), like that reported by Theberge et al. [51] for the endoglucanase from *Streptomyces lividans* 66 and lower than the optimum temperature (65°C) reported by Irdani et al. [53] for the purified endoglucanase from *Streptomyces rochei*.

The recombinant enzyme CelStrep showed a higher thermoresistance (Figure 5) than that of *S. rochei* cellulase [53], exhibiting a half life of around 24 h and 96 h at 60°C and 50°C, respectively and showing a retention of around 80% of activity after 96 h at 40°C.

CelStrep follows a Michaelis–Menten kinetics towards CMC: the $K_M$ for this substrate is 9.7 ± 0.8 mg $ml^{-1}$ similar to that reported by Theberge et al. [51], and 9-fold lower than that reported by Wittmann et al. [52], and the $v_{max}$ is 3469 ± 35 μM $min^{-1}$ corresponding to 600 IU/mg of enzyme, that is 6- and 30- fold higher than those reported by Wittmann et al. [52] and Theberge et al. [51], respectively.

## Conclusions

In this manuscript, different microorganisms isolated from compost were screened for their cellulolytic activity and the bacterium producing the highest cellulolytic activity levels was identified by 16S rRNA sequencing and designated as *Streptomyces* sp. strain G12. This strain was shown to produce the highest cellulolytic activity levels even in comparison to *Bacillus* cellulolytic strains isolated in similar conditions [54]. In order to develop a recombinant expression system for cellulases from

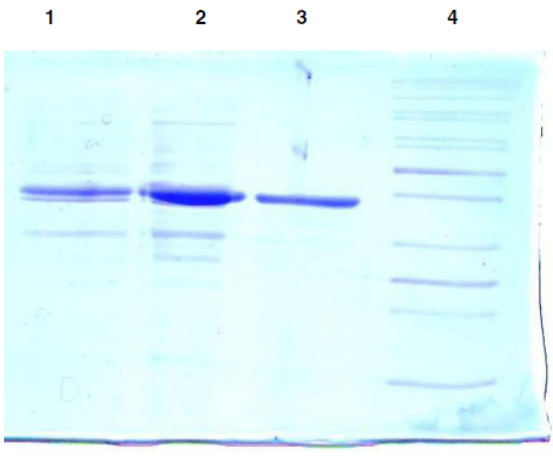


**Figure 3 SDS Polyacrylamide (12.5%) gel profiling of the purified recombinant protein CelStrep.** Lane 1, culture supernatant; Lane 2, ammonium sulfate precipitate; Lane 3, purified CelStrep (Molecular weight of 37,000 Da); Lane 4, protein molecular weight marker.

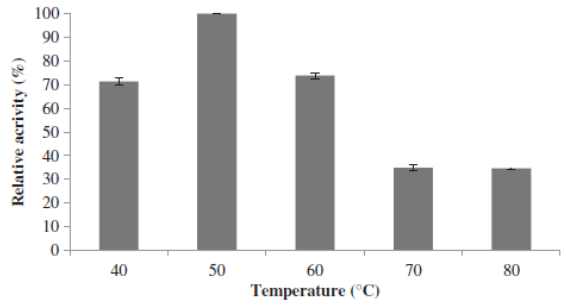


**Figure 4 Effect of temperature on the Azo-CMCase enzyme activity of the purified recombinant protein CelStrep.** The Azo-CMCase activity was measured at the temperatures ranging from 40°C to 80°C.

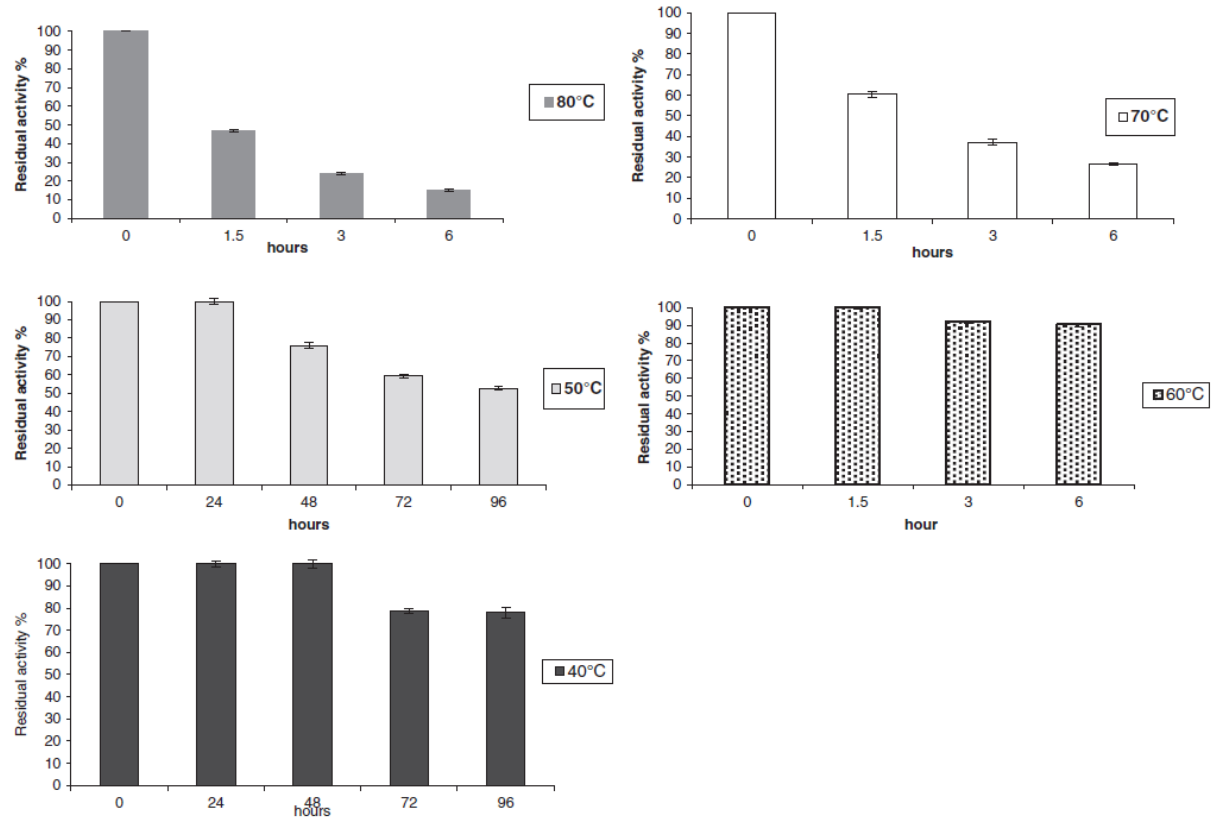


**Figure 5 Thermo-resistance of the purified recombinant protein CelStrep.** Thermo-resistance was determined after incubating the enzyme at different temperatures ranging from 40°C to 80°C, at pH 6.2 for different intervals of time, and the residual enzyme activities were determined by the Azo-CMCase assay. The results reported in this figure correspond to mean values of the three independent experiments performed in three replicates.

*Streptomyces* sp. G12, allowing their purification and characterization, the gene coding for one of the enzymes identified by proteomics as responsible for its cellulase activity was amplified and sequenced, and named *celstrep*. Analysis of the amino acid sequence of CelStrep, placed the enzyme in family 12 of the glycoside hydrolases. A heterologous expression system was set up in *E. coli* using the leader sequence of CelStrep. The active recombinant enzyme was purified from culture supernatant and characterized standing out for its thermoresistance. Besides increasing its potential of CelStrep for cellulose conversion, its thermoresistance would make this cellulase an appropriate candidate as a scaffold for directed evolution experiments aimed at developing better biocatalysts for cellulosic biofuel production, coherently with the original theory [55] that supports the direct relationship between thermostability, mutational robustness and evolutionary capacity.

Moreover, it is worth noting that the observed extracellular production of the mature active recombinant CelStrep protein in *E. coli* suggests that celstrep leader sequence could allow extracellular production of other recombinant proteins. This shows the potential of the developed *E. coli* system as a cell factory for extracellular production not only of bacterial cellulases but also of other bacterial enzymes for their characterization, highlighting general interest of these findings.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

AA carried out analysis of time course of cellulase activity in liquid cultures of the selected microorganisms, optimization of cellulase production in liquid culture by the strain G12, cloning and recombinant expression of *celstrep*. OP contributed to conceiving the study and participated in its design and coordination for the part of isolation of microorganisms, screening of cellulolytic microorganisms on solid medium, characterization of selected microorganisms and identification of the strain G12 and helped to draft the manuscript. VV carried out screening of the cellulolytic bacteria on solid media, phenotypic and molecular characterization of the selected strains, and identification of the strain G12. LB carried out the interpretation of the proteomic analyses and helped to draft the manuscript. CG carried out the proteomic analyses. VF contributed to conceiving the study and participated in its design and coordination for the part of screening of selected microorganisms in liquid cultures, optimization of cellulase production by the strain G12, cloning and recombinant expression of *celstrep*

and purification and characterization of recombinant CelStrep protein and drafted the manuscript. All authors read and approved the final manuscript.

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*Section 2-II*

### 2.2.3 Western Ghat (India) and a "maâsra" (Morocco) as a source of new (hemi)cellulolytic bacteria and fungi

This section deals with the work carried out during the research experiences in foreign laboratories. The targets were the isolation and selection of new bacteria and thermophilic fungi, in order to amplify the patrimony of microorganisms to be further investigated for their (hemi)cellulase activity production.

#### 2.2.3.1 New cellulolytic and xylanolytic bacteria.

The work carried out in the laboratory of Dr. Ashok Pandey (CSIR-NIIST,Trivandrum, Kerala) is part of the project BIOASSORT, a Marie-Curie action aimed at the "Improvement of technologies and tools, e.g. biosystems and biocatalysts, for waste conversion to develop an assortment of high added value eco-friendly and cost-effective bio-products". Particulalry, the work carried out in frame of this PhD project is correlated to the work package 2 of the BIOASSORT project, having as objectives the enlargement of the patrimony of microbes for waste valorization and the improvement of ligno(cellulo)lytic abilities, PHA production, optical purity of L-LA by genetic engineering of the new selected microorganisms.

The biodiversity of natural environment such as Western Ghat regions of Kerala, has been exploited for the isolation of new microorganisms with improved ability in cellulolytic and xylanolytic enzymes production.

50 samples of different nature (humic soil, grass land, inner part of rock, rock surface, excrement, laterite) were used for isolation of bacteria and actynomycetes. 93 purified microorganisms were screened by Congo Red assay on xylan- and CMC-containing plates for xylanase and cellulase activity detection, respectively.

Table 5 lists the samples from which positive microorganisms have been isolated and purified. The screening led to the selection of 7 xylanolytic microorganisms and 14 cellulolytic microorganisms showing activity halos on CMC containing plate assayed with Congo Red. They were divided into three groups – bacteria, *Bacillus* spp., actynomyces - on the basis of a preliminary morphological analyses.

The microorganisms were then identified by 16S rRNA sequencing. Data derived from BLAST showed that the new isolated strains have high sequence similarities with different species (Table 5). A further screening of the 21 xylanolytic and cellulolytic strains was carried out in liquid culture, using xylan- and CMC-containing media, respectively. Xylanase and CMCase activity were assayed after 6, 9 and 15 hours of incubation. In the experimental basal conditions, R5A and R55B were identified as the best producers of the cellulase activity (0.03 U $mL^{-1}$) whilst, XR50A and XR84 produced the highest levels of xylanase activity, 0.6 and 0.28 U $mL^{-1}$, respectively.

The xylanolytic strain XR84, having high similarity with *Lysinobacillus xylanolyticus,* has been further analyzed, showing the ability to produce also cellulase activity in the basal conditions tested for the screening of the cellulolytic microorganisms.

**Table 5:** Soil sample source of positive microorganisms

| Sample source area | Code | Characteristics |
|---|---|---|
| ARIKAN PARA | 5 | humic soil |
| | 7 | grass land |
| | 10 | rock surface |
| | 13 | excrement |
| | 14 | grass land |
| | 18 | grass land |
| PUCHI | 21 | grass land near tree |
| | 24 | grass land |
| | 43 | laterite |
| | 44 | humic soil + plant materials |
| | 50 | humic soil + plant materials |
| | 55 | tree bark |
| | 77 | water stream sample |
| NEELIKAL | 79 | decaying tree bark |
| | 80 | grass land |
| | 84 | laterite |
| | 103 | humic soil |

Thus, particular interest on this microorganism is driven by its ability to produce both cellulase and xylanase activities. Moreover, the strain *Lysinibacillus xylanilyticus* (isolated from forest humus of Gyeryong Mountain (Taejon, Korea)) has been very recently identified as a new taxon [35]. A *Lysinibacillus* sp. strain P5B1 isolated from soil (Mato Grosso do Sul State, Brazil) has been shown to produce both xylanase and CMCase activities by submerged fermentation on several substrates (corn straw, wheat bran, xylan, corncob, sugarcane bagasse), after 72 hours of incubation. Corn straw was shown to be the most suitable substrate for the production of both the activities of interest, with 17.8 U $mL^{-1}$ and 0.65 U $mL^{-1}$ for xylanase and CMCase, respectively [36]. More recently, Kamble et al. [37] studied the strain *Lysinibacillus fusiformis* G1-1 isolated from soil enriched with birchwood xylan. This strain was shown able to produce xylanase activity by both SSF and Smf. When grown on wheat bran, the strain produced an amount of xylanase of 48.7 U $g^{-1}$, whilst during Smf carried out in the presence of birch wood xylan as the sole carbon source, a production level of 21 U $mL^{-1}$ was evaluated.

**Table 6:** 16S rRNA partial sequencing results based on the sequence alignments performed in BLAST

| Code | % of identity whit other species |
|---|---|
| ***- Xylanolytic microorganisms*** | |
| XR13A | *Bacillus* sp.(100%) |
| XR18B | *Bacillus* sp. (100%) |
| XR24A | *Bacillus* sp. (100%) |
| XR44A | *Bacillus* sp. (100%) |
| XR80B | *Streptomyces olivochromogenes* (100%) |
| XR84A | *Lysinobacillus xylanolyticus* (100%) |
| XR50A | *Bacillus* sp. (100%) |
| ***- Cellulolytic microorganisms*** | |
| R10B | *Bacillus* sp. (100%) |
| R13A | *Bacillus* sp. (100%) |
| R13B | *Bacillus* sp. (100%) |
| R14B | *Bacillus* sp. (100%) |
| R21A | *Bacillus* sp. (100%) |
| R43B | *Bacillus* sp. (100%) |
| R77A | *Bacillus* sp. (100%) |
| R79A | *Streptomyces griseoruber* (100%) |
| R79C | *Streptomyces costaricanus* (100%) |
| R84A | *Bacillus* sp. (100%) |
| R103A | *Bacillus* sp. (100%) |
| R5A | *Bacillus* sp. (100%) |
| R7A | *Streptomyces* sp. (100 %) |
| R55B | *Paenibacillus xylanolyticus* (100 %) |

### 2.2.3.2 Thermophilic fungi for cellulase activity production

The microbial patrimony of the IRD-IMEP collection, consisting of thermopihlic fungi isolated from a “maâsra” (a rolling-stone Roman-style mill, grinding the olives into a coarse olive paste, Morocco) [38, 39], has been exploited for the selection of fungi with high levels of production of cellulase activity, during a stage performed at IRD-IMEP Institute (Marseille, France) under the supervision of Dr. Sevastianos Roussos.

Fungi are generally distinguished as the major producers of (hemi)cellulases, being the key agents acting in the decomposition of organic matter in common and cellulosic substrate of wood and soil.

Among fungi, filamentous fungi have been so far recognized as the main producers of cellulase activity, *Trichoderma reesei* being the most representative of this category [26]. As reported by Ikeda et al. [40], this fungus is able to produce high level of cellulase activity on both cellulose powder and cellulose biomass used as carbon sources, like corn fiber, wheat straw, pulp and paper waste. However, due to the enzyme compositions, particularly the low amount of β-glucosidases, *T. reesei* cellulolytic machinery is not sufficient to hydrolyze alone lignocellulosic biomass [41]. Filamentous fungi as *Penicillium brasilianum, P. janthinellum, Aspergillus* spp. and *Acremonium* have also been reported for cellulase production. Ikeda et al. [40] reported an efficient cellulase activity production by the fungus *Acremonium cellulolyticus C-1,* a hyper producer mutant of the original strain, *A. cellulolyticus* Y-94. Damaso et al. [42] performed a screening on 215 filamentous fungi isolated from soils, endophytic environment, sugarcane bagasse and poultry litter by semi-SSF. A *Penicillium* endophytic strain isolated from sugarcane bagasse and an *Aspergillus* strain isolated from a parasitic plant were selected for their ability to produce high level of CMCase, FP and β-glucosidase activities.

Recently, Khokhar et al. [43] reported a similar work based on the screening of 17 fungi belonging to the species *Trichoderma*, *Aspergillus* and *Penicillium,* isolated from different sources such as soil, industrial effluent, seeds fruit, vegetable, bread and wood. In addition, a few anaerobic fungi, such as *Orpinomyces* and *Piromyces*, were reported to produce cellulase activity [44].

The interest in thermophilic microorganisms is generally driven by the need of enzymes able to act at high temperature of reactions. Enzymes having high thermostability and high optimal activity temperatures (higher than 50°C) are strongly required in biomass-to-bioethanol process of production, due to both the extreme conditions of the biomass pretreatment, which precedes the hydrolysis step, and to the need of enzymes which can assure higher performance of hydrolysis. One more valuable advantage of performing hydrolysis of lignocellulosic material at elevated temperatures is reducing the risk of contamination by mesophiles microorganisms. In addition, higher operation temperatures increase the mass-transfer rates leading to a better substrate solubility. Several thermophilic fungi have been isolated in recent years, but only a minority of thermophilic fungal cellulases has been characterized in detail so far [45]. Thermophilic fungi have a minimal growth temperature of 20°C and a maximum growth temperature of 50°C or higher, whilst the thermotolerant forms have a temperature range of growth between 20 and 55°C. In fact, thermophily in fungi is not as extreme as in eubacteria or archaea, some species of which are able to grow near or above 100°C in thermal springs, solfatara fields, or hydrothermal vents [46]. Thermophilic fungi mainly develop in composts during the high temperature phase, and the polysaccharide constituents of the biomass, of which cellulose is the main compound, represent the best carbon source for these microorganisms. As reported by Maheshwari et al. [46],

endoglucanases (30 to 100 kDa) of thermophilic fungi are thermostable, with optimal activity between 55 and 80°C at a pH ranging between 5 and 5.5, whilst exoglucanases (40 to 70 kDa) are thermostable enzymes, optimally active from 50 to 75°C. For instance, especially elevated thermal stability is regarded as one of the main targets to improve hydrolysis technologies for biomass conversion.
These are the reasons driving the work of this project towards the selection of thermophilic fungi, for isolating and studying their (hemi)cellulase enzymes which harbor the potentialities to be exploited in lignocelluloses conversion.
150 thermophilic fungi were transferred on solid medium containing CMC and incubated at 45°C with the aim of screening them for their cellulase production ability and their apical growth rate. Apical growth rate represents filamentous fungi aptitude to colonize lignocellulose substrate. It is one of the most important parameters to take into account for filamentous fungi application in solid state fermentation, this being recognized as the best system for cellulase activity production by fungi.
Screening for cellulases production was performed by Congo Red assay added to the plates after a variable period of incubation, depending on fungi growth rate. Cellulase production was estimated measuring power index, as ratio of hydrolysis halo diameter and colony diameter. Most of the fungi grown very fast on CMC medium, produced a clear zone of activity under the mycelium, whereas the slower the strains grew, the higher the power index was.
Table 7 lists the selected fungi showing power index > 1 and the highest apical growth.

**Table 7:** Thermophlic filamentous fungi selected from IRD-IMEP collection

| Code | Strain | Growth rate (mm/days) | Power index (PI) |
|---|---|---|---|
| L-382 | *Aspergillus niger* | 1 | 2.86 |
| L-433 | *Myceliophthora thermophila* | 5.75 | 1.14 |
| L-457 | *Aspergillus fumigatus* | 5.83 | 1.12 |
| L-457 | *Aspergillus fumigatus* | 5.83 | 1.12 |
| L-521 | *Aspergillus fumigatus* | 4 | 1.36 |
| L-578 | *Malbranchea* | 3.03 | 1.36 |
| L-798 | *Aspergillus niger* | 1.57 | 2.25 |

This preliminary screening led to the selection of five *Aspergillus* strains, a *Malbranchea* strain and a *Myceliophthora thermophila* strain, that represent a potential reservoir of thermostable enzymes.
*Aspergillus niger* has been widely exploited as it possesses all the three essential components of cellulase system [47]. *A. niger* is a major source for β-glucosidases and accessory enzymes, such as α-L-arabinofuranosidase [48].
An *A. niger* strain isolated from municipal solid waste has been study for cellulase production, cellulose and CMC shown to be the best sources for this enzyme production [49].
Industrial wastes like paper and timber-saw mills (Tirupati, Andhra Pradesh) have been the source of an *A. niger* strain investigated for cellulase production. This strain has been shown able to produce a total cellulase activity of around 4 U, in submerged fermentation with paper-cellulose and saw dust as carbon source [50]. It has been revealed that the sugarcane waste at a pH of 4.5 and a temperature of

40°C, is a good source of carbon for the enhanced production of cellulase by *A. niger* [51]. Using different lignocellulosic substrates, such as grass, corncob and bagasse, the effect of temperature and pH on cellulase production by *A. niger* have been evaluated. The highest titer of endoglucanase was obtained when the strain was grown at 30°C or 35°C with an initial pH 4 of the medium, whilst the highest levels of β-glucosidase were obtained at 25 °C and pH 4 [47]. Rice husk has proved to be a good substrate for cellulase production by *A. niger* in comparison to saw dust, with a productivity of 13.702 ± 0.1U/mL/min of CMCase activity, at pH 5 and 40°C [52].

*Aspergillus fumigatus* has been shown to produce several glycosyl hydrolases in both liquid and solid state fermentation, but few reports are reported about characterization of cellulases from this fungus. *A. fumigatus* isolated from the decayed wood lying in the soil has been grown on sugarcane bagasse, wheat bran, wheat straw, rice straw and groundnut shells, and investigated for cellulase production. Sugarcane bagasse was shown to be the best substrate to achieve the highest activity levels of FPA (4.38 U $g^{-1}$), exoglucanase (14.11 U $g^{-1}$), endoglucanse (14.55 U $g^{-1}$) and β-glucosidase (21.66 U $g^{-1}$) [53].

Grigorevski-Lima et al. [54] studied the effect of carbon (brewer's spent grain, sugarcane bagasse and wheat bran) and nitrogen (corn steep liquor and sodium nitrate) sources on cellulase production by an *A. fumigatus* strain isolated from sugarcane bagasse. The highest level of endoglucanase (CMCase) activity corresponding to 365 U $L^{-1}$ was obtained using sugarcane bagasse (1%) and corn steep liquor (1.2%) in submerged fermentation within 6 days of cultivation at 30°C. High levels of FPAse, aryl-β-glucosidase, and cellobiase were produced on simple sugars, FPAse and aryl-β-glucosidase production being both highly induced by filter paper, lactose and wheat straw. Das et al. [55] studied an *A. fumigatus* isolated from the soil around a paper factory waste. This strain was shown able to produce high amounts of both cellulase and xylanase activities by SSF on a substrate prepared by mixing wheat bran and rice straw. An *A. fumigatus* isolated from mangrove soil has been selected for cellulase activity production on sawdust as lignocellulosic substrate, with a maximum level of FPase and CMCase production of around 0.255 and 5.08 U $g^{-1}$ respectively [56]. Endoglucanases secreted by *A. fumigatus* have been generally recognized to be stable and efficiently functional at high temperatures. Liu et al. [57] have recently studied two endoglucanases produced by a strain isolated from compost, showed to be highly stable in a range of temperature between 50 and 60°C, besides maintaining more than 80% of the original activity at temperatures ranged from 40 to 60°C.

Very few manuscripts have been so far reported about production and characterization of cellulases from *Myceliophthora thermophila* and *Malbranchea* spp.. Genome analyses and experimental data suggest that *Myceliophthora thermophila* is capable of hydrolyzing all major polysaccharides found in biomass, harboring a large number (>210) of glycoside hydrolases and polysaccharide lyases [58]. When grown at 50°C, the strain *Myceliophthora thermophila* D-14 was shown able to produce endoglucanase, exoglucanase, and β-glucosidase activities [59]. Badhan et al. [60] reported the production of xylanolytic and cellulolytic enzymes by an isolate *Myceliophthora* sp. strain, using a cheap medium containing rice straw and chemically defined basal medium under solid-state culture. Moretti et al. [61] isolated and characterized thermophilic and thermotolerant fungal strains from soil, decaying organic matter and sugarcane piles. *Myceliophthora thermophila* strain M.7.7 has been selected for the highest levels of xylanase activity production (up to 12 U $g^{-1}$), produced during SSF on sugarcane bagasse. The same group studied also the

production of FPase activity by the same strain, but achieving very low production levels (up to 2 U $g^{-1}$). In fact, both *Myceliophthora* and *Malbranchea* have been much more studied for xylanase activity production. Catalytic performances of two out of the four GH10 xylanases from *M. thermophila* C1 previously identified by Ustinov et al. [62], have been recently characterized towards several substrates such as wheat arabinoxylan, beech wood xylan, birch wood xylan and oat spelt xylan.
*Malbranchea pulchella* var. *sulfurea* TMD-8 was shown to be highly xylanolytic and able to grow well on the hydrolysates containing xylose, with an extracellular xylanolytic enzymes production in wheat straw and hemicelluloses extracted from it of 726 U $L^{-1}$ and 639 U $L^{-1}$, respectively [63]. The strain *Malbranchea* sp. MTCC 4887 isolate from composting soil was found able to produce an alkaline-active xylanase, with high titers up to around 140 U $mL^{-1}$ [64]. More recently, Sharma et al. [65] studied two xylanases produced by the thermophilic fungus *Malbranchea flava* MTCC 4889, showed to be optimally active at 60°C, with an half-life of 4h.

## 2.3 Material and methods

### 2.3.1 Isolation, screening and selection of bacteria

**- Isolation of samples**

Samples used in this study were obtained from three areas of Western Ghat region (Arikan para, Puchi, Neelikal), Kerala, India. Samples, mainly collected from humic soil, grass land, inner part of rock, rock surface and grass land, were aseptically stored in sterilized plastic bag at 4°C.

**- Isolation of bacteria**

Serial dilutions ($10^{-1}$–$10^{-5}$) of the samples were used to inoculate Petri dishes containing LB medium (10 g $L^{-1}$ bactotryptone, 10 g $L^{-1}$ NaCl, and 5 g $L^{-1}$ yeast extract). Plates were incubated at 37°C for a period varying from 2 to 4 days, depending on the speed of growth of the isolates. These isolates were purified, grown in liquid media and preserved in a 20% glycerol solution at -80°C.

**- Determination of cellulolytic/xylanolytic activity of bacterial isolates**

In order to determine endoglucanases and xylanases production by the isolates, pure cultures of microorganisms were individually transferred on CMC/Xylan agar plates (NaNO3 1 g $L^{-1}$, K2HPO4 1 g $L^{-1}$, KCl 1 g $L^{-1}$, MgSO4 0.5 g $L^{-1}$, Yeast extract 0.5 g $L^{-1}$, glucose 1 g $L^{-1}$, agar 17 g $L^{-1}$) containing 5 g $L^{-1}$ of CMC or Xylan, respectively. After incubation for 2-4 days, depending on the speed of growth of the isolates, the strains were assayed for their ability to degrade CMC by incubation with 0.1% Congo Red solution for 30 min followed by washing with 5 M NaCl as reported in Amore et al. [66]. All the strains with a clear halo around the colonies were chosen as positive.

**- 16S rRNA gene partial sequence**

Genomic DNA extraction, 16S rRNA gene amplification and sequencing of PCR amplification fragments were performed according to Rameshkumar and Nair [67].

**- Study of CMCase and Xylanase production in liquid medium**

Growth pre-inocolum, inoculum and CMCase activity assay were carried out according to Amore et al. [66]. Xylanase assay was performed according to Bailey et al. [68]. The reaction mixture consisting of 1.8 ml of a 1.0% (w/v) suspension of birch-wood xylan in 50 mM Na citrate at pH 5.3 and 0.2 mL of enzyme dilution (in 50 mM sodium citrate at pH 5.3) was incubated at 50 °C for 5 min. Released reducing sugars were determined by DNS method, by adding 3 mL of DNS solution and then incubating the mixture at 95°C for 5 min. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 µmol of xylose equivalent per minute.

### 2.3.2 Screening of thermophilic fungi

**- Reactivation of thermopilic fungi**

Microorganisms stored on PDA medium (potato dextrose 24 g $L^{-1}$, agar 15 $g^{-1}$) at 4°C were reactivated on the same medium, inoculating them at both 25°C and 45°C.

**- Media composition**

A modified version of Roussos' medium [69] was adopted: $(NH_4)_2SO_4$ 1 g $L^{-1}$, Urea 0.3 g $L^{-1}$, $KH_2PO_4$ 1.3 g $L^{-1}$, $Na_2HPO_4$ 0.12 g $L^{-1}$, $MgSO_4$ 0.3 g $L^{-1}$, $CaCl_2$ 0.3 g $L^{-1}$, glucose 0.5 g $L^{-1}$, agar 15 g $L^{-1}$.

**- Fungi growth**

Fungi were grown on modified Roussos' medium at 45°C. Time of incubation changed depending on the growth rate. A time of incubation from 2 to 7 days was needed to reach a colony diameter of 20-30 mm.

**- Congo Red assay**

Plates were flooded with 0.1% Congo Red and incubated for 30 minutes, then washed with 3M NaCl.

**- Power Index**

Power index (PI), as measure of cellulase activity levels, was measured as followed:
PI= hd/cd, were hd is the halo diameter and cd the colony diameter.

**- Apical growth, spores number and index of sporulation evaluation**

Apical growth rate was determined by measuring colony diameter dayily. Spores count was realized using Malassez counting chamber. For index of sporulation, 20 mL of a PDA within a 250mL-erlenmeyer flask, were inoculated with a conidiospores suspension ($10^{\wedge 6}$ spores/mL). After homogenization, the culture medium was frozen in order to solidify fastly the PDA containing the inoculum. The culture medium was incubated at 30°C for 7 days. Conidiospores were then harvested introducing 100 mL of water and Tween 80, shaking the flask on a stirrer and counted.

*Chapter 3*

# *Pleurotus ostreatus as a source of accessory enzymes for lignocellulose conversion*

## 3.1 Introduction

*Pleurotus ostreatus* is a white rot basidiomycete fungus having industrial relevance mainly because of its ability to produce high amount of ligninolytic enzymes, such as laccases [70, 71, 72], that find application in several industrial fields.

*P. ostreatus* genome has been fully sequenced [73] and the fungus shows to possess several genes coding for glycoside hydrolases (cellulases, xylanases, arabinofuranosidases, mannanase, pectinases), thus pushing the interest towards its exploitation for new enzymes discovery (Table 8).

Recently, agro-processing wastes, such as tomato processing waste and apple pomace, have been shown to be suitable substrates for production of xylanases through solid state fermentation of the fungus [74, 75].

This chapter deals with the description of the studies performed on a new family GH51 α-L-arabinofuranosidase, showed to be the enzyme responsible for the xylanase activity previously detected during *Pleurotus ostreatus* SSF on tomato processing waste.

Particularly, in section 3-I, **paper V "A family GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus*: identification, recombinant expression and characterization"** describes the characterization of the new α-L-arabinofuranosidase; section 3-II regards the study of rPoAbf mechanism of reaction and section 3-III deals with the design and production of rPoAbf site-direct mutants for the study of structure/function relationships.

**Table 8:** Representative genes coding for glycosyl hydrolase in *Pleurotus ostreatus*

| *GENE* | *PROTEIN FAMILY* | *ACTIVITY* |
|---|---|---|
| **fgenesh1_pm.05_#_466** | Glycoside hydrolase, 38 | α-mannosidase activity |
| **estExt_Genewise1Plus.C_020468** | Glycoside hydrolase, 53 | Glucosidase activity |
| **e_gw1.06.236.1** | α-L-arabinofuranosidase | L-arabinose metabolic process |
| **e_gw1.10.496.1** | Glycoside hydrolase, 1 | Carbohydrate metabolic process |
| **fgenesh1_pm.10_#_226** | Glycoside hydrolase, 11 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **estExt_Genewise1Plus.C_012234** | Glycosyl hydrolase, 13 | α-amylase activity |
| **estExt_Genewise1.C_061283** | Glycoside hydrolase, 16 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **estExt_Genewise1.C_051360** | Glycoside hydrolase, 5 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **fgenesh1_pm.08_#_111** | Glycoside hydrolase, 10 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **e_gw1.10.719.1** | Glycoside hydrolase, 31 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **fgenesh1_pm.C_scaffold_6000012** | Glycoside hydrolase, 47 | Mannosyl-oligosaccharide 1,2-α-mannosidase activity |
| **estExt_Genewise1Plus.C_50361** | Glycoside hydrolase, 28 | Polygalacturonase activity |
| **fgenesh1_pm.C_scaffold_3000790** | Glycoside hydrolase, 45 | Cellulase activity |
| **gm1.10469_g** | Glycoside hydrolase, 12 | Cellulase activity |
| **e_gw1.03.1789.1** | Glycoside hydrolase, 45 | Cellulase activity |
| **gm1.2707_g** | Glycoside hydrolase, 6 | 1, 4-β cellobiohydrolase activity |
| **e_gw1.06.1045.1** | Glycoside hydrolase, 7 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **fgenesh1_pm.11_#_111** | Glycoside hydrolase, 32 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **estExt_Genewise1.C_041725** | Glycoside hydrolase, 17 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **fgenesh1_pm.03_#_282** | Glycoside hydrolase, 18, chitinase active site | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **e_gw1.07.1651.1** | Glycoside hydrolase, 27 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **e_gw1.03.175.1** | Glycoside hydrolase, 3 | Hydrolase activity, hydrolyzing O-glycosyl compounds |
| **fgenesh1_pm.C_scaffold_1000458** | Glycoside hydrolase, 3 | 1, 4-β cellobiohydrolase activity |
| **fgenesh1_pm.01_#_114** | Glycoside hydrolase, 3 | 1, 4-β cellobiohydrolase activity |
| **estExt_fgenesh1_pg.C_100264** | Glycoside hydrolase, 11 | Cellulose-binding domain |
| **estExt_Genewise1Plus.C_080884** | Glycoside hydrolase, 5 | Cellulose-binding domain |
| **e_gw1.05.1493.1** | Glycoside hydrolase, 5 | Cellulose-binding region |
| **e_gw1.11.1427.1** | Glycoside hydrolase, 18 | Chitinase activity |
| **estExt_Genewise1Plus.C_040177** | Glycosyl hydrolase, 92 | α-1,2-mannosidase, putative |
| **genemark.584_g** | Glycoside hydrolase, 62 | Arabinanase/levansucrase/invertase |
| **gm1.6978_g** | Glycoside hydrolase, 35 | β-galactosidase activity |
| **e_gw1.07.166.1** | Glycoside hydrolase, 37 | α,α-trehalase activity |
| **e_gw1.7.421.1** | Glycoside hydrolase, 35 | β-galactosidase activity |
| **estExt_Genewise1Plus.C_071521** | Glycoside hydrolase, 15 | Glucan 1,4-α-glucosidase with starch-binding domain |
| **fgenesh1_pm.C_scaffold_5000102** | Glycoside hydrolase, 12 | Cellulase activity |
| **e_gw1.11.1195.1** | Glycoside hydrolase, 28 | Polygalacturonase activity, Pectin lyase |
| **e_gw1.05.1559.1** | Glycoside hydrolase, 28 | polygalacturonase activity |
| **estExt_fgenesh1_pm.C_50572** | Glycoside hydrolase, 2 | β-Galactosidase/glucuronidase domain |
| **estExt_Genewise1Plus.C_041816** | Glycoside hydrolase, 2 | β-Galactosidase/glucuronidase domain |
| **e_gw1.05.2326.1** | Glycoside hydrolase | α-L-arabinofuranosidase |
| **e_gw1.08.941.1** | Glycoside hydrolase | α-L-arabinofuranosidase |

## 3.2 Results

### *Section 3-I*
### 3.2.1 A family GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus* (Paper V)

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

# A family GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus*: identification, recombinant expression and characterization

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**Abstract** An α-L-arabinofuranosidase produced by *Pleurotus ostreatus* (PoAbf) during solid state fermentation on tomato pomace was identified and the corresponding gene and cDNA were cloned and sequenced. Molecular analysis showed that the *poabf* gene carries 26 exons interrupted by 25 introns and has an open reading frame encoding a protein of 646 amino acid residues, including a signal peptide of 20 amino acid residues. The amino acid sequence similar to the other α-L-arabinofuranosidases indicated that the enzyme encoded by *poabf* can be classified as a family 51 glycoside hydrolase. Heterologous recombinant expression of PoAbf was carried out in the yeasts *Pichia pastoris* and *Kluyveromyces lactis* achieving the highest production level of the secreted enzyme (180 mg $L^{-1}$) in the former host. rPoAbf produced in *P. pastoris* was purified and characterized. It is a glycosylated monomer with a molecular weight of 81,500 Da in denaturing conditions. Mass spectral analyses led to the localization of a single *O*-glycosylation site at the level of Ser160. The enzyme is highly specific for α-L-arabinofuranosyl linkages and when assayed with *p*-nitrophenyl α-L-arabinofuranoside it follows Michaelis–Menten kinetics with a $K_M$ of 0.64 mM and a $k_{cat}$ of 3,010 $min^{-1}$. The optimum pH is 5 and the optimal temperature 40°C. It is worth noting that the enzyme shows a very high stability in a broad range of pH. The more durable activity showed by rPoAbf in comparison to the other α-L-arabinofuranosidases enhances its potential for biotechnological applications and increases interest in elucidating the molecular bases of its peculiar properties.

**Keywords** α-L-arabinofuranosidase · Fungus · Recombinant expression · *Pichia pastoris*

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## Introduction

The α-L-arabinofuranosidases (EC 3.2.1.55) are enzymes that degrade hemicelluloses, such as arabinoxylan, arabinogalactan and L-arabinan, cleaving α-L-arabinofuranosidic linkages and acting synergistically with other enzymes to allow the complete hydrolysis of hemicelluloses and pectins. The α-L-arabinofuranosidases can be found in plant, bacteria and fungi.

There is a growing interest into α-L-arabinofuranosidases due to their application in various industrial processes, such as the production of L-arabinose to be used as food additive, increase of wine aroma by hydrolysis of monoterpenols,

clarification of fruit juices, improvement of the overall quality of bread by delaying the bread staling, improvement of animal feed digestibility, and enhancement of pulp delignification (Numan and Bhosle 2006).

The α-L-arabinofuranosidases are glycoside hydrolases that cleave terminal non-reducing α-L-1,2-, α-L-1,3- and/or α-L-1,5-arabinofuranosyl residues from polymers that contain L-arabinose. Some act on short oligosaccharides, while others are capable of hydrolyzing side-chain arabinose residues from polymeric substrates. α-L-arabinofuranosidases are classified into families GH3, GH43, GH51, GH54 and GH62 of the classification of glycoside hydrolases based on amino acid sequence similarity reported in the CAZy database (http://www.cazy.org/) (Cantarel et al. 2009).

In many cases, microorganisms that utilize hemicelluloses possess α-L-arabinofuranosidases with various substrate specificities and biochemical properties. Some fungal species produce several α-L-arabinofuranosidase isoenzymes.

The white-rot fungus *Pleurotus ostreatus* has been widely studied for its ligninolytic activity due to the expression of a laccase multigene family (Pezzella et al. 2009), while only recently its hemicellulolytic capability has been reported with detection of xylanase activity during solid state fermentation (SSF) on tomato pomace and the observed correlation between production times of xylanase and laccase activities suggested their synergistic action in waste transformation (Iandolo et al. 2011).

In this manuscript an α-L-arabinofuranosidase was identified by proteomic analyses of the enzymes responsible for xylanase activity produced by *P. ostreatus* at the eighth day of SSF on tomato pomace. The gene and cDNA coding for this enzyme named PoAbf were cloned and sequenced. A heterologous expression system was set up for PoAbf in the yeasts *Pichia pastoris* and *Kluyveromyces lactis* achieving the highest production level of the secreted enzyme in the former host. rPoAbf produced in *P. pastoris* was purified and characterized.

## Materials and methods

### Non-denaturing *polyacrylamide gel electrophoresis*

The zymogram analysis was performed by a modified version of method reported by Ratanakhanokchai et al. (1999). Samples from *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida; ATCC MYA-2306) suspended in sodium phosphate buffer (50 mM, pH 6.8) were subjected to electrophoresis in an *sodium dodecyl sulfate* (SDS)-10% polyacrylamide gel containing 0.1% birchwood xylan. After electrophoresis, the gel was soaked in 25% (*v*/*v*) isopropanol by gently shaking to remove the SDS and renature the proteins in the gel. The gel was then washed four times for 30 min at 4°C in 0.1 M acetate buffer (pH 5.5). After further incubation for 60 min at 50°C, the gel was soaked in 0.1% Congo red solution for 30 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After, the gel was submerged in 0.5% acetic acid; the background turned dark blue and the activity bands were observed.

### In situ mass spectrometry analyses

Slices of interest from the non-denaturing *polyacrylamide gel electrophoresis* (PAGE) on samples from *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida; ATCC MYA-2306) were destained by washes with 0.1 M $NH_4HCO_3$ pH 7.5 and acetonitrile, reduced for 45 min with 100 µL of 10 mM dithiothreitol in 0.1 M $NH_4HCO_3$ buffer pH 7.5 and carboxyamidomethylated for 30 min 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 h at 4°C. The buffer solution was then removed and 50 µL of 10 mM $NH_4HCO_3$ pH 7.5 were added and incubated for 18 h 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, USA) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed on 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 400 nL/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 1,800 *m*/*z*) followed by MS/MS scans of the five most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50,000 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 protein identification with a licensed version of MASCOT 2.1 (Matrix Science, Boston, MA, USA). Raw data from nanoLC–MS/MS analyses were used to query the *P. ostreatus* database (http://genome.jgi-psf.org/PleosPC15_2/PleosPC15_2.home.html; http://genome.jgi-

psf.org/PleosPC9_1/PleosPC9_1.home.html) 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 of <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-HPLC grade was from Carlo Erba (Milan, Italy). All other reagents and solvents were of the highest purity available from J. T. Baker (Phillipsburg, NJ, USA).

## Isolation and sequencing of gene and cDNA

Chromosomal high-molecular weight DNA from *P. ostreatus* was prepared as described by Raeder and Broda (1988). Total RNAs were extracted from lyophilized mycelia by using QIAGEN RNeasy Plant (QIAGEN, Italy) and following manufacturer's instructions. Reverse transcription reaction was performed using MultiScribe™ Reverse Transcriptase (Applied Biosystems, Branchburg, NJ, USA) and the oligonucleotide dT-NotI as primer. Products of the PCR experiments, performed using the gene-specific oligonucleotides (Table 1), were cloned into the p-GEM-T Easy Vector (Promega, Italy). Sequencing by dideoxy chain termination method was performed by PRIMM Sequencing Service (Naples, Italy) using universal and specific oligonucleotide primers.

## Analysis of gene and protein sequences

Sequence of gene and cDNA were deposited with the EMBL Data Library under accession numbers HE565355 and HE565356, respectively. Alignments of DNA and of deduced amino acid sequences were generated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Signal peptide prediction was achieved using SignalP V2.0 (http://www.cbs.dtu.dk/services/). Potential *N*-glycosylation sites (Asn-XXX-Ser/Thr) were computed on the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), and *O*-GalNAc (mucin type) glycosylation sites were predicted on the OGPET 1.0 server (http://ogpet.utep.edu/OGPET/).

## Heterologous expression

### *Strains, media and plasmids*

The *Escherichia coli* strain Top 10 (F-mcrA D (mrrhsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 D (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG) was used in all DNA manipulations. *E. coli* was grown in Luria–Bertani (LB) medium (in grams per liter: 10 bacto tryptone, 10 NaCl, and 5 yeast extract) supplemented, when required, with 100 μg $ml^{-1}$ of ampicillin.

The *K. lactis* strain used for heterologous expression was CMK5 (a *thr lys pgi1 adh3 adh1URA3 adh2URA3*) (Saliola et al. 1999). Plasmid pYG132 (Saliola et al. 1999) was engineered from pKD1, a plasmid originally isolated from *Kluyveromyces drosophilarum* (Falcone et al. 1986) that can replicate stably in *K. lactis*. Both *K. lactis* strain and plasmid were kindly given by Prof. C. Falcone (University "La Sapienza", Rome, Italy). Insert expression is controlled by the ethanol-inducible *KlADH4* promoter (Mazzoni et al. 1992) and the *Saccharomyces cerevisiae* phosphoglycerate kinase (*PGK*) terminator. *K. lactis* was grown in different media, supplemented, when required, with 100 μg $ml^{-1}$ of geneticin G418: YPPG (in grams per liter: 10 yeast extract, 40 bactotryptone and 20 galactose), YPPD (in grams per liter: 10 yeast extract, 40 bactotryptone and 20 Glucose), YPPA (in grams per liter: 10 yeast extract, 40 bactotryptone,

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

| Primer | Nucleotide sequences | Annealing temperature (°C) |
|---|---|---|
| 5′ arabin-FW1 | ATGTATCGACTCGCTGCACT | 60°C |
| 5′ arabin-FW2 | ATGTAGGCCTTGGCCCTTG | 60°C |
| 5′ arabin-FW3 | ATGTTTGAGGTAATGACAATGC | 60°C |
| 5′ arabin-FW4 | ATGCACGACTCTCCTAGTATCAC | 62°C |
| 5′ arabin-FW5 | ATGTTCATTCTGAATGATATGCA | 60°C |
| 5′ arabin-REV | GCAGCCCCATCGATCGTCAC | 66°C |
| Intarabin-FW | GACGATCGATGGGGCTGC | 62°C |
| Intarabin-REV | GGCGACCGTTTGCAGGCG | 62°C |
| 3′ arabin-FW | GCCACGCAATGGAATCACC | 60°C |
| 3′ arabin-REV | CTACGACGCCTTGATAGTGA | 60°C |
| contr pYG132 FW | GGGAGAGCTCCAGCAGGGC | 66°C |
| contr pYG132 REV | GGTTCTACCAGCAGGGACGG | 66°C |

and 20 L-arabinose), YPPX (in grams per liter: 10 yeast extract, 40 bactotryptone and 20 xylose) and YPPWS (in grams per liter: 10 yeast extract, 40 bactotryptone and 20 wheat straw).

The "Easy Select Pichia Expression kit" (Invitrogen, Carlsbad, CA, USA) was used for expression in *P. pastoris* strain X33 following the manufacturer's instructions. pPICZB was chosen as expression vector. Its expression cassette includes the *AOX1* promoter that allows methanol-inducible high level expression and the *AOX1* transcriptional terminator. *P. pastoris* transformants were grown in BMGY (1% yeast extract, 2% peptone, 1%(*v*/*v*) glycerol, 0.00004% biotin, 1.34% yeast nitrogen base with ammonium sulphate and 0.1 M potassium phosphate, pH 6.0) and BMMY, the same as BMGY except that it contains 0.5% (*v*/*v*) methanol instead of glycerol.

*Vector construction*

The vectors pYG-abf and pPICZ-abf used for recombinant expression of PoAbf in *K. lactis* and *P. pastoris*, respectively were prepared cloning *poabf* cDNA in pYG132 (in *Hin*dIII) under control of the ethanol-inducible *KlADH4* promoter (Piscitelli et al. 2005) and in pPICZB (in *Eco*RI/*Xba*I) under control of methanol-inducible *AOX1* promoter.

*Yeasts transformation, cultivation and enzyme production*

*K. lactis* transformation was performed by electroporation with a Bio-Rad MicroPulser apparatus, as specified by the manufacturer. The cells were spread on a YPPG medium containing 100 μg $ml^{-1}$ of geneticin G418, after an overnight incubation at 28°C in YPPG. Agar plate assays on YPPG supplemented with 100 μg $ml^{-1}$ of geneticin G418, 0.5% ethanol and 20 μg $ml^{-1}$ of the chromogenic substrate 4-methylumbelliferyl-a-L-arabinofuranoside (MUA) (Carbosynth, Compton, Berkshire, UK) were used to select transformants. The Abf-expressing clones were identified under UV light by the presence of fluorescence around the colonies after incubation at 28°C for 3 days. 100 uL of ethanol were added each day to the lid of the plate to compensate for its evaporation. The transformed clones were screened for PoAbf production by growing them in 10 mL of selective medium (YPPG containing geneticin) supplemented with 0.5% ethanol, at 28°C on a rotary shaker (150 rpm). Ethanol (0.5%) was added daily and samples were taken at intervals to measure optical density and ABF activity. The best producing clone was chosen to study PoAbf production. Inocula grown on selective YPPG medium were used to seed flasks (250 ml) containing 50 ml of selective medium (YPPG, YPPD, YPPA, YPPX or YPPWS) starting from 0.6 OD600. Cultures were grown at 28°C or 20°C on a rotary shaker for 7 days.

The recombinant plasmid pPICZB–abf was linearized by *Pme*I hydrolysis and introduced into *P. pastoris* strain X33 by electroporation. The electroporation mixture was plated onto YPD–zeocin agar medium. After 72 h at 28°C, the transformant colonies were screened for activity production on plates containing the chromogenic substrate MUA. *P. pastoris* transformants were grown at 30°C in 20 mL of BMGY in an incubator shaker until cell density (OD600) reached the value of 20. The cells were inoculated in 50 mL BMMY in a 250-mL flask to an OD600=1 to start the induction. The culture was kept in an incubator–shaker at 28°C for 15 days (200 rpm) with the addition of 0.25 mL of methanol once a day to maintain induction. For optimizing production, the best producing clone, after growth at 28°C in 20 mL BMGY to an OD600=20, was inoculated in 50 mL of two modified versions of BMMY containing 1% or 1.5% (v/v) methanol and incubated at 28°C or in BMMY at 20°C.

Enzyme activity assays

*Xylanase activity assay*

Xylanase activity assay was performed according to Bailey et al. (1992). The reaction mixture consisting of 1.8 mL of a 1.0% (*w*/*v*) suspension of birchwood xylan in 50 mM Na citrate at pH 5.3 and 0.2 mL of enzyme dilution (in 50 mM sodium citrate at pH 5.3) was incubated at 50°C for 5 min. Released reducing sugars were determined by DNS method, by adding 3 ml of DNS solution and then incubating the mixture at 95°C for 5 min. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 μmol of xylose equivalent per minute.

*α-L-arabinofuranosidase activity assay*

α-L-Arabinofuranosidase activity was determined according to Yanay and Sato (2000). The activity was measured by spectrophotometric method with *p*-nitrophenyl α-L-arabinofuranoside (pNPA) (Gold Biotechnology, St Louis, MO, USA) as substrate. The assay mixture contained 200 μL of the substrate solution (2 mM pNPA in 50 mM sodium phosphate buffer pH 6.5) and 200 μL of appropriately diluted enzyme solution. After incubation at 30°C for 10 min, the reaction was stopped by the addition of 1.6 mL of 1 M $Na_2CO_3$. The liberated *p*-nitrophenol in the mixture was measured by spectrophotometry at 405 nm ($\varepsilon$=17,000 $M^{-1}$ $cm^{-1}$). One unit of α-L-arabinofuranosidase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute in the reaction mixture under these assay conditions.

### PoAbf purification

Proteins secreted by rPoAbf expressing *P. pastoris* were precipitated from the medium (after cell removal by centrifugation at 5,000×*g* for 10 min) by the addition of $(NH_4)_2SO_4$ up to 80% saturation at 4°C and centrifugation at 10,000×*g* for 30 min. The precipitate was resuspended in 0.02 M Tris–HCl pH 7.5 and loaded on HiTrap Phenyl FF high sub (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A (0.02 M Tris–HCl, 1.2 M $(NH_4)_2SO_4$, pH 7.5), and the proteins were eluted isocratically with buffer B (0.02 M Tris–HCl pH 7.5). Fractions containing activity were combined and concentrated on an Amicon PM-10 membrane and analyzed by SDS-PAGE.

### Protein concentration determination

Protein concentration was determined by the method of Lowry et al. (1951), using the BioRad Protein Assay (BioRad Laboratories S.r.l., Segrate, MI, Italy), with bovine serum albumin as standard.

### Determination of molecular mass

The molecular mass of rPoAbf produced by *P. pastoris* was determined by using a Superdex 200 HR 10/30 column (GE Healthcare, Uppsala, Sweden). The column was eluted with 50 mM Tris–HCl buffer, pH 7.0, containing 150 mM NaCl. The calibration was performed with thyroglobulin (669 kDa), apoferritin (440 kDa) alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa) as standards.

### Optimum temperature and temperature stability

To determine the optimum temperature of the purified enzyme, the substrate of the activity assay (2 mM pNPA) was dissolved in 50 mM sodium phosphate buffer pH 6.5 and the incubation (10 min) was performed at 30°C, 40°C, 50°C, 55 C and 60°C. The temperature stability of rPoAbf was studied by incubating the purified enzyme preparation in 50 mM citrate phosphate buffer pH 5.0, at 30°C, 40°C, 50°C and 60°C. The samples withdrawn were assayed for residual α-arabinosidase activity performing incubation (10 min) at 40°C. The experiments were performed in duplicate and reported values are representative of three experiments.

### Optimum pH and pH stability

To determine the optimum pH of the purified enzyme, the substrate of the activity assay (2 mM pNPA) was dissolved in citrate phosphate buffers (McIlvaine 1921) with pH values between 3.0 and 7.0 and in 50 mM Tris–HCl with pH values 7.0 and 8.0 and the incubation (10 min) was performed at 40°C. The pH stability of the purified enzyme preparation was studied by diluting it in citrate phosphate buffers, pH 3–8 and incubating at 25°C. From time to time, samples were withdrawn and immediately assayed for residual α-arabinosidase activity performing incubation (10 min) at 40°C. The experiments were performed in duplicate and reported values are representative of three experiments.

### Assays of enzyme specificity

Activity of PoAbf was assayed against the substrates *p*NP-α-L-arabinofuranoside (Carbosynth, Compton, Berkshire, UK), *p*NP-β-D-xylopyranoside (Carbosynth, Compton, Berkshire, UK), *p*NP-α-D-glucopyranoside (Carbosynth, Compton, Berkshire, UK) *p*NP-β-D-glucopyranoside (Carbosynth, Compton, Berkshire, UK) and *o*NP-β-D-galactopyranoside (Carbosynth, Compton, Berkshire, UK) at concentrations in the range 0.1–6 mM in citrate phosphate buffer (McIlvaine, 1921) at pH 5.

Activity against the natural substrates CM-linear arabinan and larch arabinogalactan, and the arabinooligosaccharides 1,5-α-arabinotriose and 1,5-α-arabinohexaose (Megazyme International Ireland, Co. Wicklow, Ireland) was assayed measuring the liberation of arabinose as equivalent of galactose, by means of the D-galactose/lactose kit (Megazyme International Ireland, Co. Wicklow, Ireland), following the manufacturer's instructions. 600 mU (measured on pNPA) of PoAbf were incubated with 0.2% arabinans in 100 mM sodium acetate buffer pH 4.6, at 37°C for 72 h (final volume, 300 μL). Arabinooligosaccharides were dissolved in 100 mM sodium acetate buffer pH 4.6 and incubated with 7 mU (measured on pNPA) of PoAbf at 37°C for 1 h (final volume 300 μL). The activity of PoAbf against AZO-wheat arabinoxylan (Megazyme International Ireland, Co. Wicklow, Ireland) was assayed following supplier's instructions. The experiments were performed in duplicate and reported values are representative of three experiments.

### Determination of $k_{cat}$ and $K_M$

For determination of the Michaelis–Menten constants $K_M$ and $k_{cat}$, the activity assay was performed at pNPA concentrations from 0.1 mM to 2.0 mM at pH 5.0 performing incubation of 10 min at 40°C. The experiments were performed in duplicate and reported values are representative of three experiments.

### Mass spectrometry analyses of rPoAbf

α-L-arabinofuranosidase was dissolved in denaturant buffer (Tris 300 mM pH 8.0, urea 6 M, EDTA 10 mM), and disulfide bridges were reduced with DTT (tenfold molar

excess on the Cys residues) at 37°C for 2 h and then alkylated by adding IAM (fivefold molar excess on thiol residues) at room temperature for 30 min in the dark. Protein sample was desalted by size exclusion chromatography on a Sephadex G-25M column (GE Healthcare). Fractions containing protein were lyophilized. Lyophilized fractions were dissolved in 10 mM AMBIC buffer pH 8.0. Enzymatic digestion was performed using both trypsin and V8 protease using an enzyme/substrate ratio of 1/50 (*w*/*w*) at 37°C for 16 h. MALDI–MS experiments were performed on a Voyager-DE STR MALDITOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) equipped with a nitrogen laser (337 nm). Of the total mixture, 1 μL was mixed (1/1, *v*/*v*) with a 10-mg $mL^{-1}$ solution of α-cyano-4-hydroxycinnamic acid in ACN/50 mM citrate buffer, 70/30 (*v*/*v*). Spectra were acquired using a mass (*m*/*z*) range of 400–5,000 amu.

## Results

### Enzyme identification

The native PAGE showed that two different xylanase bands can be detected analyzing the second and the eighth days of *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida; ATCC MYA-2306)-SSF on tomato processing residues, when maximum level of xylanase activity—related to any enzyme acting on any part of xylan—was reached (Iandolo et al. 2011). The activity positive protein band with lower electrophoretic mobility produced at the eighth day was excised from a preparative, non-denaturing gel and analyzed by proteomics. By comparison with genome sequences of the monokaryon strains *P. ostreatus* PC15 (http://genome.jgi-psf.org/PleosPC15_2/PleosPC15_2.home.html) and PC9 (http://genome.jgi-psf.org/PleosPC9_1/PleosPC9_1.home.html), the protein was identified as an α-L-arabinofuranosidase—having the ID 29657 in *P. ostreatus* PC15 and 94793 in *P. ostreatus* PC9 strains—that was named PoAbf. The fact that, even if xylanase activity had been found, an arabinofuranosidase was identified can be explained considering that arabinoxylan is used as a substrate and that this substrate contains arabinose side branches on which the enzyme seems to act. The time course of α-L-arabinofuranosidase activity was then analyzed during *P. ostreatus* SSF on tomato processing waste by using the pNPA as substrate, thus demonstrating the presence of a peak of around 0.18 $Ug^{-1}$ dry matter at the sixth day of fermentation.

### Sequences of the *poabf* gene and cDNA and its derived protein

Analysis of genome sequence of the monokaryon *P. ostreatus* strain PC15 showed that the gene with ID 29657 is interrupted at the 5′ region and does not contain any start codon. On the other hand, a complete coding sequence was identified in the monokaryon *P. ostreatus* strain PC9 for the gene with ID 94793. However, analysis of the deduced amino acid sequence corresponding to the latter gene according to the annotated version showed that it lacks the signal peptide, in spite of the demonstrated extracellular nature of the analyzed α-L-arabinofuranosidase. The analysis of genome sequence of PC9 strain upstream of the annotated start codon allowed design of different oligonucleotides to be used in PCR experiments to amplify cDNA coding for the investigated protein in the dikaryon strain *P. ostreatus* ATCC MYA-2306. This cDNA was synthesized by PCR on RNA extracted from *P. ostreatus* grown on tomato processing residues at the eighth day of SSF using as primers oligonucleotides (Table 1) whose sequences were designed on the basis of genome sequence to amplify three overlapped fragments. By carrying out PCR experiments of the 5′ region with different primer oligonucleotides at the 5′ end (5′ arabin-FW1-5) in combination with the same oligonucleotide at the 3′ end (5′ arabin-REV), it was possible to ascertain the start codon of *poabf*, considering which oligonucleotide gave the most 5′ extended region. *poabf* gene was also synthesized by PCR on *P. ostreatus* ATCC MYA-2306 genome.

The cDNA coding for PoAbf has an open reading frame of 1,941 bp and it codes for a protein of 646 amino acids. A unique signal peptide sequence of 20 amino acids could be singled out, which satisfied criteria for a “minimal” signal sequence (von Heijne 1985).

The mature protein is 626 amino acids in length and has a calculated molecular mass of 68,912.51 Da. The coding sequence is interrupted by 25 introns as detected when the cDNA and gene sequences were compared. The intron size ranges from 46 to 112 bp. All introns are flanked by the consensus sequences GT.AG (Kupfer et al. 2004). No potential *N*-glycosylation site (Asn-X-Ser) was found in PoAbf deduced amino acid sequence, while Ser160 and Thr187 were identified as potential *O*-GalNAc (mucin type) glycosylation sites within the patterns T-n-GST-n-VS and T-n-RTT-n-PS, respectively.

Similarity searches were performed with Basic Local Alignment Search Tool (BLAST) using the deduced amino acid sequence of PoAbf as the query and a library made with the sequences of experimentally characterized family GH51 enzymes (http://www.cazy.org/GH51_characterized.html) extracted from the Carbohydrate-active enzymes database (http://www.cazy.org/, Cantarel et al. 2009). The best hit (62% identities) was with an α-L-arabinofuranosidase from *Meripilus giganteus* (EMBL accession CAL81200.1; Sørensen et al. 2006), followed by several enzymes from *Aspergillus* spp. (EMBL accessions BAB96815.1, BAB21568.2, AAC41644.1, etc.) displaying ~35% identities

with PoAbf. Plant and bacterial GH51 ABFs gave lower scores, typically less than 30% identities. The distribution of genes encoding enzymes similar to PoAbf was examined by performing a BLAST search (conducted on Sept 22, 2011) on the non-redundant protein sequence databank of the NCBI. The results revealed that only a limited number of fungi have GH51 enzymes with over 50% identity to PoAbf, namely *Leucoagaricus gongylophorus*, *Serpula lacrymans*, *Schizophyllum commune*, *Postia placenta* and *Coprinopsis cinerea*. All other fungal hits showed less than 40% sequence identity with PoAbf, hinting at possible differences in substrate or product specificities.

The family GH51 glycoside hydrolases have a catalytic mechanism with retention of configuration with two glutamates involved in catalysis (one acting as an acid/base and the other as a nucleophile) (Henrissat et al. 1995). Three-dimensional structures of family GH51 ABFs have been obtained for two bacterial enzymes, namely from *Geobacillus stearothermophilus* (Hövel et al. 2003) and *Clostridium thermocellum* (Taylor et al. 2006). The catalytic domain is a (β/α)8 barrel, and the active site glutamates are found at the C-termini of strands β-4 (acid/base) and β-7 (nucleophile) (Taylor et al. 2006). Based on this information and the sequence alignment, the proposed catalytic glutamates of PoAbf are located at positions 364 (acid/base) and 471 (nucleophile).

## Recombinant expression system

The enzyme α-L-arabinofuranosidase secreted by *P. ostreatus*, PoAbf, was expressed in *K. lactis* and *P. pastoris*. The *poabf* cDNA sequence was optimized for yeast recombinant expression by adapting it to the yeast codon usage and avoiding its hydrolysis by the endonucleases adopted for the cloning. Synthesis of this cDNA was performed by MrGene (http://mrgene.com).

### *K. lactis* transformants

When the production of recombinant α-L-arabinofuranosidase in *K. lactis* was assayed on plates containing MUA as substrate, around 20% of transformants caused a fluorescence emission after 3 days. Eighteen recombinant clones were grown in liquid cultures and evaluated for Abf production by assay with pNPA. The amount of extracellular α-L-arabinofuranosidase activity produced after 7 days ranged from (0 to 3.50)$\times 10^{-3}$U mL$^{-1}$. The best producing clone was used to optimize production of rPoAbf and study the time course of recombinant α-L-arabinofuranosidase. Cultures were carried out to improve α-L-arabinofuranosidase production, varying the growth conditions. Decreasing the growth temperature from 28°C to 20°C led to a 10-fold increase of activity production. Comparison of growth time courses at the two temperatures showed only a slight delay of yeast growth due to the temperature decrease. Glucose, galactose, L-arabinose, xylose and wheat straw were tested as carbon sources. The maximum activity level was obtained at the seventh day with all the tested substrates. The highest production of secreted activity was achieved at 20°C in the medium containing glucose ($62.0\times10^{-3}$U mL$^{-1}$), followed by those containing xylose ($49.72\times10^{-3}$U mL$^{-1}$), galactose and wheat straw ($35.55\times10^{-3}$U ml$^{-1}$) and arabinose ($20.36\times10^{-3}$U mL$^{-1}$).

### *P. pastoris* transformants

All the *P. pastoris* transformant clones were proven to secrete recombinant α-L-arabinofuranosidase activity as

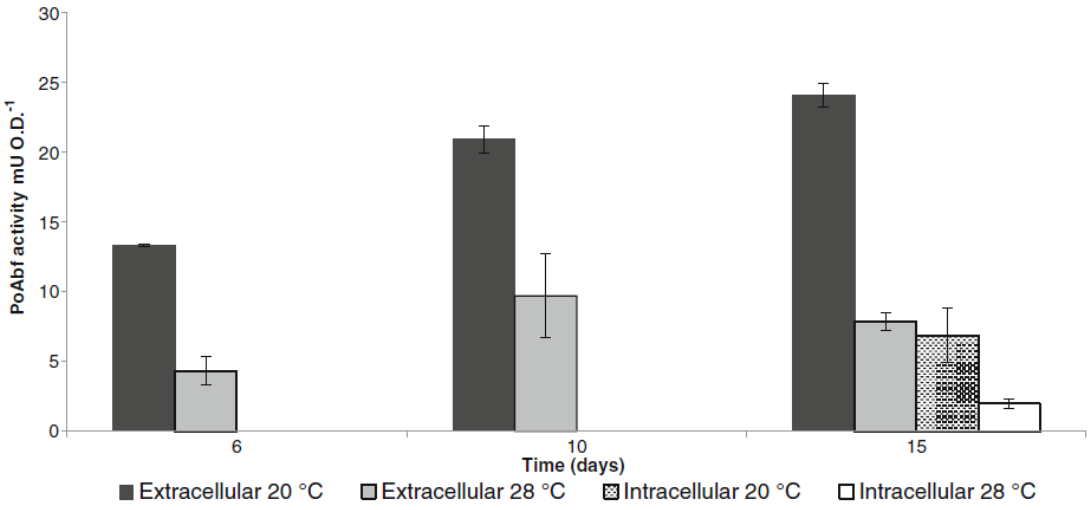


**Fig. 1** Effect of growth temperature on α-arabinofuranosidase activity levels produced by *P. pastoris* recombinant clone

shown by assay on plates containing MUA as substrate, giving fluorescence emission after 3 days. Seventeen recombinant clones were grown in liquid cultures and evaluated for Abf production. The maximum amount of α-L-arabinofuranosidase activity produced after 15 days was of $30\times10^{-2}$U $mL^{-1}$, corresponding to 8.3 mU $O.D.^{-1}$, in culture supernatant and of 1.2 mU $O.D.^{-1}$ in the cellular extracts.

The best producing clone was used to optimize rPoAbf production, study the time course of α-L-arabinofuranosidase production and characterize the recombinant enzyme. It was shown that rPoAbf production increases linearly for at least 1 month. Cultures were carried out to improve α-L-arabinofuranosidase production, varying the growth conditions. Increasing methanol concentration from 0.5 to 1 and

**Table 2** MALDI–MS analysis of the reduced and carboxamidomethylated rPoAbf digested with trypsin

| Peptide | *m/z* | Sequence |
|---|---|---|
| 1-3 | 469.28 | MYR |
| 352-357 | 643.40 | RAALGR |
| 104-110 | 747.43 | FTVPAGR |
| 255-261 | 758.46 | GQTVPTR |
| 543-551 | 1033.60 | YIPSTLPSR |
| 481-489 | 1041.53 | AAYMIGMER |
| 383-391 | 1073.65 | WPAFVNALR |
| 552-561 | 1095.60 | TGTAFWGVTR |
| 244-254 | 1297.68 | FFRFPGGNNLE |
| 91-103 | 1369.79 | SVPVSNSLPNSLR |
| 226-239 | 1447.74 | ANGMRDDIATALAE |
| 156-170 | 1546.90 | TTSGSTIVSQTVPIR |
| 111-125 | 1547.76 | TGQVGFANSGFFGMK |
| 247-261 | 1586.81 | FPGGNNLEGQTVPTR |
| 171-186 | 1717.95 | GASTSWQQISVSLTPR |
| 358-372 | 1723.92 | AQPFKLTYVEIGNED |
| 628-644 | 1786.95 | TFTFAAAGFSVNVITIK |
| 240-254 | 1782.00 | MKPKFFRFPGGNNLE |
| 226-243 | 1931.99 | ANGMRDDIATALAEMKPK |
| 44-61 | 1945.01 | DISHSGDGGLYAELLQNR |
| 450-468 | 1965.03 | YAAISTNPNDIFGSPANGR |
| 534-551 | 2027.07 | LFSTNMGNEYIPSTLPSR |
| 244-261 | 2037.06 | FFRFPGGNNLEGQTVPTR |
| 552-571 | 2136.10 | TGTAFWGVTRNTATGEIIIK |
| 448-468 | 2151.11 | GEYAAISTNPNDIFGSPANGR |
| 441-468 | 2961.42 | NGVTFYEGEYAAISTNPNDIFGSPANGR |
| 469-489 | 2281.07 | LTFSTMQSASGEAAYMIGMER |
| 104-125 + Oxidized Met | 2292.19 | FTVPAGRTGQVGFANSGFFGMK |
| 134-155 | 2345.26 | GSFFVKFPTASSFTGNLIVGLR |
| 363-382 | 2404.19 | LTYVEIGNEDFFASDTYTYR |
| 372-398 | 3167.65 | DFFASDTYTYRWPAFVNALRGAFPNLK |
| 140-170 + GalNacHex | 3572.62 | FPTASSFTGNLIVGLRTTSGSTIVSQTVPIR |
| 140-170 + $GalNacHex_2$ | 3734.80 | FPTASSFTGNLIVGLRTTSGSTIVSQTVPIR |
| 490-525 | 3865.11 | NSDIVFAASYAPLLGHVAGSQWTPNLIAFD TGNVYR |
| 187-223 | 3967.06 | TTPPSTDNQFFVTIDGAAASGQTINFAMFS LFPPTFK |
| 51-90 | 4111.13 | GGLYAELLQNRALQQVTPNTAASLNAWSAI NGGQISVVAD |
| 62-103 | 4247.44 | ALQQVTPNTAASLNAWSAINGGQISVVADS VPVSNSLPNSLR |
| 126-170 | 4722.30 | IVASSTYKGSFFVKFPTASSFTGNLIVGLR TTSGSTIVSQTVPIR |
| 441-489 | 5224.16 | NGVTFYEGEYAAISTNPNDIFGSPANGRLT FSTMQSASGEAAYMIGMER |

Ser160 in bold is glycosylated

1.5% causes a decrease of rPoAbf production by 2.5- and 4-fold, respectively, while decreasing the growth temperature from 28°C to 20°C lead to an increase of activity production (up to 1.1 $UmL^{-1}$ after 15 days) (Fig. 1). Enzyme activity was also found in intracellular protein fraction at both analyzed temperatures (Fig. 1).

## rPoAbf characterization

The enzyme purified to apparent homogeneity was subjected to structural and functional characterization as described below. The estimated molecular weight deduced from SDS-PAGE is 81,500 Da, considerably larger than that deduced from cDNA sequence (data not shown), probably due to an anomalous hydrodynamic behavior during electrophoretic separation on SDS-PAGE as expected from a glycosylated protein (see below). Using chromatography in a calibrated Superdex 200 HR 10/30 column (GE Healthcare, Uppsala, Sweden), a molecular weight of 39,000 Da was obtained for the native enzyme (data not shown). These results indicate that PoAbf is a monomer. The reason for the smaller apparent molecular weight in gel filtration could be retarded migration of the α-arabinosidase in the gel matrix, as was also reported for the α-arabinosidases and xylanases of *Trichoderma reesei* (Lappalainen 1986; Poutanen 1988). The presence of a galactose-binding domain like in the enzyme could be the reason for its binding to the column material.

Mass spectrometry analyses of rPoAbf allowed us to map 77.9% of the total amino acid sequence and the results are summarized in Table 2. Mass spectral analyses led to the localization of a single *O*-glycosylation site at level of Ser160, while Thr187 was found unmodified. Figure 2 shows the partial MALDI–MS spectrum obtained from the analysis of the tryptic peptide mixture of rPoAbf. The spectrum shows the occurrence of a signal at *m/z* 3572.62 assigned to peptide 140-170 modified with an oligosaccharide chain containing a GalNac and an Hexose moiety; moreover it is evident that the presence of a signal at *m/z* 3743.80 was attributed to the same peptide carrying a GalNacHex2 oligosaccharide moiety thus indicating the heterogeneity of *O*-glycosylation at Ser160.

The enzyme follows Michaelis–Menten kinetics towards pNPAra: the $K_M$ for this substrate is 0.64±0.11 mM and the $k_{cat}$ 3,010±145 $min^{-1}$. The pH optimum for the enzyme (assayed in a range from 3–8) is 5 (Fig. 3a) and the optimal temperature is 40°C (Fig. 3b). PoAbf shows a half life of 17 h at 50°C and few minutes at 60°C, while it retains 100% of its activity for at least 1 week at 30°C and 40°C. PoAbf shows very high stability in a broad range of pH ($T_{1/2}$ pH 3=24 days; $T_{1/2}$ pH 5=51 days; $T_{1/2}$ pH $7_{citrate–phosphate\ buffer}$=38 days; $T_{1/2}$ pH $7_{Tris–HCl\ buffer}$=42 days; $T_{1/2}$ pH 8=38 days).

The hydrolyzing ability of rPoAbf was tested versus a series of other nitrophenyl glycosides: *p*NP-β-D-xylopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, and *o*NP-β-D-galactopyranoside. Among these compounds, only *p*NP-β-D-glucopyranoside was recognized by the

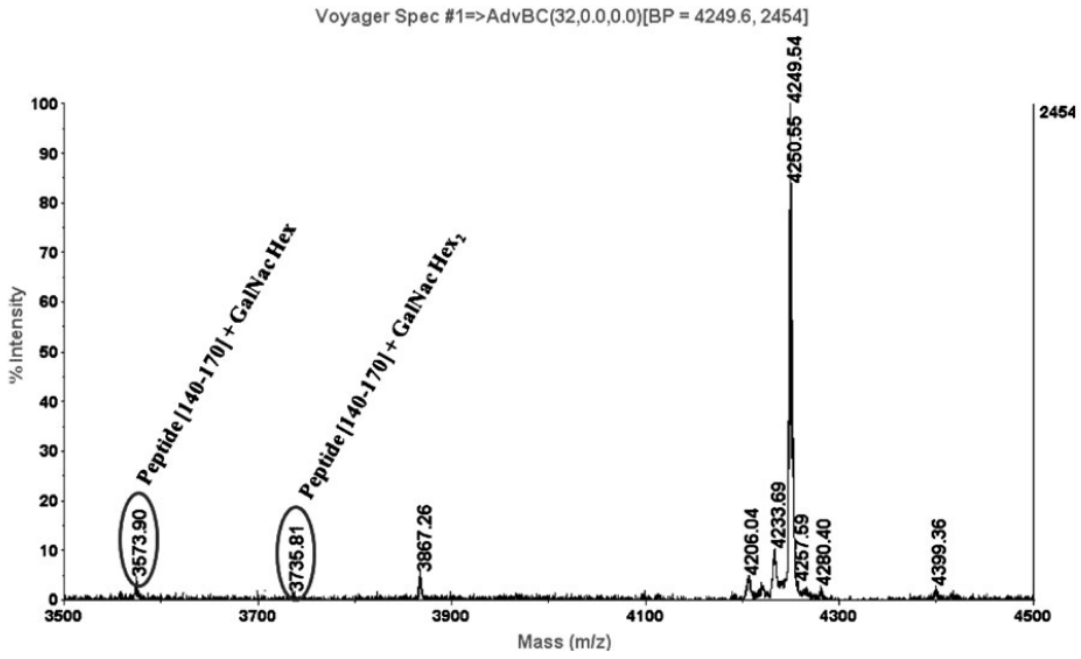


**Fig. 2** Partial MALDI–MS spectrum obtained from the analysis of the tryptic peptide mixture of rPoAbf

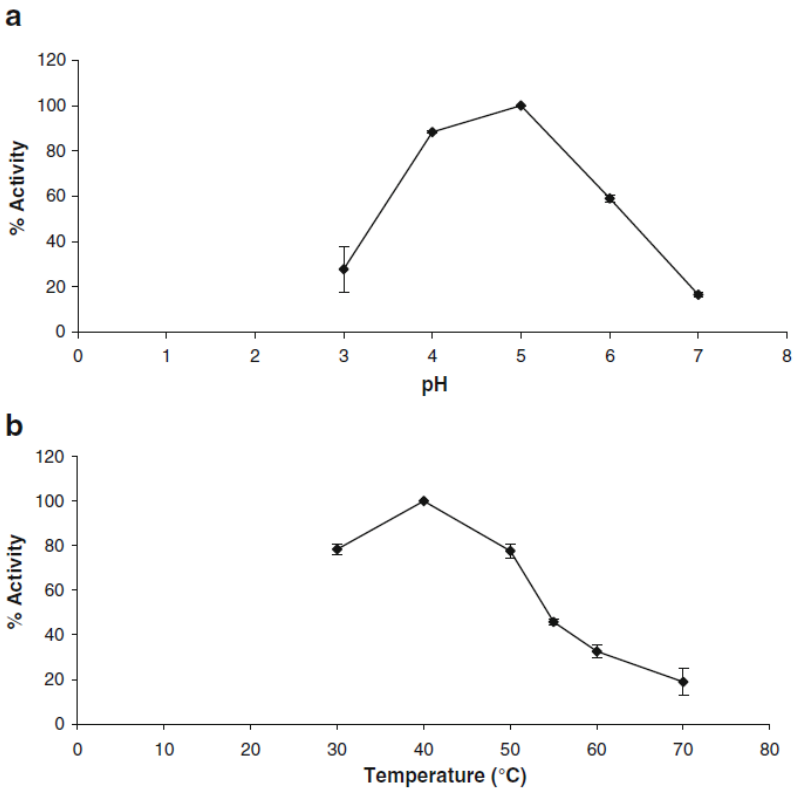


**Fig. 3** Effect of pH (**a**) and temperature (**b**) on the activity of rPoAbf towards pNPA as substrate. The percent activity reported in **a** and **b** was calculated as ratio to the maximum activity at optimum pH (5) and temperature (40°C), respectively

enzyme and a $K_M$ of 3.79±0.12 mM and a $k_{cat}$ of 9.3±0.5 $min^{-1}$ were measured for this substrate.

The enzyme was shown able to hydrolyze both the tested arabinooligosaccharides 1,5-α-arabinotriose and 1,5-α-arabinohexaose (Table 3). PoAbf is active on CM-linear arabinan (Table 3), while it does not hydrolyze larch arabinogalactan. Moreover, when the enzyme was incubated with the AZO-wheat arabinoxylan, it was shown to possess an endo-1,4-β-xylanase activity of around 0.55±0.08 $UmL^{-1}$.

## Discussion

To the best of our knowledge, this is the first report on the cloning, recombinant expression, purification and characterization of α-L-arabinofuranosidase from the genus of *Pleurotus*. This α-L-arabinofuranosidase was identified analyzing the enzymes responsible for xylanase activity produced by *P. ostreatus* SSF on tomato pomace. The gene and cDNA coding for this enzyme named PoAbf were cloned and sequenced. Analysis of the amino acid sequence of PoAbf places the enzyme in family 51 of the glycoside hydrolases.

A heterologous expression system was set up in the yeasts *P. pastoris* and *K. lactis* using the leader sequence of PoAbf in both the expression systems and the highest production level of the secreted enzyme (180 mg $L^{-1}$) was achieved in the former host. Decreasing growth temperature from 28°C to 20°C caused an increase of recombinant expression level of extracellular enzyme of around three-fold. Comparison of production levels of intracellular recombinant enzyme at the analyzed temperatures allowed us to demonstrate that the low temperature favors the production but it does not improve protein secretion. Many studies have demonstrated that temperature is a critical parameter in *P. pastoris* growth and recombinant protein production, in both batch and fed-batch systems and it has been reported that reducing induction temperature to 20°C or even lower values is beneficial for the efficient expression of heterologous proteins in *P. pastoris*. A common opinion accounting for this effect lies in the fact that lowering temperature could at least help to reduce cell

**Table 3** Arabinose liberation from natural substrates and arabinooligosaccharides

| Substrate | Amount of released sugar (μg/mL) |
|---|---|
| Larch arabinogalactan | 0 |
| CM-linear arabinan | 327±24 |
| Arabinotriose | 112±18 |
| Arabinohexaose | 102±26 |

Equivalents of galactose were measured as described in section "Material and methods"

skeleton lysis and protease secretion when cultivated on methanol, besides reducing cell death. Jin et al. (2010) reported that lowering induction temperature from 30°C to 20°C causes a 100-fold increase of recombinant production of Porcine interferon-α in *P. pastoris*. A reduction of temperature from 30°C to 25°C or 20°C resulted in a 2- and 3-fold increase of the specific productivity of the 3H6 Fab fragment, respectively (Dragosits et al. 2009). Li et al. (2001) showed that the increase of the recombinant production of herring antifreeze protein at lower temperature might benefit from the better protein folding. In fact, cultivating the cells at lower growth temperatures induces the rate of protein synthesis and thus may allow the nascent peptide chains to fold properly. By lowering the induction temperature to 22°C, also recombinant polygalacturonate lyase activity exhibited a 2.9-fold increase compared to cultivation at 30°C (Wang et al. 2009).

In all the other expression systems so far set up in *P. pastoris*, α-L-arabinofuranosidase was expressed under the control of yeast signal peptide. When compared with the other α-L-arabinofuranosidase recombinant expression systems, the system hereby reported showed similar or better yields, in terms of both units per milliliter and milligrams per liter. Particularly, our system was proven to be more efficient than the other recombinant expression systems so far reported in *P. pastoris*, where an expression yield of 20 mg $L^{-1}$ for the α-L-arabinofuranosidase B from *Aspergillus kawachii* (Miyanaga et al. 2004) and of 0.2 $UmL^{-1}$ for the α-L-arabinofuranosidase from *Trichoderma koningii* (Wan et al. 2007) was achieved. Yields obtained in *S. cerevisiae* expression systems ranged from 0.013 $UmL^{-1}$ for the α-L-arabinofuranosidase from *Aureobasidium pullulans* (Wan et al. 2007) to 1.40 $UmL^{-1}$ for *Aspergillus niger* α-L-arabinofuranosidase (Crous et al. 1996). Similar activity levels were achieved with *E. coli* expression systems, giving 0.94 $UmL^{-1}$ (Lee et al. 2011) for the α-L-arabinofuranosidases from *Penicillium* sp. LYG 0704, 1.48 $Umg^{-1}$ for the α-L-arabinofuranosidase from *Fusarium oxysporum* f. sp. *dianthi* (Chacòn-Martìnez et al. 2004) and 1.2 $UmL^{-1}$ (Shi et al. 2010) for the α-L-arabinofuranosidase from *Streptomyces* sp. S9.

rPoAbf produced in *P. pastoris* was purified and characterized, and it was shown to have some properties similar to the other members of family 51 ABFs (Numan and Bhosle 2006). Their subunit molecular weight generally ranges from 56–81 kDa. All enzymes show an acidic pH optimum and a value of $K_M$ for pNPAra ranging from 0.5–0.8 mM.

PoAbf revealed to be a versatile enzyme able to work on arabinooligosaccharides, with a higher affinity for the shorter ones, and on the natural polysaccharides linear arabinan and arabinoxylan, displaying both exo- and endoxylanase activities. It is worth noting that PoAbf shows very high stability in a broad range of pH, mostly at acidic pH, while most of other α-L-arabinofuranosidases were proven to be less stable, such as recombinant arabinofuranosidase from *Streptomyces sp. 9* that totally lost its activity after incubation for 1 h at pH 4 (Shi et al. 2010), or *A. pullulans* α-L-arabinofuranosidase that shows a half life of 29 min at pH 3 (Wan et al. 2007). PoAbf stability at pH 3 is also higher than stability of α-L-arabinofuranosidase from *T. reesei* (Poutanen 1988). The highly durable activity of rPoAbf enhances its potential for biotechnological applications and increases interest in this enzyme as a candidate for mutagenesis experiments aimed at elucidating the molecular bases of its peculiar properties.

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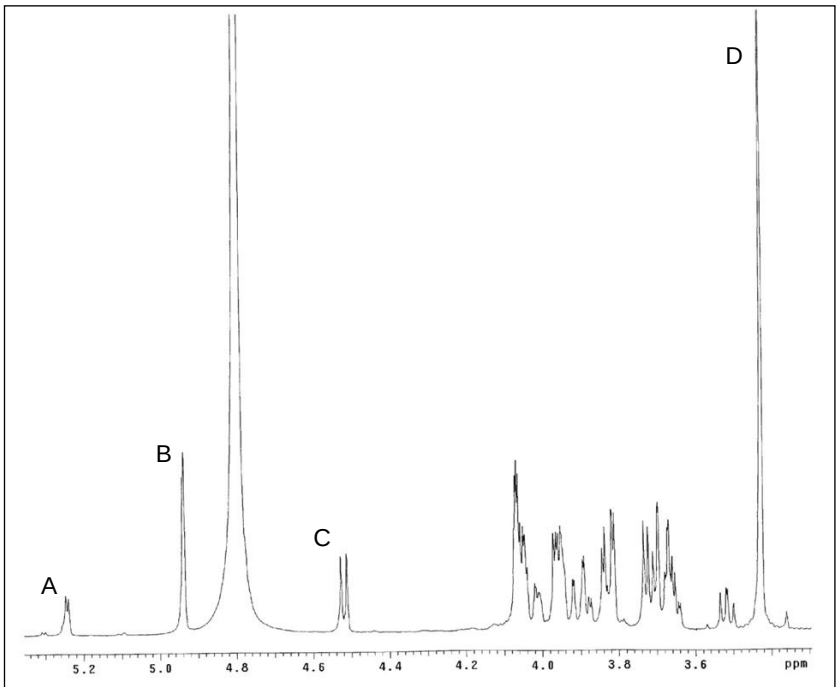
***Section 3-II***

### 3.2.2 Stereochemical study of rPoAbf wt hydrolysis

Direct $^{1}H$ and $^{13}C$ NMR analysis in $D_2O$ of the water-soluble fraction from the lyophilized residue evidence the presence of methyl α-arabinofuranoside together with the α- and the β-arabinopyranose forms. Figure 5 shows the $^{1}H$ NMR of this mixture, with the attribution of the most significant signals. Generation of methyl α-arabinofuranoside evidences the occurrence of a retaining mechanism with the nucleophilic attack of methanol (or water) to a β-configured glycosyl-enzyme intermediate formed in a previous step. Exclusive identification of L-arabinose in its pyranose forms also highlights the fast mutarotation of the initially generated α-arabinofuranose.




*Legenda*

A: H-1 beta AraPyr

B: H-1 Methyl α–AraFur

C: H-1 α–AraPyr

D: -$OCH_3$ Methyl α–AraFur

**Figure 5:** $^{1}H$ NMR of the crude mixture from hydrolysis of 4-nitrophenyl arabinofuranoside by PoAbf wild type

*Section 3-III*

### 3.2.3 Study of PoAbf structure/function relationships

Design of PoAbf mutants (Table 9) was carried out in order to study the structure/function relationships. Glycine was used for substituting the target residues, being commonly used to replace aminoacids that are considered to have a key structural/catalytic role. Particularly, rPoAbf S160G was prepared with the aim of elucidating the role of glycosylation on the enzyme stability. rPoAbf E364G and rPoAbf E471G were prepared for confirming the catalytic role of E364 and E471 as acid/base and nucleophlici residues, respectively, hypothesized on the base of sequence alignments.

**Table 9:** Design of PoAbf mutants

| PoAbf mutants |
|---|
| S160G |
| E364G |
| E471G |

#### 3.2.3.1 rPoAbf mutants design and production

Production of the above reported rPoAbf mutants has been performed in the yeast *Pichia pastoris*, previously shown to be the best host for this enzyme production. The production was studied at both 28°C and 20°C, the latter being the optiml temperature for enzymes production. Thus, growth cultures at 20°C have been performed to compare the activity production levels among wild-type and mutants. It is worth noting a strong decrease of the level of activity produced by E364G and E471G mutants, which suggests the hypothesized role of the two residues. However, further analyses will be carried out to confirm this hypothesis, such as the addition of external nucleophiles for trying reactivating the activity. (Figure 6).

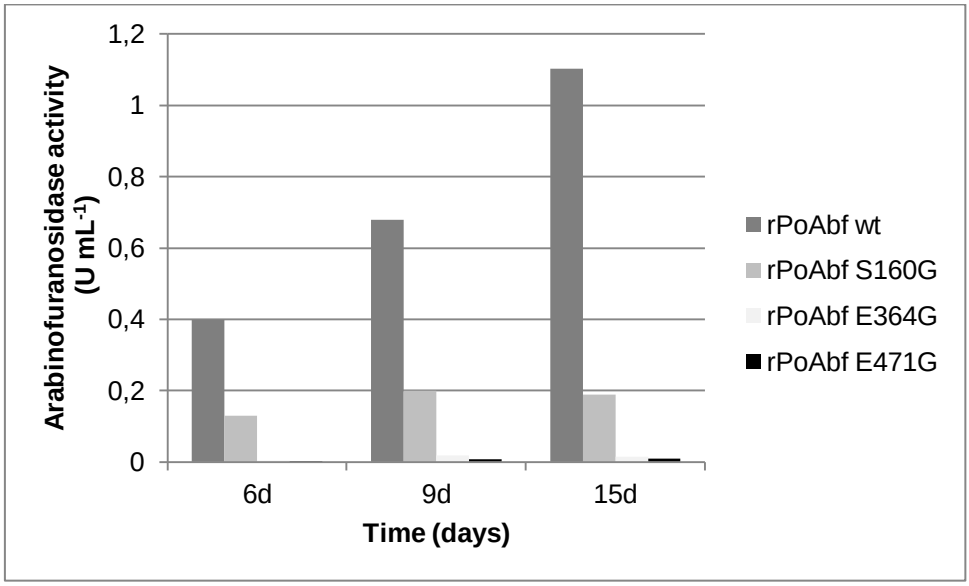



**Figure 6:** Comparison of rPoAbf wt and its mutants levels of production

### 3.2.3.2 rPoAbf S160G characterizaztion

***- Catalytic properties***

rPoAbf S160G has been over-produced, purified and studied for its catalyc properties. It follows a Michaelis-Menten kinetics when incubated with p-nitrophenil–α–L-arabinofuranoside, with a $K_M$ of 0.89±0.19 mM and a $k_{cat}$ of 2,590± 165 $min^{-1}$. Optimum pH and optimal temperature of reaction have been exstimated to be 5 and 40°C, respectively, similarly to the wild-type enzyme. Glycosilation has been shown to affect rPoAbf stability at 50°C and pH 8, conditions in which rPoAbf S160G has an half-life ($t_{1/2}$) much lower than rPoAbf wt (Table 10).

**Table 10:** rPoAbf S160G *versus* rPoAbf wt.

| | **rPoAbf wt *versus* rPoAbf S160G** | |
|---|---|---|
| **$K_M$** | 0.64 ± 0.11 mM | 0.89±0.19 mM |
| **$k_{cat}$** | 3,010 ± 145 $min^{-1}$ | 2,590± 165 $min^{-1}$ |
| **Optimal temperature** | 40°C | 40°C |
| **Optimum pH** | 5 | 5 |
| **Thermo-resistance** | $t_{1/2=}$17 h a 50°C | $t_{1/2=}$3 h a 50°C |
| **pH stability** | $t_{1/2=}$38 gg a pH 8 | $t_{1/2}$= 2.5 h a pH 8 |

Many studies indicate that glycosylation strongly affects glycosyl hydrolase function and biomass conversion. However, few manuscripts deeply investigated the effect of glycosylation on the large GH families involved in lignocellulosic biomass degradation [77]. However it is well known that glycosylation impacts the function, structural framework, and stability of the enzymes. As reported by Jeoh et al. [78], most of the *T. reesei* cellulases are glycoproteins, where the extent and type of glycan can strongly vary. Cel7A, the major cellulase produced by *T. reesei* is highly O- and N-glycosylated, and an altered level of N-linked glycosylation was observed to negatively impact the activity and cellulose binding affinity. Zouh et al. [79] computationally annotated the glycosylated residues in all known cellulases, and conducted a systematic analysis of the distributions of the N-linked and O-linked glycosylated residues in these enzymes. However, deglycosylation not always negatively affect glycosyl hydrolase activity. For instance, the effect of N-linked glycosylation on secretion, activity, and stability of α-amylase from *Aspergillus oryzae* were studied by Eriksen et al. [80] showing that deglycosylation did not led to the loss of enzyme stability.

***- Structural properties***

Circular Dichroism (CD) analyses have been performed to study and compare the structure of rPoAbf wt and its mutant S160G. Spectra between 190-250 nm were recorded, showing for rPoAbf wt a minimum at 210 nm, typical of the beta-sheets structure. As far as rPoAbf S160G is concerned, this minimum is shifted, suggesting the presence of an unfolded strucutre (Figure 7). Particularly, for the mutant an increase of the unfolded structure % (from 36 to 44%) and a decrease of the beta-

sheets structure % (from 35 to 24 %) have been recorded by using the software Dichroweb, thus suggesting the important role of the glycosilation in rPoAbf stability, according to the loss of stability in a broad range of pH and temperature.

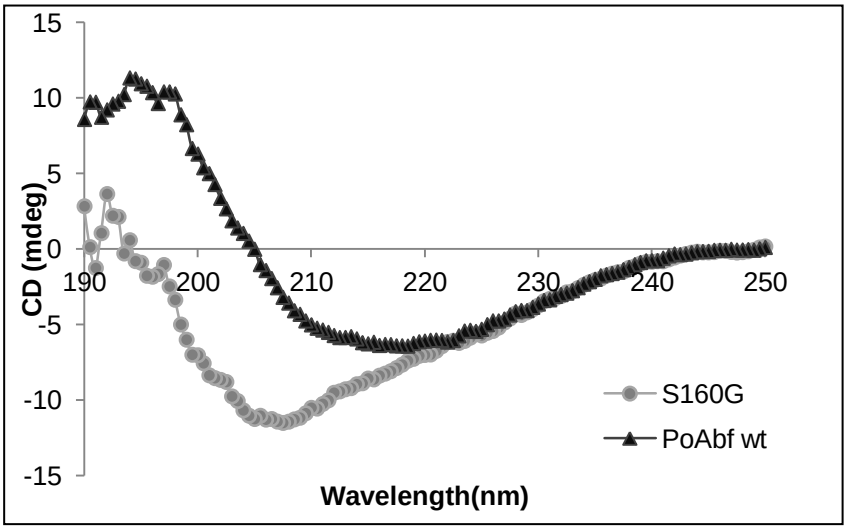



**Figure 7:** CD spectra for rPoAbf wt and rPoAbf S160G structure analyses

### 3.3 Material and methods

**- Site-directed mutants recombinant expression**

The pPICZ-abf vector containing the cDNA encoding for PoAbf (EMBL Data Library accession number HE565356) was used for recombinant expression in *P. pastoris,* as previously reported [77]. Site-directed mutagenesis was performed using the QuikChange sitedirected mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic primers for mutated genes preparation are following reported (the mutated nucleotides are underlined):

| Mutant | Primer Lenght | Sequence |
|---|---|---|
| S160G | 30 | GAACCACTTCTGGT**GGC**ACTATCGTTTCCC |
| E367G | 34 | CAAATTGACCTACGTC**GGG**ATCGGTAATGAGGAC |
| E480G | 35 | GCAATCTGCTTCCGGA**GGG**GCTGCCTACATGATTG |

The mutated genes were sequenced to confirm that only the desired mutations were inserted. Sequencing was performed by PRIMM Sequencing Service (Naples, Italy) using universal and specific oligonucleotide primers. The wild type and mutated proteins were overexpressed, purified and assayed as previously described [77].

**- Study of the catalytic properties of rPoAbf mutants**

Optimum temperature and temperature stability, optimum pH and pH stability catalytic constants $k_{cat}$ and $K_M$ were evaluated following the protocol reported in Amore et al. [77].

**- Spectroscopy Techniques**

Far-UV CD spectra were recorded on a Jasco J715 spectropolarimeter equipped with a Peltier thermostatic cell holder in a quartz cell (0.1 cm light path) from 190 to 250 nm. The temperature was kept at 25 °C and the sample compartment was continuously flushed with nitrogen gas. The final spectra were obtained by averaging three scans, using a bandwidth of 1 nm, a step width of 0.5 nm, and a 4 s averaging per point. The spectra were then corrected for the background signal using a reference solution without the protein.

- **Stereochemical study of rPoAbf wt hydrolysis**

For $1^H$ NMR characterization, the enzymatic reaction included wild type PoAbf (0.2 mg), 4-NPAF (20 mg), and methanol (2.5 M) in a final volume of 5.5 mL. After 10 min at 40 °C, the reaction was quenched by the addition of 2 mM $HgCl_2$ and lyophilized.

This PhD project was aimed at the development of new biocatalysts, namely cellulase, xylanase and arabinofuranosidase, involved in lignocelluloses conversion into fermentable sugars for second generation bioethanol production.
Two bacterial cellulases, CelB31C and CelStrep, were identified from the new isolates *Bacillus amyloliquefaciens* B31C and *Streptomyces* sp. G12, respectively, whilst a new arabinofuranosidase, PoAbf, was identified from the fungus *Pleurotus ostreatus*. The production of the new enzymes has been optimized by setting up and optimizing recombinant expression systems in *E. coli* and *P. pastoris*, in the case of CelStrep and PoAbf, respectively. On the other hand, conditions for native CelB31C production and purification were investigated.
The new recombinant and native enzymes were purified and characterized from the catalytic point of view, and tables 11A and 11B list the catalytic properties of the new cellulases and new arabinofuranosidase.

**Table 11:** Catalytic properties of cellulases from *Bacillus amyloliquefaciens B31C and Streptomyces* sp.*G12* (A) and rPoAbf from *Pleurotus ostreatus* (B)

**A)**

| | **rCellStrept** | **CelB31C** |
|---|---|---|
| **Optimal temperature** | 50°C | 50-70°C |
| **Thermoresistance** | $t_{1/2}$= 24h at 60°C<br>$t_{1/2}$= 96h at 40°C | 90 % of activity retained at 40°C after 6 days |

**B)**

| | **rPoAbf** |
|---|---|
| **Optimal temperature** | 40°C |
| **Optimum pH** | 5 |
| **pH Stability** | $t_{1/2}$= 51 days at pH 5 |
| **Thermoresistance** | $t_{1/2}$= 7days at 30°C/40°C |

As far as PoAbf is concerned, experiments of site-directed mutagenesis were also performed in order to elucidate the role of glycosilation on the enzyme stability and to investigate the role of E364 and E471 as nucleophile and acid/basic catalytic residues, respectively.
It has been shown that site-directed mutagenesis of E364 and E471 residues, causes a strong decrease of the production levels of arabinofuranosidase activity in the correspondent mutants, thus suggesting the hypothesized role of these aminoacids in the catalytic mechanisms of PoAbf.

It has been demonstrated that glycosilation affects rPoAbf stability at 50 °C and pH 8, conditions in which rPoAbf S160G has an half-life much lower than rPoAbf wt.
Moreover, in this work four thermophilic filamentous fungi have been selected from a collection of 150 thermophilic fungi isolated from a “maâsra” in Morocco, based on their cellulase activity production on cellulose-containing solid medium and their attitude to colonize substrates (apical growth). Their cellulolytic and xylanolytic systems will be studied and solid state fermentation processes on agro-industrial wastes will be set up for producing the enzymes of interest.
93 microorganisms have been isolated from natural habitats already exploited for isolation of new microorganisms with biotechnological interest, namely Western Ghat. Their screening for cellulase activity production in both solid and liquid medium was carried out, leading to the selction of 7 xylanolytic microorganisms and 14 cellulolytic microorganisms. Amone these new isolates, a *Lysinobacillus xylanolyticus* (XR84) strain was shown to produce both cellulase and xylanase activities in the basal liquid growth conditions tested in this study, thus pushing towards the interest to further characterize the potential role of this microorganism and its hydrolytic enzymes in lignocellulose conversion.
The new biocatalysts will be explored for their potential role in lignocellulosic biomass conversion, as components of minimal enzymatic cocktail finely designed for hydrolysis of lignocellulose, thus evaluating their potential contribution to improve cost efficiency of second generation bioethanol production.
Moreover, efforts from this project support the use of lignocellulosic waste as a source for industrial enzyme production, that is an alternative disposal, generally consisting in incineration, land filling and use for animal feed production. The use of the whole lignocellulose is also outlooked by this work, enhancing the exploitation of all the components of the biomass, towards the development of a biorefinery where the production of fermentable sugars is combined to the production of other bio-based products.

***Aknowledgements***



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**Appendix I**

***List of Communications***

1. Vincenza Faraco, **Antonella Amore**, Yitzhak Hadar, Tomer Salame and Giovanni Sannia, "**Optimization of *Pleurotus ostreatus* transformation for laccase promoter analysis**", Lignobiotech ONE Symposium, 1st Symposium on biotechnology applied to lignocellulose, March 28th – April 1st 2010.
2. Donata Iandolo, **Antonella Amore**, Giovanni Sannia and Vincenza Faraco, " **Xylanase and laccase production by fungal solid state fermentation"**, Lignobiotech ONE Symposium, 1st Symposium on biotechnology applied to lignocellulose, March 28th – April 1st 2010.
3. Valeria Ventorino, **Antonella Amore**, Vincenza, Giuseppe Blaiotta, Olimpia Pepe, **"Selection of cellulolytic bacteria for processing of cellulosic biomass"**, 14th International Biotechnology and Exhibiotion, 14-18 September 2010.
4. **Antonella Amore** and Vincenza Faraco. "Exploring biodiversity to advance cellulosic ethanol production from biowaste: new cellulolytic microorganisms and their enzymes" 15th INTERNATIONAL TRADE FAIR OF MATERIAL AND ENERGY RECOVERY AND SUSTAINABLE DEVELOPMENT, ECOMONDO, November 9th to 12th, 2011 Rimini, Italy.
5. **Antonella Amore** and Vincenza Faraco. "α-L-arabinofuranosidase from *Pleurotus ostreatus* for food and beverage industries", 5th INTERNATIONAL CONFERENCE ON NEW HORIZONS IN BIOTECHNOLOGY (NHBT-2011) November 21-24, 2011, Trivandrum, India.
6. **Antonella Amore**, Angela Amoresano, Leila Birolo, Vincenza Faraco. "A high-added value product from tomato pomace conversion: α-L-arabinofuranosidase from *Pleurotus ostreatus* for lignocellulose conversion", Environmental Microbiology and Biotechnology 2012 (EMB2012) April 10-12, 2012, Bologna, Italy.
7. **Antonella Amore**, Angela Amoresano, Leila Birolo, Vincenza Faraco. "High-added value products from agro-industrial wastes: ligninolytic and hemicellulolytic activities production and application", Renewable Resources and Biorefineries (RRB-8) June 4-6, 2012, Toulose, France.
8. **Antonella Amore**, Yoichi Honda, Vincenza Faraco. "A new system for in vivo analyses of *Pleurotus ostreatus* promoters of laccases for food and beverages industries", International Conference on Industrial Biotechnology (ICIB2012), November 21-23, 2012, Patiala, India
9. Vincenza Faraco, **Antonella Amore** and Simona Giacobbe. "Rational and random mutagenesis of a family GH51 α-L-arabinofuranosidase for food and beverage industries", International Conference on Industrial Biotechnology (ICIB2012), November 21-23, 2012, Patiala, India
10. Vincenza Faraco, Olimpia Pepe, **Antonella Amore** and Valeria Ventorino. "New microorganisms and enzymes for conversion of wastes and eco-friendly and eco-efficient production of high added values products", IFIB 2012 Italian Forum on Industrial Biotechnology and Bioeconomy, October 23-24, 2012, Milan, Italy

***List of Publications***

1. Iandolo D*, **Amore A***, Birolo L, Leo G, Olivieri G and Faraco V. "**Fungal solid state fermentation on agro-industrial wastes for acid wastewater decolourization in a continuous flow packed-bed bioreactor"** Bioresource Technology 2011, 102 (16):7603-7607
   *These authors equally contributed to this manuscripts
2. **Amore A**, Amoresano A, Birolo L, Henrissat B, Leo G, Palmese A, Faraco V**.** "**A family GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus*: identification, recombinant expression and characterization"** Appl Microbiol Biotechnol 2011, 94(4):995-1006.
3. Karp SG, Faraco V, **Amore A**, Birolo L, Giangrande C, Soccol VT, Pandey A, Soccol CR. "**Characterization of laccase isoforms produced by *Pleurotus ostreatus* in solid state fermentation of sugarcane bagasse"** Bioresource Technology 2012, 114:735-9.
4. Castanera R, Pérez G, Omarini A, Alfaro M, Pisabarro A, Faraco V, **Amore A** and Ramírez L. **"Transcriptional and enzymatic profiles of *Pleurotus ostreatus* laccase genes in submerged and solid state fermentation and their relation to the mycelia growth rate"** Applied and Environmental Microbiology 2012, 78(11):4037-45.
5. **Amore A** and Faraco V. "**Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production" Renew. Sust. En. Rev**. 2012; 16:3286-3301.
6. **Amore A**, Honda Y, Faraco V. " **Copper induction of enhanced green fluorescence protein expression in *Pleurotus ostreatus* driven by laccase *poxa1b* promoter"** FEMS Microbiol Lett 2012, 337(2):155-6**3**
7. **Amore A**, Honda Y, Faraco V. "**Enhanced green fluorescent protein expression in *Pleurotus ostreatus* for *in vivo* analysis of fungal laccase promoters.**" Appl Biochem Biotechnol. 2012, 168(4):761-9.
8. **Amore A**, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V, **"Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces sp.* G12 isolated from compost."** Microbial Cell factories 2012, 11(1):164.
9. **Amore A,** Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V, **"Industrial waste based compost as a source of novel cellulolytic strains and enzymes"** FEMS microbiology letters 2012.
10. Piscitelli A, **Amore A** and Faraco V, **"Last advances in synthesis of added value compounds and materials by laccase-mediated biocatalysis "** Curr Org Chem, 2012, 16:2508-2524.
11. **Amore A**, Pepe O, Ventorino V, Aliberti A and Faraco V, "**Cellulolytic *Bacillus* strains from natural habitats - A review**" accepted for publication in Chemistry TODAY

***Chapters in book***

1. **Amore A** and Faraco V. **"Chemico-physical factors affecting food and beverage processing fermentations"** within the volume on *ENGINEERING ASPECTS OF FERMENTATION PROCESSES IN THE FOOD INDUSTRY* (Editors: Carlos Ricardo Soccol, Ashok Pandey, Christian Larroche) for the Taylor & Francis Book series: Contemporary Food Engineering (*In press*)

***Experiences in other laboratories***

- Stage at **Department of Food Sciences, University of Naples "Federico II"**,Portici (Napoli) for the isolation and selection of cellulolytic microorganisms from industrial waste based compost (Janaury - March 2010), ). The work was supervised by Prof. Olimpia Pepe.

***Experiences in foreign laboratories***

- Stage at **NIIST-CSIR Institute, Trivandrum (Kerala, India)** for isolation, selection and characterization of cellulolytic and xylanolytic microorganisms, and set up of solid state fermentation processes (August-December 2012). The work was supervised by Dr. Ashok Pandey and Dr. Binod Paramaswaren.

- Stage at **IMEP-IRD Institute, Marseille (France)** for screening and selection of thermopile cellulolytic fungi (November-December 2010). The work was supervised by Dr. Sevastianous Roussos.

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# ATTESTATION DE STAGE

Je soussigné, Sévastianos ROUSSOS, Directeur Adjoint de l'IMEP, atteste que Mlle Antonella AMORE, étudiante italienne, boursière sur projet, a réalisé un stage de formation du 13 novembre au 18 décembre 2010 au Laboratoire d'Ecologie Microbienne et Biotechnologie de l'Institut Méditerranéen d'Ecologie et Paléoécologie, Université Paul Cézanne à Marseille. Au cours de son stage sous ma responsabilité scientifique et l'encadrement technique de Martine Martinez et de Yoan Labrousse, techniciens au laboratoire, elle a travaillé sur le criblage de 150 souches de champignons filamenteux thermophiles pour la recherche de souches à haute activité cellulosique en vue de la valorisation biotechnologique des substrats lignocellulosiques. A la fin de son stage elle a fourni un rapport écrit sur les techniques utilisées et les souches criblées. En particulier elle a utilisé un milieu minéral de base et des colorants spécifiques pour détecter l'activité, elle a pu être sensibilisée sur la physiologie de sporulation des souches et sur la mise en œuvre de la technique de Fermentation en milieu solide, en utilisant le respiromètre PNEO. Son séjour à la Faculté des Sciences et Techniques de Saint Jérôme à Marseille lui a permis de sélectionner une souche hyper productrice de cellulases à 50°C.

Mlle Antonella AMORE, est une personne très aimable, intelligente, motivée par le travail , pouvant travailler d'une manière autonome au laboratoire et avec beaucoup d'initiative. Je serai très heureux de pouvoir l'accueillir à nouveau pour un stage beaucoup plus long, sur les Fermentations en milieu solide pour la production de cellulases sur des substrats urbains solides.

*Fait à Marseille le 15 décembre 2010*

S. Roussos
Directeur Adjoint de l'IMEP

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19th December 2012

This is to certify that Ms. Antonella Amore, Researcher, University of Naples "Federico II" , Italy, carried out research work in my Laboratory from 27th August to 27th November 2012. During this period she worked on isolation and screening of novel cellulase and xylanase producing microbes which might be having potential application in second generation bioethanol production.

Dr. Binod Parameswaran

Dr. BINOD PARAMESWARAN
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***Others***

- **Best Poster Award at "Renewable Resources and Biorefineries (RRB-8) Conference"**, June 4-6, 2012, Toulose, France.

- **Best Poster Award at "International Conference on Industrial Biotechnology (ICIB2012)"**, November 21-23, 2012, Patiala, India.

## Appendix II

## Other Publications

Bioresource Technology 102 (2011) 7603–7607

Contents lists available at ScienceDirect

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Short Communication

# Fungal solid state fermentation on agro-industrial wastes for acid wastewater decolorization in a continuous flow packed-bed bioreactor

Donata Iandolo [a,1,3], Antonella Amore [a,3], Leila Birolo [a,b], Gabriella Leo [a,2], Giuseppe Olivieri [b,c], Vincenza Faraco [a,b,*]

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ABSTRACT

This study was aimed at developing a process of solid state fermentation (SSF) with the fungi *Pleurotus ostreatus* and *Trametes versicolor* on apple processing residues for wastewater decolorization. Both fungi were able to colonize apple residues without any addition of nutrients, material support or water. *P. ostreatus* produced the highest levels of laccases (up to 9 U $g^{-1}$ of dry matter) and xylanases (up to 80 U $g^{-1}$ of dry matter). A repeated batch decolorization experiment was set up with apple residues colonized by *P. ostreatus*, achieving 50% decolorization and 100% detoxification after 24 h, and, adding fresh wastewater every 24 h, a constant decolorization of 50% was measured for at least 1 month. A continuous decolorization experiment was set up by a packed-bed reactor based on colonized apple residues achieving a performance of 100 mg dye $L^{-1}$ $day^{-1}$ at a retention time of 50 h.



## 1. Introduction

Wastewaters from the textile industry can be considered as the most polluting among all industrial sectors, and their treatment is greatly challenging. The white rot basidiomycetes fungi are, so far, the microorganisms most efficient in degrading synthetic dyes. This property is due to the production of extracellular lignin-modifying enzymes – manganese peroxidases (MnP), lignin peroxidases (LiP), and laccases (Lac) – which are able to degrade a wide range of xenobiotic compounds, including dyes. Laccase is the main enzyme involved in dye decolorization by cultures of *Phlebia tremellosa*, *Pleurotus sajor-caju* and *Pleurotus ostreatus* and several recent studies have been focused on dye degradation by laccases. Most studies dealing with ligninolytic enzymes have been performed in submerged conditions, whereas solid state fermentation (SSF) would be more suited for cultivating basidiomycetes fungi, because it provides conditions very similar to the environment in which they have evolved and it shows several advantages over liquid fermentation systems (Singhania et al., 2009). Many studies reported the exploitation of SSF to give an extra value to agro-industrial residues (Rodriguez Couto, 2008; Sharma and Arora, 2010; Cha et al., 2010) and different reactors for SSF were reported (Singhania et al., 2009).

In this study, a process of SSF of the basidiomycetes fungi *P. ostreatus* and *Trametes versicolor* on apple processing residues was developed for production of ligninolytic and (hemi)cellulolytic enzymes and their use in a packed-bed reactor for decolorization of textile wastewaters.

## 2. Methods

### 2.1. Lignocellulosic substrates

Commercially available wheat, employed for fungal pre-culture, was prepared as previously described (Iandolo et al., 2011). Apple residues were collected in a local apple processing farm of Campania (Italy). The residues (peels, seeds and stems) were reduced to small pieces and sieved to have 0.8–2.0 mm dimension particles, and stored by freezing them at −20 °C. Before sterilizing in autoclave at 110 °C for 1 h, 1.5% (w/w) $CaCO_3$ was added to reach a final pH of 6.0. As far as the percentage of humidity is concerned, the starting value of 71% was kept, without further water addition.

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### 2.2. Fungal strains

The basidiomycetes fungi used in this study were a strain of the white rot fungus *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC MYA-2306) from ATCC, the Global Bioresource Centre and *T. versicolor* (NBRC4937) from the fungal collection Nite Biological Resource Center (Department of Biotechnology National Institute of Technology and Evaluation, Japan).

### 2.3. Solid state fermentation

Fungal growth on wet apple residues prepared as above described and samples preparation for analyses were carried out as previously described (Iandolo et al., 2011).

### 2.4. Analytical determinations

Evaluation of humidity and dry matter content, analysis of soluble reducing sugars content and assays of laccase, manganese peroxidase, lignin peroxidase, filter paper and xylanase activity, and evaluation of total protein content were performed as previously described (Iandolo et al., 2011).

### 2.5. Non-denaturing PAGE

Non-denaturing PAGE was performed as previously described (Iandolo et al., 2011). The gels were stained to visualize laccase activity by using ABTS as the substrate.

### 2.6. Decolorization batch experiments

Batch decolorization experiments on previously formulated model wastewaters containing acid industrial dyes (Faraco et al., 2009) were performed with apple wastes after three days of fungal fermentation and crude enzymatic extracts from these samples. An amount of colonized waste (0.3–0.5 or 0.6–1 g) corresponding to a laccase activity of 1 or 2 U or a volume of crude enzymatic extracts corresponding to 1 U of laccase activity were incubated with wastewater models up to a final volume of 4 mL, at room temperature under vigorous agitation by a rotary shaker. Decolorization was evaluated recording UV–VIS absorption spectra at different times (10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 7 h, 9 h, 24 h) of incubation and calculated as the extent of decrease of spectrum area recorded between 380 and 740 nm in comparison with the corresponding area of the spectrum of the untreated wastewater model. To evaluate the extent of color absorption on mycelium and agro-industrial residues, incubation of colored wastewaters with residues after mycelium inactivation by autoclave treatment at 120 °C for 20 min, and measurement of decolorization at different times were performed.

A repeated sequencing batch decolorization experiment was set up by using 0.3–0.5 g of apple residues colonized by *P. ostreatus* at the third day of SSF corresponding to 1 U of laccase activity within a dialysis tube (cut off of 12,000–14,000 KDa) (Delchimica scientific glassware srl, Italy). Each decolorization cycle consisted of addition of 4 ml of the wastewater to the apple residues. A new cycle was initiated after 24 h when no further change in the wastewater decolorization was measured. Decolorization was evaluated as described above. The experiments were performed in three-replicates and reported values are representative of three experiments.

### 2.7. Continuous packed-bed reactor

For setting up the fixed bed reactor for acid bath decolorization, 12 g of apple processing residues colonized by *P. ostreatus* at the 3rd day of SSF were homogenized in a blender in the presence of 12 mL of 50 mM Na phosphate pH 7 and packed in a dialysis membrane (~480 mm × 4.5 mm, cut off 12,000–14,000 KDa) (Delchimica scientific glassware srl, Italy). The dialysis membrane was placed within a glass column (495 mm × 9 mm) and surrounded by glass beads (500 μm mean diameter) in order to optimize flux distribution around the dialysis membrane (working volume 30 mL) (Fig. 1). The reactor system was operated at room temperature. Absorption at 590 nm (corresponding to the wavelength of maximum absorption of Acid Blue 62) was continuously measured, and at regular time periods of 24 h decolorization % was evaluated as described above (Section 2.6). Reported values are representative of three experiments.

### 2.8. Detoxification experiments

Detoxification was evaluated on the acid bath (4 mL) after 24 h of treatment with apple processing residues colonized by *P. ostreatus* at the third day of SSF within the dialysis tube (final activity 1 U) and on the acid bath treated in the continuous packed-bed reactor. Toxicity was determined as previously described (Palmieri et al., 2005). The experiments were performed in duplicate and reported values are representative of at least two experiments.

### 2.9. Protein identification by mass spectrometry

Protein identification by mass spectrometry was performed on slices of interest from the non-denaturing PAGE as previously described (Lettera et al., 2010).

## 3. Results and discussion

### 3.1. Development of solid state fermentation process on apple residues

A process of SSF with *P. ostreatus* and *T. versicolor* was developed on apple residues, demonstrating the ability of both fungi to colonize the waste quickly and extensively. It is worth noting that addition of either a material support or water was not necessary to allow fungal colonization of the waste. During SSF, no significant changes in humidity and reducing sugars level were measured (data not shown). A continuous reduction of dry mass was assessed for both fungi (Table 1), possibly due to waste conversion into volatile compounds by fungal metabolism.

### 3.2. Time courses of ligninolytic and glycosyl hydrolytic enzymes

The production of ligninolytic (Lac, LiP and MnP) and glycosyl hydrolytic (xylanase and cellulase) enzymes was evaluated during *T. versicolor* and *P. ostreatus* SSFs (Table 1). As far as laccase activity levels are concerned, *P. ostreatus* reached similar levels to those produced on tomato pomace, while *T. versicolor* achieved a ten fold lower maximum value with respect to tomato pomace SSF (Iandolo et al., 2011). In comparison with SSF processes previously reported with bith other *P. ostreatus* strains (Elisashvili et al., 2008; Stajic et al., 2006; Kurt and Buyukalaca, 2010) and other fungi (Elisashvili et al., 2008; Moldes et al., 2003; Rosales et al., 2002), SSF of the *P. ostreatus* strain tested in this study allowed the achievement of similar values, although neither optimization of culture conditions nor nutrient/water addition were performed in this study. As far as peroxidase production is concerned, only *T. versicolor* secreted detectable levels of MnP activity, while neither *P. ostreatus* nor *T. versicolor* produced appreciable values of LiP. Among the investigated glycosyl hydrolases, only xylanolytic enzymes were produced at detectable levels by both fungi, measuring lower values than those previously reported in other studies on SSFs with fungi

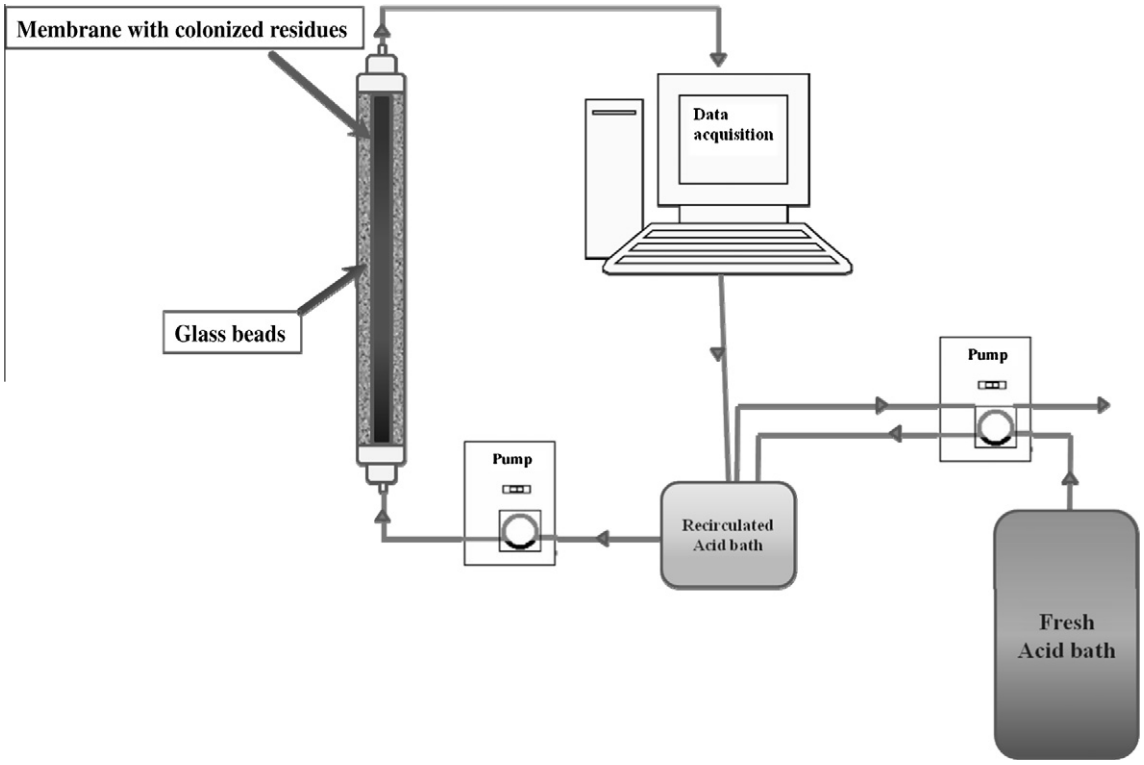


**Fig. 1.** Schematic diagram of the packed-bed reactor based on apple residues colonized by *P. ostreatus* employed, operating in a continuous mode.

**Table 1**
Dry mass content and enzyme activity values during SSF. Results of the analytical determinations reported correspond to mean values of three-replicates of two independent experiments.

| Time (days) | Dry mass content (g) | MnP activity (U/g d.m.) | Laccase activity (U/g d.m.) | Xylanase activity (U/g d.m.) |
|---|---|---|---|---|
| *Pleurotus ostreatus* | | | | |
| 0 | 17.6 ± 0.3 | n.d. | 0 | 0 |
| 1 | 14.6 ± 0.4 | n.d. | 1.27 ± 0.15 | 44.08 ± 4.83 |
| 2 | 14.3 ± 1.1 | n.d. | 2.76 ± 0.14 | 55.55 ± 2.10 |
| 3 | 12.5 ± 1.1 | n.d. | 9.05 ± 0.97 | 45.26 ± 8.12 |
| 4 | 13.6 ± 0.5 | n.d. | 5.88 ± 0.93 | 76.94 ± 10.12 |
| 5 | 13.9 ± 0.8 | n.d. | 6.27 ± 0.31 | 46.87 ± 3.42 |
| 7 | 11.7 ± 0.9 | n.d. | 3.32 ± 0.69 | 36.60 ± 1.99 |
| *Trametes versicolar* | | | | |
| 0 | 15.1 ± 0.3 | 0 | 0 | 0 |
| 1 | 15.2 ± 0.7 | 0 | 1.11 ± 0.17 | 36 ± 1 |
| 2 | 15.0 ± 0.4 | 0.47 ± 0.06 | 3.82 ± 0.07 | 31.30 ± 0.35 |
| 3 | 14.6 ± 0.3 | 0.56 ± 0.03 | 4.12 ± 0.36 | 36.85 ± 1.43 |
| 4 | 13.5 ± 0.2 | 0.60 ± 0.08 | 1.94 ± 0.00 | 18.10 ± 1.70 |
| 5 | 14.1 ± 0.4 | 0.89 ± 0.06 | 2.70 ± 0.18 | 30.15 ± 1.00 |
| 6 | 13.7 ± 1.9 | 0.64 ± 0.04 | 1.44 ± 0.38 | 34.15 ± 1.04 |
| 7 | 13.0 ± 0.2 | 0.65 ± 0.03 | 1.80 ± 0.11 | 24.24 ± 3.38 |

n.d.: not detectable.

*A. terreus* (Gawande and Kamat, 1999), *P. dryinus* and *P. ostreatus* (Elisashvili et al., 2008), *Penicillium* sp. (Nair et al., 2008). Hence, both fungi were shown to be able to produce hemicellulolytic but not cellulolytic activities under the analysed conditions. Moreover, for both fungi, the correlation between production times for laccase and xylanase activities (Table 1) suggests their synergistic action in waste transformation.

### 3.3. Wastewater decolorization

Crude enzymatic extracts from apple residues colonized by *P. ostreatus* or *T. versicolor* at the 3rd day (corresponding to the maximum laccase activity production) were tested for decolorization ability on model wastewater containing acid dyes. A maximum decolorization of 40% was achieved after 24 h of incubation with 1 U of laccase activity from *T. versicolor* while just 3 h of incubation were needed to gain the same effect with 1 U of laccase activity from *P. ostreatus*. The latter result is similar to the decolorization level previously achieved with extracellular enzymes containing laccase (2 U $mL^{-1}$) from *P. ostreatus* liquid culture after 24 h of incubation (Faraco et al., 2009). Experiments of dye decolorization with crude enzymatic extracts from fungi cultivated under solid state conditions have been previously reported for *T. hirsuta* (Couto and Sanroman, 2006), *T. pubescens* (Osma et al., 2007) and *T. versicolor* (Xin and Geng, 2011), producing not better performances than those reported hereby.

Apple residues colonized by *P. ostreatus* and *T. versicolor* at the 3rd day were also used in decolorization experiments. As far as treatment of acid bath with *P. ostreatus* colonized residues is concerned, around 43% of decolorization was achieved in only 10 min of incubation with an amount of colonized waste corresponding to a laccase activity of 0.25 U $ml^{-1}$ of wastewater, and 34% of the decolorization obtained accounts for enzymatic degradation. After 24 h of incubation of wastewater with colonized apple residues, 68% of total decolorization, with a 24% due to enzyme activity, was achieved, showing an increasing contribution of absorption over enzymatic degradation when incubation goes on. Similarly, when apple residues colonized by *T. versicolor* at the 3rd day were tested, 60% and 71% of total decolorization (with 39% and 21% due to enzymatic degradation, respectively) were achieved after 10 min and 24 h of incubation, respectively. When a fresh sample of acid wastewater was added to *P. ostreatus* colonized apple residues that had been recovered after the first 24 h decolorization round, a total decolorization of 58% including an enzymatic contribution of 18% was achieved after a new 24 h cycle. The leaching of about 60% of laccase activity in the acid wastewater sample removed after the first decolorization round can explain the lower enzymatic performance at the second decolorization round. Therefore, a repeated sequencing batch decolorization experiment was set up using the apple residues within a dialysis membrane to avoid the leaching of enzymes during the acid bath incubation. These experiments allowed reaching a 50% decolorization due to enzymatic degradation in 24 h without loss of enzymatic activity in the wastewater. Every 24 h, fresh wastewater was added and a decolorization of 50% due to enzymatic degradation was kept constant for at least 1 month. Complete detoxification of the wastewater was also achieved after 24 h incubation.

### 3.4. Acid bath decolorization in the continuous packed-bed reactor

In order to carry out decolorization experiment in continuous mode, a fixed-bed reactor was set up by packing an amount of apple processing residues (after three days of fungal colonization) corresponding to a laccase activity of 40 U in a dialysis tube within a glass column surrounded by glass beads. 100 mL of acid bath were recirculated through the column at a flux of 2 mL/minute, corresponding to a passage time of 15 min, up to reaching a stationary decolorization of 70% as reduction of $Abs_{590nm}$ and 38% as reduction of the extent of spectrum area between 380 and 740 nm after 7 days (data not shown). Then, a continuous wastewater feeding was started by adding fresh wastewater to the reservoir at a flux of 0.01 mL/minute corresponding to a retention time of around 2 days (Fig. 1) and the decolorization was then kept constant, with a performance of 100 mg dye $L^{-1}$ $day^{-1}$. Another packed-bed bioreactor for dye continuous decolorization based on laccases produced by fungal SSF has so far been developed with the fungus *T. hirsuta* (Couto et al., 2006) grown on orange peelings. In this study, a worse performance was achieved for the dyes Methyl Orange and Poly R-47816 (16 and 28 mg $L^{-1}$ $day^{-1}$, respectively), using a similar retention time (3 days).

The main aspect of our bioreactor is that continuous decolorization was carried out by using a low cost equipment and a simple reactor design, without a strict control of operative conditions that is required for fungal solid state fermenters instead. The absence of living biomass avoids problems associated with requirement of continuous substrate feeding to sustain the growth, biomass wash-out or bioreactor clogging. The only drawback of our bioreactor can be found in the expected decrease of the enzymatic activity due to laccase denaturation. However, our system was shown able to work for at least one month. The high ratio between the retention time of wastewater feeding and the passage time due to the liquid recirculation (about 100) assures the good mixing of the flow packed bed. Consequently, dead zones inside the packing are avoided and mass transfer to the dialysis membrane is enhanced.

### 3.5. Enzymes identification

When fractionation on native PAGE and staining of bands for laccase activity were carried out on extracts from apple residues that have been colonized by the fungus at the 3rd day, the pattern of laccase isoenzymes was proved similar to that obtained during SSF on tomato pomace (Iandolo et al., 2011). The activity positive protein bands were excised and subjected to protein identification by MS/MS ion search on a MASCOT server against the annotated *P. ostreatus* genome (http://genome.jgi-psf.org/PleosPC15_1). POXA3 and POXC could be unambiguously identified (2 peptides with 6% sequence coverage, and 5 peptides with 16% of sequence coverage, respectively) as well as the Dye-decolorizing peroxidase (6 peptides with 10% of sequence coverage) as already previously identified (Faraco et al., 2007). Hence, POXA3 and POXC laccases and the Dye-decolorizing peroxidase are the main responsible for decolorization by apple residues colonized by *P. ostreatus* after three days of SSF.

## 4. Conclusions

The developed SSF showed to be an effective system for apple residues upgrading by production of high xylanase activity levels and development of a laccase-based system for continuous dye decolorization. The developed system was very suitable to its application for dye decolorization, since it was able to operate in a continuous mode giving a performance of 100 mg dye $L^{-1}$ $day^{-1}$ with a retention time of 50 h, very low equipment cost and no operational problems. Considering that the tested wastewater simulates a real textile effluent, this work indicates the possibility of implementing the developed techniques for the treatment of textile-dyeing wastewaters.

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# Enhanced Green Fluorescent Protein Expression in *Pleurotus ostreatus* for In Vivo Analysis of Fungal Laccase Promoters

**Antonella Amore · Yoichi Honda · Vincenza Faraco**

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**Abstract** The laccase family of *Pleurotus ostreatus* has been widely characterized, and studies of the genes coding for laccase isoenzymes in *P. ostreatus* have so far led to the identification of four different genes and the corresponding cDNAs, *poxc*, *pox1*, *poxa1b* and *poxa3*. Analyses of *P. ostreatus* laccase promoters *poxc*, *pox1*, *poxa1b* and *poxa3* have allowed identification of several putative response elements, and sequences of metal-responsive elements involved in the formation of complexes with fungal proteins have been identified in *poxc* and *poxa1b* promoters. In this work, development of a system for in vivo analysis of *P. ostreatus* laccase promoter *poxc* by enhanced green fluorescent protein expression is performed, based on a poly ethylene glycol-mediated procedure for fungal transformation. A quantitative measurement of fluorescence expressed in *P. ostreatus* transformants is hereby reported for the first time for this fungus.

**Keywords** Fungi · Laccases · Transformation · Promoters · Fluorescence

## Introduction

*Pleurotus ostreatus* is known to be able to degrade plant cell wall lignin extensively by secreting the extracellular enzymes manganese peroxidases and laccases, whose ability to degrade a variety of organic pollutants including polychlorinated biphenyls, polycyclic aromatic hydrocarbons and dioxins was also demonstrated [1–5]. The laccase family of *P.*

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*ostreatus* has been widely characterized. Besides the typical laccase isoenzyme POXC [6], representing the most abundantly produced laccase from *P. ostreatus*, the fungus excretes the white laccase isoenzyme POXA1w, having a singular metal content [7], the heterodimeric laccase isoenzyme POXA3 [8–10] and the laccase POXA1b notably stable at alkaline pH, with a $t_{1/2}$ of 30 days at pH 10 [11]. Studies of the genes coding for laccase isoenzymes in *P. ostreatus* have also led to the identification of six different genes and of the corresponding cDNAs, *poxc* (previously named *pox2*) [6], *pox1* (which codes for a laccase isoenzyme not identified yet) [12], *poxa1b* [11], *poxa3* [8], *pox3* and *pox4* and the gene *pox5* [13].

The addition of copper sulphate to *P. ostreatus* growth medium causes a marked increase of total laccase activity and a transcription induction of *poxc* and *poxa1b* genes [14]. Analyses of *P. ostreatus* laccase promoters have allowed identification of several putative response elements [8, 13, 15], such as metal-responsive elements (MREs) [16], heat shock elements [17] and xenobiotic-responsive elements [18]. Moreover, MRE sequences involved in formation of complexes with fungal proteins have been identified in the *poxc* and *poxa1b* promoters [15].

Development of an efficient transformation system of the fungus *P. ostreatus* is needed to perform in vivo analysis of laccase promoters. Only rare examples of green fluorescent protein expression have been so far reported for *P. ostreatus*, but the green fluorescent protein gene was only transiently expressed in the fungus [19], or quantitative measurement of intracellular fluorescence was not reported [20].

This work was aimed at setting up an effective and stable system for in vivo analysis of laccase promoters in *P. ostreatus*. Development of a system for enhanced green fluorescent protein expression under the control of laccase promoter *poxc* was achieved in *P. ostreatus*, based on a poly ethylene glycol (PEG)-mediated transformation procedure for fungal transformation. Besides integration of *egfp* gene within genomic DNA of the *P. ostreatus* transformants, expression of enhanced green fluorescent protein was demonstrated by fluorescence detection in hyphae of the transformants through fluorescence microscopy and quantitative measurement of fluorescence emission by intracellular protein extracts of the transformants.

## Materials and Methods

### Strain and Culture Media

*P. ostreatus* dikaryotic strain #261 (ATCC 66376) was used as the host strain for transformation experiments. Maintenance of the strain was performed on PDY [2.4 % potato dextrose (Difco, Detroit, MI, USA), 0.5 % yeast extract (Difco, Detroit, MI, USA) 1.5 % agar (Difco)] medium at 28 °C.

Liquid cultures of *P. ostreatus* transformants were prepared pre-inoculating 75 ml of PDY broth in 250-mL Erlenmeyer flasks with six agar plugs (11 mm diameter) of *P. ostreatus* mycelium, from 7-day-old agar culture, in a temperature-controlled incubator at 28 °C on rotary shaker (at 125 rpm). The cultures were prepared by inoculating 72 mL of PDY broth in a 250-mL Erlenmeyer flask with 8 ml of 5-day-old pre-inocula.

### Vector Construction

The EMBL accession number of the promoter sequence of the *P. ostreatus* laccase *poxc* [6] reported in this paper is Z40075. To prepare the vector pEGFPec (Fig. 1) designed to study the

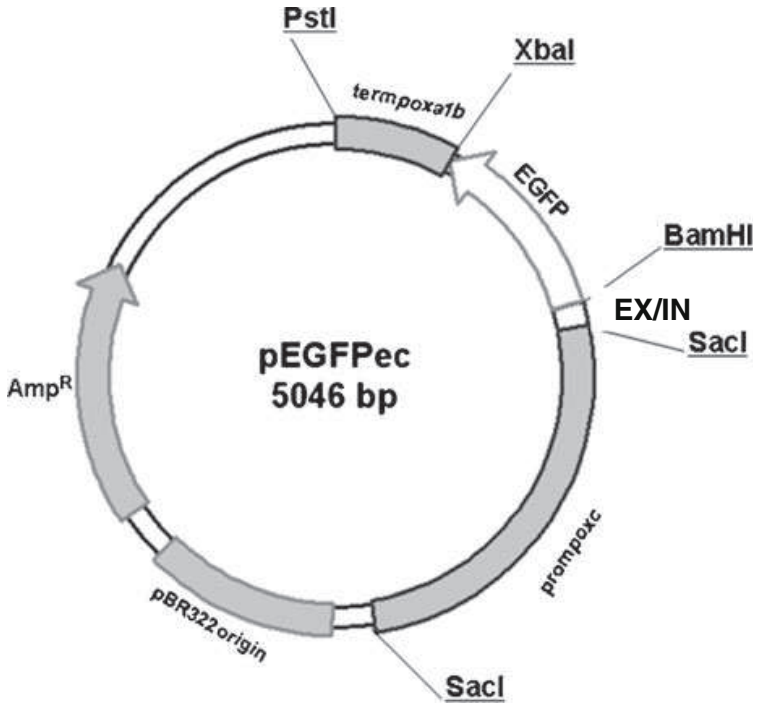


**Fig. 1** Map of the vector pEGFPec. This vector was adopted to study the *poxc* promoter through enhanced green fluorescent protein gene expression in *P. ostreatus* by co-transformation with pTM1 vector

*poxc* promoter through enhanced green fluorescent protein gene expression in *P. ostreatus*, the *poxa1b* terminator and the *poxc* promoter were amplified by polymerase chain reactions (PCR) using plasmid vectors selected from the *P. ostreatus* genomic library [6, 11, 12] as templates and the gene-specific oligonucleotides Term poxa1b XbaI/Term poxa1b PstI and Prom poxc SacI rev/Prom poxc SacI fw (Table 1) as primers, respectively. The amplified fragment of *poxa1b* terminator was subjected to hydrolysis with the restriction enzymes *Xba*I and *Pst*I and ligated into the *Xba*I/*Pst*I digested pUC13 vector, giving the vector pA1BTERM.

An intron/exon fragment was prepared by annealing of the synthetic oligomers EGFP1dir and EGFP1rev having complementary sequences including *poxc* gene intron number XIX

**Table 1** List of the primers used in the amplification experiments (oligonucleotides of rows 1–6) and oligonucleotides for intron/exon fragment preparation (oligonucleotides of rows 7–8)

| | Oligonucleotide | Nucleotide sequences |
|---|---|---|
| 1 | Term poxa1b XbaI | GC*TCTAGA***TTATAATTTGACTT** |
| 2 | Term poxa1b PstI | CATTGGTT*CTGCAG***CCAGCGT** |
| 3 | Prom poxc SacI rev | AATTGC*GAGCTC***CGGGCACCT** |
| 4 | Prom poxc SacI fw | TTAACC*GAGCTC***CGTTGGTAG** |
| 5 | EGFP 3dir | CG*GGATCC***GTGAGCAAGGGCGAGGAG** |
| 6 | EGFP 5rev | GC*TCTAGA***CTTGTACAGCTCGTCCATG** |
| 7 | EGFP 1dir | *C***ATGCCACCTG**gtatgcatacttccccctgcggccc**gtattgctgctaacctgcatttctccagCCGCG***G** |
| 8 | EGFP 1rev | *GATCC***CGCGG**ctggagaaatgcaggttagcagcaata**cgggccgcaggggaagtatgcatacCAGGTGGCA**T*GAGCT* |

Nucleotides in bold are complementary to the sequences to be amplified. Nucleotides in italics are recognized or left by restriction enzymes, and nucleotides in lower case belong to intron sequences

flanked by two amino acids at the 5′ end and three at 3′ end [11] and the sticky ends features of the restriction enzymes *Sac*I and *Bam*HI, followed by digestion by *Sac*I and *Bam*HI.

The *egfp* gene was amplified by PCR reaction using the plasmid vector pEGFP-C1 (Clontech Laboratories, Inc., CA, USA) as template and the gene-specific oligonucleotides EGFP3dir/EGFP5rev as primers (Table 1). The plasmid vector pA1BTERM was subjected to hydrolysis by the endonucleases *Sac*I and *Xba*I and ligation reaction among the amplified *egfp* gene, the intron/exon fragment and the linearized pA1BTERM vector was carried out. The vector thus obtained was subjected to *Sac*I hydrolysis and ligated to the amplified *poxc* promoter fragment after *Sac*I digestion, giving the pEGFPec vector.

## Transformation of *P. ostreatus*

Liquid cultures of *P. ostreatus* for protoplasting were set up by inoculating 60 mL YMG broth [1 % glucose, 0.4 % yeast extract (Difco, Detroit, MI, USA), 1 % malt extract] in 250-mL cotton-plugged Erlenmeyer flasks with six agar plugs (11 mm diameter) of *P. ostreatus* mycelium, grown on PDA [2.4 % potato destrose (Difco, Detroit, Michigan, USA)] medium. The inocula were incubated in a temperature-controlled incubator at 28 °C on a rotary shaker (at 120 rpm) for 5 days. The biomass was homogenized and retrieved in a 100-mL single flask. Recovered mycelium was incubated for 5 h in a temperature-controlled incubator at 28 °C on rotary shaker (at 120 rpm). The biomass was transferred in two 50-mL Falcon conical tubes. The samples were washed twice with deuterium-depleted water and twice with 0.5 M sucrose by centrifuging at 450×*g* for 8 min. The pellets were recovered into one tube. Enzyme digestion solution consisted of 200 mg lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich SRL, Milano, Italy) and 20 mg chitinase from *Trichoderma viride* (Sigma-Aldrich SRL, Milano, Italy) dissolved in 10 mL of 0.5 M sucrose and filtered by a 0.22-μm PVDF membrane (Millipore S.p.A., Vimodrone Italy). Enzyme digestion solution was added to the sample that was incubated at 31 °C for 3 h on a rotary shaker (at 50 rpm). Next, 0.5 M sucrose was added to the sample up to 50 mL. The sample was centrifuged at 450×*g* for 8 min and washed twice with STC [0.5 M sucrose, 0.05 mM Tris–HCl (pH 8.0) solution with 18.2 % sorbitol and 2.22 % $CaCl_2$ anhydrate] to remove enzymatic solution. Protoplasts were resuspended in 4 mL of STC solution.

For transformation, 200 μL of this protoplast solution was gently mixed with 15 μL of heat-denaturated λ phage DNA (0.3 γ/λ, Fermentas) and transforming DNA (1 μg of pTM1, or 1 μg of pTM1 and 5 μg of pEGFPec). Samples were incubated on ice for 40 min. Then, 1 mL of PTC [0.5 M sucrose, 0.05 mM Tris–HCl (pH 8.0) solution with 40 % PEG#4000 (Sigma-Aldrich SRL, Milano, Italy), 17.2 % sucrose, 8.88 % $CaCl_2$ anhydrate] was added. The sample was mixed gently at room temperature (RT) and then incubated at RT for 20 min. Protoplast solution (600 μL) was spread on regeneration medium (1 % glucose, 0.4 % yeast extract, 1 % malt extract, 17.1 % sucrose and 1.5 % agar) containing 2 μg/ml of carboxin (Sigma-Aldrich). Plates were incubated at 28 °C.

## Extraction of Intracellular Proteins

*P. ostreatus* 7-day-old liquid cultures prepared as described in the first paragraph of the "Materials and Methods" section, in the presence of 2 μg/ml of carboxin, were filtered through sterilized cotton lint to retrieve suspended mycelia. Recovered mycelium was frozen and then lyophilized. Mycelium was crushed in porcelain mortar and then suspended in the extraction buffer containing 100 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, 7 mM β-

mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 % Triton (Sigma-Aldrich). After centrifuation at 15,000×*g* at 4 °C for 15 min, the supernatant was recovered for further assay. Protein concentration was determined by the method of Lowry et al. (1951), using the Bio-Rad Protein Assay (Bio-Rad Laboratories S.r.l., Segrate, MI, Italy), with bovine serum albumin as standard.

### Intracellular GFP Determination

The crude supernatant was diluted to 0.05 mg of protein per milliliter with the extraction buffer, and a fluorescence spectrum (500 to 600 nm) was determined using a 460-nm excitation wavelength with a LS 50B Fluorescence Spectrometer (Perkin-Elmer). Maximum fluorescence occurred at 520 nm. The entity of fluorescence emission was measured as difference between spectrum area recorded between 500 and 550 nm for the transformant and that of the control sample (not transformed fungus). The experiments were performed in three replicates, and reported values are representative of two experiments.

### Fluorescence Microscopy

*P. ostreatus* mycelia were grown on microscope coverslips and observed in a Nikon Eclipse TE 2000U microscopic system with appropriate fluorescein isothiocyanate filters (Nikon Corporation, Tokyo, Japan). Normal phase-contrast images of each sample were used as controls. The digital image was further processed using Photoshop 5.0 (Adobe).

### Extraction of Genomic DNA from Mycelium of *P. ostreatus* and PCR Analyses

Chromosomal high molecular weight DNA from *P. ostreatus* was prepared as described by Raeder and Broda [21]. Amplification experiments were carried out on 50 ng of genomic DNA in a 50-μl total volume, using the gene-specific oligonucleotides EGFP 3dir and EGFP 5rev (Table 1) as primers and *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). PCR conditions consisted of 30 cycles of 94 °C (1 min), 58 °C (45 s) and 72 °C (2 min) plus an additional final chain elongation step at 72 °C for 10 min.

## Results and Discussion

### Expression Plasmids and *P. ostreatus* Transformation

In this work, it was demonstrated that a green fluorescent protein (GFP) system faithfully reports gene expression when it is driven by *poxc* promoter. GFP of the jellyfish *Aequorea victoria*, which can be directly visualized as a result of the emission of green light when excited with long UV or blue light [22], fulfils the prerequisite of a versatile reporter gene [23].

In this work, the vector pEGFec for in vivo analysis of *P. ostreatus* laccase promoters was constructed by using the gene coding for enhanced green fluorescent protein (EGFP). The *gfp* gene has been developed as a tool to monitor gene expression in situ and in vivo for many reasons. The fluorescence results from its intrinsic chromophore structure and does not require any substrate or cofactor. Moreover, because of the fluorescence properties of GFP, it has been widely used in the gene engineering of plants, mammalian cells, viruses and filamentous fungi. Despite these advances, GFP has been successfully expressed in only

four homobasidiomycetes, *Schizophyllum commune* [24], *Phanerochaete chrysosporium* [25], *Agaricus bisporus* and *Coprinus cinereus* [26]. Transgene expression of GFP in basidiomycetes appears to be hampered by a number of factors. In the model species, *S. commune*, transforming DNA was inactivated by preferential methylation [27]. AT-rich sequences inactivated gene expression [28, 29], and introns were needed for mRNA accumulation to occur [24, 29]. Efficient GFP expression in *A. bisporus* and *C. cinereus* required introns [26].

In this work, the *P. ostreatus poxc* promoter region of 1,305 bp was used as *cis*-regulatory element to drive expression of EGFP. An intron/exon fragment containing an intron/exon sequence of the *poxc* gene was included between the *poxc* promoter and the *egfp* gene, considering previous results showing that efficient GFP expression in *A. bisporus* and *C. cinereus* [26] and *P. chrysosporium* requires introns [25].

A homologous selection marker, the mutant gene cassette $Cbx^R$, encoding a modified iron-sulfur protein Ip subunit of succinate dehydrogenase with an aminoacidic substitution (His239 to Leu) and conferring resistance to systemic fungicide carboxin [30] was adopted. Co-transformation with pTM1 vector conferring carboxin resistance and pEGFPec vector containing *egfp* gene under the control of *poxc* promoter region was carried out, by adopting an adapted version of transformation protocol reported by Salame and coworkers [31].

Transformants were firstly screened for carboxin resistance. The carboxin-resistant colonies were subjected to at least four rounds of selection by transferring on fresh selection medium. Around 50 carboxin-resistant transformants were obtained per microgram of DNA per $10^7$ viable protoplasts in a transformation. Hence, these transformants were further analysed for *egfp* integration in genomic DNA and fluorescence emission.

Transformation of *P. ostreatus* with the green fluorescent protein gene by using a PEG-mediated transformation system has been reported by Li and coworkers [19], but green fluorescence disappeared 30 h after transformation. Recently, the expression of fused *egfp-hph* gene under the control of *gpd* promoter was carried out in *P. ostreatus* using *Agrobacterium*-mediated infection [20]. However, no quantitative measure of protein expression was reported by these authors.

## Molecular Analyses

Carboxin-resistant transformants were analysed by PCR to verify the integration of the transforming DNA in genomic DNA. The PCR reaction was performed on genomic DNA extracted from transformants mycelia. Ten transformants showed the expected 0.7-kb target band, indicating *egfp* gene integration in genomic DNA (Fig. 2).

## Intracellular GFP Determination

All the *P. ostreatus* transformants showing *egfp* gene integration in genomic DNA by PCR analysis were analysed for intracellular fluorescence emission by measuring emission of fluorescence of intracellular protein extracts from 7-day-old mycelium, in comparison to the control (not transformed mycelium). In Fig. 3, intensity of fluorescence of three of the selected transformants (11, 17 and 33) is reported. An intensity of fluorescence emission up to 13,000 units was measured for the analyzed protein solution (0.05 mg/ml). The expression of GFP in each of these transformants has proved stable over a 6-month period of repeated subculturing on selective media (data not shown).

Difference in intracellular fluorescence emission of different transformants could be ascribed to the different copy numbers and loci of exogenous DNA integration within the

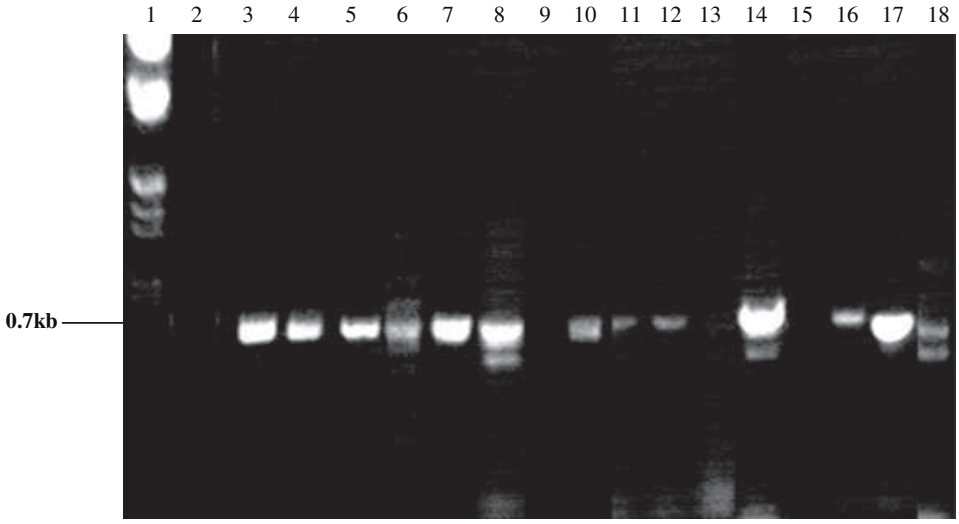


**Fig. 2** Electrophoretic analysis of PCR products obtained on genomic DNA extracted from transformants. The analysis was performed on 1 % agarose gel. *Lane 1* DNA Molecular Weight Marker III (Roche Applied Science, Indianapolis, USA), *lane 2* PCR products obtained on genomic DNA extracted from mycelium containing the plasmid pTM1, *lanes 3–18* PCR products obtained on genomic DNA extracted from mycelium containing the plasmids pEGFPec and pTM1

fungal genome. Variation in GFP concentration among independent fungal transformants has been observed by other authors [32, 33].

EGFP Visualization

Detection of fluorescence was performed in vivo on 2-day grown transformants mycelia on microscopic slides. In Fig. 4, phase-contrast micrographs of transformants (A1 and A2) and the corresponding fluorescence micrographs (B1 and B2) are shown. Untransformed mycelium did not show any fluorescence. Scanned images show a positive fluorescence emission with respect to untransformed control. Fluorescence emission extended to entire hyphae, especially to clamp connections.

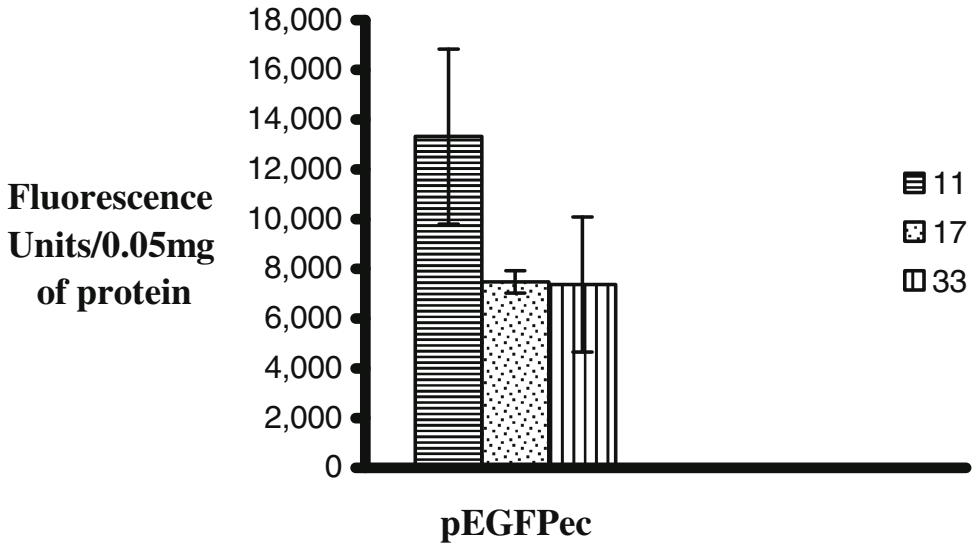


**Fig. 3** Bar graph showing the fluorescence emission intensity at 500–550 nm of three of the transformants (transformant numbers 11, 17 and 33) carrying the construct pEGFPec shown in Fig. 1 after subtracting the background fluorescence emission of control (untransformed fungus)

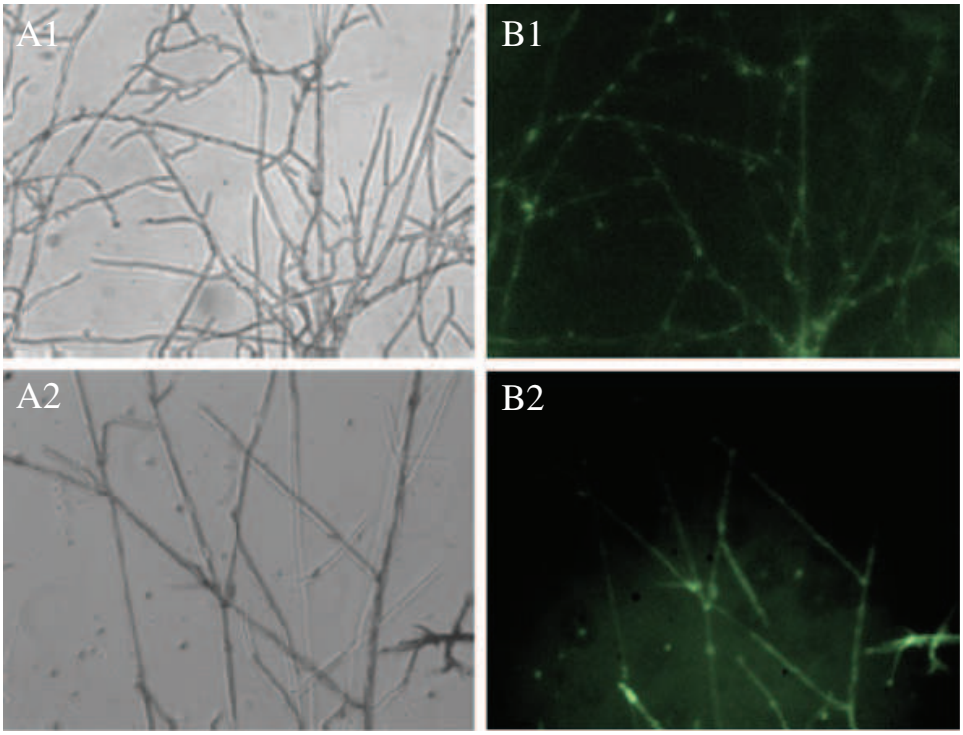


**Fig. 4** Phase-contrast images (**a**) and fluorescence micrographs (**b**) of transformed mycelium containing the plasmids pEGFPec (*1* transformant 11, *2* transformant 33). The green colour in the mycelium is due to GFP

## Conclusions

In this manuscript, development of an effective and stable system for in vivo analysis of laccase promoters in *P. ostreatus* by enhanced green fluorescent protein expression is reported. The promoter of the laccase gene *poxc* was chosen among the different *P. ostreatus* laccase promoters as a model for setting up this system since POXC is the most typical among the *P. ostreatus* laccases, and it is the most abundantly produced isoenzyme in all the analysed conditions. Co-transformation with pTM1 vector conferring carboxin resistance and pEGFPec vector containing *egfp* gene under the control of *poxc* promoter region was carried out. *egfp* gene integration in genomic DNA was demonstrated for most of the carboxin-resistant transformants, and an intracellular fluorescence emission up to 13,000 units/0.05 mg of protein in comparison to the untransformed mycelium was measured.

The most relevant significance of this work is that a quantitative measure of fluorescence expressed in *P. ostreatus* transformants was reported for the first time. Therefore, the developed system will allow both a quantitative analysis of effects of different transcriptional inducers on expression driven by *poxc* promoter and investigation of the role of putative response elements present in this promoter.

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RESEARCH LETTER

# Copper induction of enhanced green fluorescent protein expression in *Pleurotus ostreatus* driven by laccase *poxa1b* promoter

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## Abstract

*In silico* analyses of several laccase promoter sequences have shown the presence of many different responsive elements differentially distributed along the promoter sequences. Analysis of *Pleurotus ostreatus* laccase promoter *poxa1b* extending around 1400-bp upstream of the start codon showed the presence of several putative response elements, such as 10 metal-responsive elements. Development of a system for *in vivo* analysis of *P. ostreatus* laccase promoter *poxa1b* by enhanced green fluorescent protein expression was carried out, based on a polyethylene glycol–mediated procedure for fungal transformation. Quantitative measurement of fluorescence expressed in *P. ostreatus* transformants grown in the presence and in the absence of copper sulfate was performed, demonstrating an increase in expression level induced by the metal.

## Introduction

Twelve putative laccase genes have been identified in the recently sequenced *Pleurotus ostreatus* genome (http://www.jgi.doe.gov/sequencing/why/50009.html), one of which is annotated as a ferroxidase-like. The promoter regions of all the 11 *P. ostreatus* laccase genes, extending 500-bp upstream of the start codon, have been analyzed, revealing the presence of several putative response elements, differentially distributed along the promoter sequences (Piscitelli *et al.*, 2011). All the analyzed *P. ostreatus* laccase promoters contain putative metal-responsive elements (MREs) with sequence homology to those reported in ascomycetous yeast. *Cis*-acting MREs have been discovered in multiple copies in the *Saccharomyces cerevisiae* metallothionein promoter where they are essential for efficient metal-inducible transcription (Thiele, 1992). Sequences similar to MREs have been also found in several laccase promoters of basidiomycetous fungi such as the promoter region of the gene coding for the major laccase isoenzyme LAP2 from *Trametes pubescens* (Galhaup *et al.*, 2002), the promoter region of the copper-inducible LAC2 laccase from *Gaeumannomyces graminis* (Litvintseva & Henson, 2002), the promoter region of the strongly copper-induced *lac4* gene from *Pleurotus sajorcaju* (Soden & Dobson, 2003), and the promoters of three laccase genes (*lacA*, *lacB*, and *lacC*) from *Trametes* sp. AH28-2 (Xiao *et al.*, 2006).

The presence of putative MREs in *P. ostreatus* laccase promoters is consistent with the observation that the level of laccase activity production by the fungus increases substantially in copper-supplemented cultures and the copper induction on expression of POX isoenzymes acts at the level of gene transcription (Palmieri *et al.*, 2000). It is worth noting that *poxa1b* mRNA was the most abundant induced transcript at all of the growth times analyzed. Analyses of the region *P. ostreatus poxa1b* promoter extending around 500-bp upstream of the ATG had allowed individuation of four putative MREs (Piscitelli *et al.*, 2011), all being recognized by fungal proteins as





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shown by electromobility shift assays (Faraco *et al.*, 2003). MRE-like sequences involved in formation of complexes with fungal proteins have been identified by footprinting analyses of the *poxa1b* promoter that showed the occurrence of a large protected region including a1bMRE2 and a1bMRE3 sites with opposite orientations (Faraco *et al.*, 2003). Besides increasing expectation of their roles in regulation of laccase expression, no physiological function of these putative MREs could be confirmed, because of lack of appropriate promoter assay systems in basidiomycetes. Indeed, development of an efficient transformation system of the fungus *P. ostreatus* is needed to perform *in vivo* analysis of these laccase promoter elements, in view of their mutagenesis for laccase overproduction.

In this work, a system for enhanced green fluorescent protein (GFP) expression under the control of laccase promoter *poxa1b* in *P. ostreatus* was developed, based on a polyethylene glycol (PEG)–mediated fungal transformation procedure. Analysis of effect of copper sulfate addition to fungal growth medium on fluorescence expression driven by *poxa1b* promoter in *P. ostreatus* showed an increase in expression level induced by the metal.

## Materials and methods

### Strain and culture media

*Pleurotus ostreatus* dikaryotic strain #261 (ATCC 66376) was used as the host strain for transformation experiments. Maintenance of the strain was performed on PDY [2.4% potato dextrose (Difco, Detroit, Michigan), 0.5% yeast extract (Difco), 1.5% agar (Difco)] medium at 28 °C.

Liquid cultures of *P. ostreatus* transformants were prepared pre-inoculating 75 mL of PDY broth in 250-mL Erlenmeyer flasks with six agar plugs (11 mm diameter) of *P. ostreatus* mycelium, from 7-day-old agar culture, in a temperature-controlled incubator at 28 °C on rotary shaker (at 125 rpm). The cultures were prepared by inoculating 80 mL of PDY broth in 250-mL Erlenmeyer flask with 8 mL of 5-day-old pre-inocula. When indicated, $CuSO_4$ (150 μM) was added to the cultures.

### Vector construction

The GenBank accession number of the sequence of the *P. ostreatus* laccase *poxa1b* gene (Giardina *et al.*, 1999) reported in this paper is AJ005017.

To prepare the vector pEGFPea1b (Fig. 1a) designed to study the *poxa1b* promoter through enhanced GFP gene expression in *P. ostreatus*, the *poxa1b* terminator and the *poxa1b* promoter were amplified by PCR using plasmid vectors selected from the *P. ostreatus* genomic library

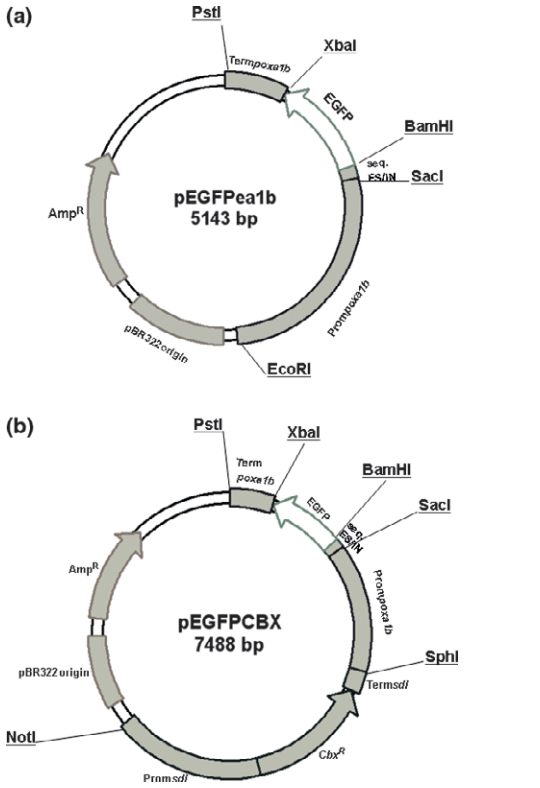


**Fig. 1.** Map of the vectors pEGFPea1b (a) and pEGFPCBX (b), adopted to study the *poxa1b* promoter through enhanced GFP gene expression in *Pleurotus ostreatus* with and without cotransformation with pTM1 vector, respectively.

(Giardina *et al.*, 1995, 1996, 1999) as templates and the gene-specific oligonucleotides Termpoxa1bXbaI/Termpoxa1bPstI and Prompoxa1SacIrev/Prompoxa1SacIfw (Table 1) as primers, respectively. The amplified fragment of *poxa1b* terminator was subjected to hydrolysis with the restriction enzymes XbaI and PstI and ligated into the XbaI-/PstI-digested pUC13 vector, giving the vector pA1BTERM.

An intron/exon fragment was prepared by annealing of the synthetic oligomers EGFP1dir and EGFP1rev having complementary sequences including *poxc* gene intron number XIX flanked by two amino acids at the 5′ end and three at 3′ end (Giardina *et al.*, 1996) and the sticky ends features of the restriction enzymes SacI and BamHI, followed by digestion by SacI and BamHI.

The *egfp* gene was amplified by PCR using the plasmid vector pEGFP-C1 (Clontech Laboratories, Inc., CA) as template and the gene-specific oligonucleotides EGFP3-dir/EGFP5rev as primers (Table 1). The plasmid vector pA1BTERM was subjected to hydrolysis by the endonucleases SacI and XbaI, and ligation reaction among the amplified *egfp* gene, the intron/exon fragment, and the linearized pA1BTERM vector was carried out. The vector thus obtained was subjected to SacI/EcoRI hydrolysis and

**Table 1.** List of the primers used in the amplification experiments (oligonucleotides of the rows 1–6) and oligonucleotides for intron/exon fragment preparation (oligonucleotides of the rows 7–8)

| | Oligonucleotide | Nucleotide sequences |
|---|---|---|
| 1 | Termpoxa1b XbaI | GC*TCTAGA*TTATAATTTGACTT |
| 2 | Termpoxa1b PstI | CATTGGTT*CTGCAG*CCAGCGT |
| 3 | Prompoxa1 SacI | AATTGC*GAGCTC*GGGGAGATC |
| 4 | Prompoxa1 EcoRI | G*GAATTC*GGCGCATCAATACC |
| 5 | EGFP 3dir | CG*GGATCC*GTGAGCAAGGGCGAGGAG |
| 6 | EGFP 5rev | GC*TCTAGA*CTTGTACAGCTCGTCCATG |
| 7 | EGFP 1dir | *C***ATGCCACCTGgtatgcatacttccccctgcggcccgtattgctgctaacctgcatttctccag****CCGCG***G* |
| 8 | EGFP 1rev | *GATCC***CGCGGctggagaaatgcaggttagcagcaatacgggccgcaggggggaagtatgcatac****CAGGTGGCA***TGAGCT* |

Nucleotides in bold are complementary to the sequences to be amplified. Nucleotides in italics are recognized or leaved by restriction enzymes, nucleotides in lower-case type belong to intron sequences

ligated to the amplified *poxa1b* promoter fragment after SacI/EcoRI digestion, giving the pEGFPea1b vector.

The vector pEGFPCBX (Fig. 1b) was constructed by cloning the DNA fragment resulting from NotI/SphI hydrolysis of pTM1 into pEGFPea1b vector. This fragment includes the gene $cbx^R$ and its own promoter and terminator. To include the desired restriction sites NotI/SphI within pEGFPea1b, this vector was hydrolyzed by the enzymes SphI–EcoRI, and an oligonucleotide whose sequence contains the polylinker EcoRI–NotI–SphI was then ligated. Ligation between the DNA fragment excised from pTM1 and pEGFPea1b hydrolyzed by NotI and SphI was then carried out.

## Transformation of *P. ostreatus*

Liquid cultures of *P. ostreatus* for protoplasting were set up by inoculating 60 mL YMG broth [1% glucose, 0.4% yeast extract (Difco), 1% malt extract] in 250-mL cotton plugged Erlenmeyer flasks with six agar plugs (11 mm diameter) of *P. ostreatus* mycelium, grown on PDA [2.4% potato dextrose (Difco)] medium. The inocula were incubated in a temperature-controlled incubator at 28 °C on a rotary shaker (at 120 rpm). The biomass was homogenized and retrieved in a 100-mL single flask. Recovered mycelium was incubated for 5 h in a temperature-controlled incubator at 28 °C on rotary shaker (at 120 rpm). The biomass was transferred in two 50-mL Falcon conical tubes. The samples were washed twice with deuterium-depleted water and twice with 0.5 M sucrose by centrifuging at 450 ***g*** for 8 min. The pellets were recovered into one tube. Enzyme digestion solution consisting of 200 mg of lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich SRL, Milano, Italy) and 20 mg of chitinase from *Trichoderma viride* (Sigma-Aldrich SRL) was dissolved by ultrasonic machine in 10 mL of 0.5 M sucrose and filtered by 0.22-μm PVDF membrane (Millipore S.p.A., Vimodrone, Italy). Enzyme digestion solution was added to the sample that was incubated at 31 °C for 3 h on a rotary shaker (at 50 rpm). Next, 0.5 M sucrose was added to the sample up to 50 mL. The sample was centrifuged at 450 ***g*** for 8 min and washed twice with STC [0.5 M sucrose, 0.05 mM Tris–HCl (pH 8.0) solution with 18.2% sorbitol and 2.22% $CaCl_2$ anhydrate] to remove enzymatic solution. Protoplasts were resuspended in 4 mL of STC solution.

For transformation, 200 μL of this protoplast solution was gently mixed with 15 μL of heat-denaturated λ phage DNA (0.3 γ/λ; Fermentas) and transforming DNA (1 μg of pTM1 or 1 μg of pTM1 and 5 μg of pEGFPea1b or 1 μg pEGFPCBX). Samples were incubated on ice for 40 min. Then, 1 mL of PTC [0.5 M sucrose, 0.05 mM Tris–HCl (pH 8.0) solution with 40% PEG#4000 (Sigma-Aldrich SRL), 17.2% sucrose, 8.88% $CaCl_2$ anhydrate] was added. The sample was mixed gently at RT, then incubated at RT for 20 min. Protoplast solution (600 μL) was spread on regeneration medium (1% glucose, 0.4% yeast extract, 1% malt extract, 17.1% sucrose, 1.5% agar) containing 2 μg $mL^{-1}$ of carboxin (Sigma-Aldrich). Plates were incubated at 28 °C.

## Extraction of intracellular proteins

*Pleurotus ostreatus* 7-day-old liquid cultures prepared as described in the first paragraph of this section in the presence of 2 μg $mL^{-1}$ of carboxin were filtered through sterilized cotton lint to retrieve suspended mycelia. Recovered mycelium was frozen and then lyophilized. Mycelium was crushed in porcelain mortar and then suspended in the extraction buffer containing 100 mM Tris–HCl pH 7.5, 2.5 mM EDTA, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton (Sigma-Aldrich). After centrifuging at 15 000 ***g*** at 4 °C for 15 min, supernatant was recovered for further assays. Protein concentration was determined by the method of Lowry *et al.* (1951), using the BioRad Protein Assay

(BioRad Laboratories S.r.l., Segrate, MI – Italy), with bovine serum albumin as standard.

### Intracellular GFP determination

The crude supernatant was diluted to 0.05 mg of protein per mL with the extraction buffer above reported, and a fluorescence spectrum (500–600 nm) was determined using a 460 nm excitation wavelength with a LS 50B Fluorescence Spectrometer (Perkin-Elmer). Maximum fluorescence occurred at 520 nm. The entity of fluorescence emission was measured as difference between spectrum area recorded between 500 and 550 nm for the transformant and that of the control sample (nontransformed fungus). The experiments were performed in three replicates, and reported values are representative of two experiments.

### Fluorescence microscopy

*Pleurotus ostreatus* mycelia were grown on microscope coverslips and observed in a NIKON ECLIPSE TE 2000-U microscopic system with appropriate fluorescein isothiocyanate filters (Nikon Corporation, Tokyo, Japan). Normal phase-contrast images of each sample were used as controls. The digital image was further processed using PHOTOSHOP 5.0 (Adobe).

### Detection of the introduced sequence in the transformants

Chromosomal high-molecular weight DNA from *P. ostreatus* was prepared as described by Raeder & Broda (1988). Amplification experiments were carried out on 50 ng of genomic DNA in a 50 μL total volume, using the gene-specific oligonucleotides EGFP 3dir and EGFP 5rev (Table 1) as primers and *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) conditions consisted of 30 cycles of 94 °C (1 min), 58 °C (45 s), and 72 °C (2 min) plus an additional final chain elongation step at 72 °C for 10 min.

### Southern hybridization analysis of the transformants

Genomic DNA from the transformants was isolated (Raeder & Broda, 1988), digested with the restriction enzymes EcoRI, BamHI, and PstI (Promega, Italy), and after electrophoresis on 0.8% agarose gel, transferred to a Hybond-NX nylon membrane (GE Healthcare). The membrane was hybridized using the PCR-amplified egfp sequence as radioactive probe, as previously described (Palmieri *et al.*, 2000).

### Detection of *egfp* transcript in the transformants

Total RNAs were extracted from lyophilized mycelia of transformants using Qiagen RNeasy Plant (Qiagen, Italy) and following manufacturer's instructions. Reverse transcription reaction was performed using MultiScribeTM Reverse Transcriptase (Applied Biosystems, Branchburg, NJ) and the oligonucleotide dT-NotI as primer. Products of the PCR experiments, performed using the gene-specific oligonucleotides EGFP3dir/EGFP5rev (Table 1), were analyzed on 1% agarose gel.

## Results and discussion

### In silico analysis of *poxa1b, poxc,* and *poxa3* promoters

Analysis of the *P. ostreatus poxa1b*, *poxc*, and *poxa3* promoter regions extending around 1400-bp upstream of the ATG was performed searching for the putative response elements heat shock element (HSE, repeated NGAAN motif; Mager & De Kruijff, 1995), NIT2 binding site (TATCT; Marzluf, 1997), antioxidant response element (ARE, TGACNNNGC; Soden & Dobson, 2003), putative response elements PRE (ATATC and TGGGT motifs; Soden & Dobson, 2003), MRE (TGCRCNC; Thiele, 1992), xenobiotic responsive elements (XRE TNGCGTG; Xiao *et al.*, 2006), Cre-A-binding site (GCGGGG; Litvintseva & Henson, 2002), and stress-responsive element (STRE, CCCCT; Galhaup *et al.*, 2002). Several putative response elements were identified differentially distributed along the promoter sequences (Fig. 2). The highest number (10) of putative MREs was identified within the *poxa3* and *poxa1b* promoters, in the latter case consistently with previous data of *poxa1b* transcription induction by copper addition to fungal growth medium (Palmieri *et al.*, 2000).

### Expression plasmids and *P. ostreatus* transformation

Considering the high number of putative MREs present in *poxa1b* promoter, it can be considered as a good candidate as regulated promoter to be used to drive homologous recombinant expression in the fungus *P. ostreatus*. To develop a system for *in vivo* analysis of *poxa1b* promoter and its metal regulation, the gene-encoding GFP was adopted as reporter gene putting its expression under the control of 1400-bp-long *poxa1b* promoter region. GFP of the jellyfish *Aequorea victorea* emits fluorescence as a result of its intrinsic chromophore structure, not requiring any substrate or cofactor (Chalfie *et al.*, 1994), and it represents a versatile reporter gene (Cubitt *et al.*, 1995).

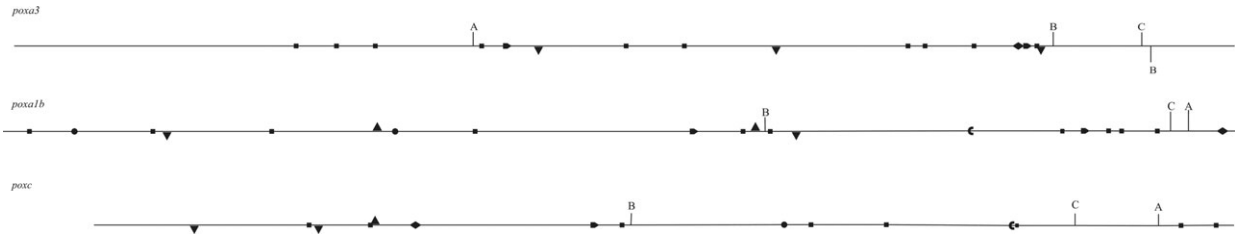


**Fig. 2.** Distribution of putative cis-acting elements in the promoter regions of *Pleurotus ostreatus* laccase genes *poxc*, *poxa1b*, and *poxa3* around 1400-bp upstream of the start codons: (a) TATA box; (b) CAAT box; (c) GC box; (◗) HSE; (▲) NIT2; (▬) ARE; (■) MRE; (●) XRE; (▼) Cre-A-binding site; (◆) STRE.

The vector pEGFPea1b for *in vivo* analysis of *P. ostreatus* laccase promoters was constructed using the gene coding for enhanced GFP (*EGFP*). A *P. ostreatus poxa1b* promoter region of 1336 bp was used as *cis*-regulatory element to drive expression of *EGFP*. An intron/exon fragment containing an intron/exon sequence of the *poxc* gene was included between the *poxa1b* promoter and the *egfp* gene, considering previous results showing that efficient GFP expression in *Agaricus bisporus* and *Coprinus cinereus* (Burns *et al.*, 2005) and *Phanerochaete chrysosporium* (Ma *et al.*, 2001) requires introns.

A homologous selection marker, the mutant gene cassette $Cbx^R$, encoding a modified iron–sulfur protein Ip subunit of succinate dehydrogenase with an aminoacid substitution (His239 to Leu) and conferring resistance to systemic fungicide carboxin (Honda *et al.*, 2000), was adopted.

Cotransformation with pTM1 vector conferring carboxin resistance and pEGFPea1b vector containing *egfp* gene under the control of *poxa1b* promoter region was carried out, by adopting an adapted version of transformation protocol reported by Salame *et al.* (2010).

Moreover, an unique vector containing both the mutant gene cassette $Cbx^R$ and the reporter cassette *poxa1b* promoter-*egfp* gene was constructed and adopted for transformation.

Transformants were firstly screened for carboxin resistance. The carboxin-resistant colonies were subjected to at least four rounds of selection by transferring on fresh selection medium. Around 50 carboxin-resistant transformants were obtained per μg of pTM1 DNA per $10^7$ viable protoplasts in a transformation with pTM1 and pEGFPea1b, and five carboxin-resistant transformants were obtained per μg of pEGFPCBX DNA per $10^7$ viable protoplasts in a transformation with this vector. Hence, cotransformation with vectors containing gene cassette $Cbx^R$ and the reporter cassette *poxa1b* promoter-*egfp* gene allowed a 10-fold higher transformation efficiency than transformation with an unique vector containing both cassettes. This could be ascribed to the larger size of the latter construct. The carboxin-resistant transformants were further analyzed for checking the presence of *egfp* and fluorescence emission.

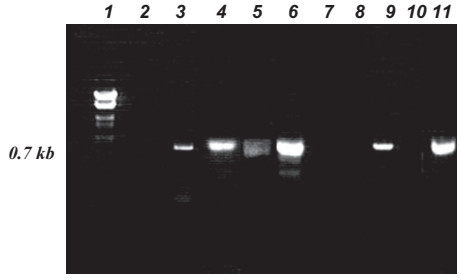


**Fig. 3.** Electrophoretic analysis of PCR products obtained on genomic DNA extracted from *Pleurotus ostreatus* transformants. The analysis was performed on 1% agarose gel. Lane 1: DNA Molecular Weight Marker III (Roche Applied Science, Indianapolis, IN); lane 2: PCR products obtained on genomic DNA extracted from nontransformed mycelium; lanes 3–9: PCR products obtained on genomic DNA extracted from mycelium cotransformed with the vectors pEGFPea1b and pTM1 (clone n.1, n.5, n.6, n.7, n.8, n.10); lanes 9–11: PCR products obtained on genomic DNA extracted from mycelium containing the vector pEGFPCBX (clone n.2a, n.2a*, n.43).

## Molecular analyses

Carboxin-resistant transformants were analyzed by PCR to verify the presence of the transforming DNA. The PCR was performed on genomic DNA extracted from transformants mycelia. Six of the nine analyzed transformants showed the expected 0.7-kb target band, indicating the presence of the *egfp* gene in the transformants (Fig. 3).

Southern hybridization analysis of the transformants 5 and 43 was carried out to analyze the mode of integration of the transforming DNA (Fig. 4). The non transformed mycelium does not show any hybridization. The transformants 5 and 43 showed a different pattern of bands. The transformant 43 showed single bands in each digestion. For the transformant 5, several bands of various sizes were observed. These results demonstrated that the introduced sequence was integrated ectopically into the

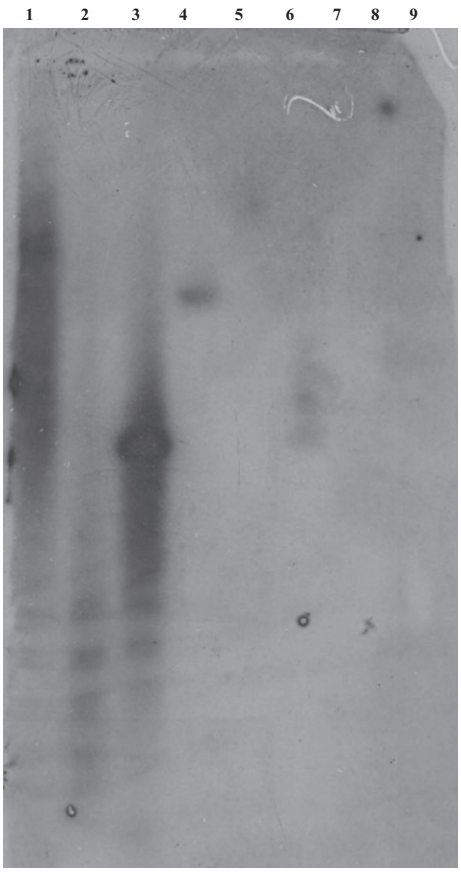


**Fig. 4.** Southern hybridization analysis of the transformants. Lanes 1, 2, and 3: EcoRI-, BamHI-, PstI-digested DNA extracted from the transformant 5; lanes 4, 5, and 6: EcoRI-, BamHI-, PstI-digested DNA extracted from the transformant 43; lanes 7, 8, and 9: EcoRI-, BamHI-, PstI-digested DNA extracted from the nontransformed mycelium.

chromosomal DNA with one or more copy numbers in these transformants.

Transcription of *egfp* in the transformants 5 and 43 was demonstrated by RT-PCR (Fig. 5).

## EGFP visualization

Detection of fluorescence was performed *in vivo* on 2 days grown transformants mycelia on microscopic slides. In Fig. 6, phase-contrast micrographs of transformants (a) and the corresponding images under UV light (b) are shown. Nontransformed mycelium did not show any fluorescence. Scanned images show a positive fluorescence emission with respect to untransformed control. Fluorescence emission extended to entire hyphae, especially to clamps connection. Similar phenomenon was also observed when *poxc* promoter-driven reporter plasmid was used for transformation (to be published elsewhere).

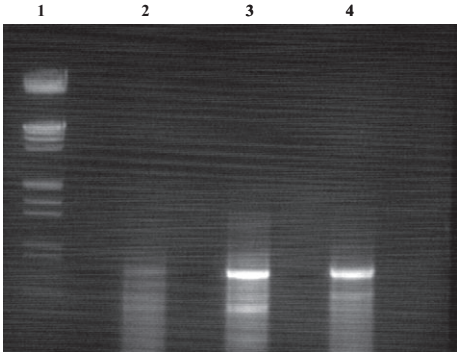


**Fig. 5.** Electrophoretic analysis of RT-PCR products obtained on total RNA extracted from *Pleurotus ostreatus* transformants. The analysis was performed on 1% agarose gel. Lane 1: DNA Molecular Weight Marker III (Roche Applied Science); lane 2: PCR products obtained on RNA extracted from nontransformed mycelium; lanes 3–4: PCR products obtained on RNA extracted from the transformants 43 and 5, respectively.

## Intracellular GFP determination

The *P. ostreatus* transformants 1, 5, 2, and 43 were analyzed for intracellular fluorescence emission by measuring emission of fluorescence of intracellular protein extracts from 7-day-old mycelium in comparison with the control (nontransformed mycelium; Fig. 7). The entity of fluorescence emission was measured as difference between spectrum area recorded between 500 and 550 nm for the transformant and that of the control sample (nontransformed fungus). The expression of GFP in each of the transformants has proved stable over a 6-month period of repeated subculturing on selective media (data not shown).

Difference in intracellular fluorescence emission was revealed for different transformants that could be ascribed to the different copy numbers and loci of exogen DNA integration within the fungal genome. Variation in GFP concentration among independent fungal transformants has been observed by other authors (Chalfie *et al.*, 1994; Cubitt *et al.*, 1995).

Comparison of intracellular fluorescence emission by transformants growth in the presence and in the absence of copper sulfate showed that metal addition causes an increase in green fluorescence driven by the *poxa1b* promoter, up to fourfold (20 000 fluorescence unit per 0.05 mg of proteins).

It is worth noting that an induction of transcription from a particular promoter sequence was hereby demonstrated by quantitative measurement of fluorescence emission for the first time in basidiomycetes.

GFP has been successfully expressed in the homobasidiomycetes *Schizophyllum commune* (Lugones *et al.*, 1999), *P. chrysosporium* (Ma *et al.*, 2001), *A. bisporus*, and

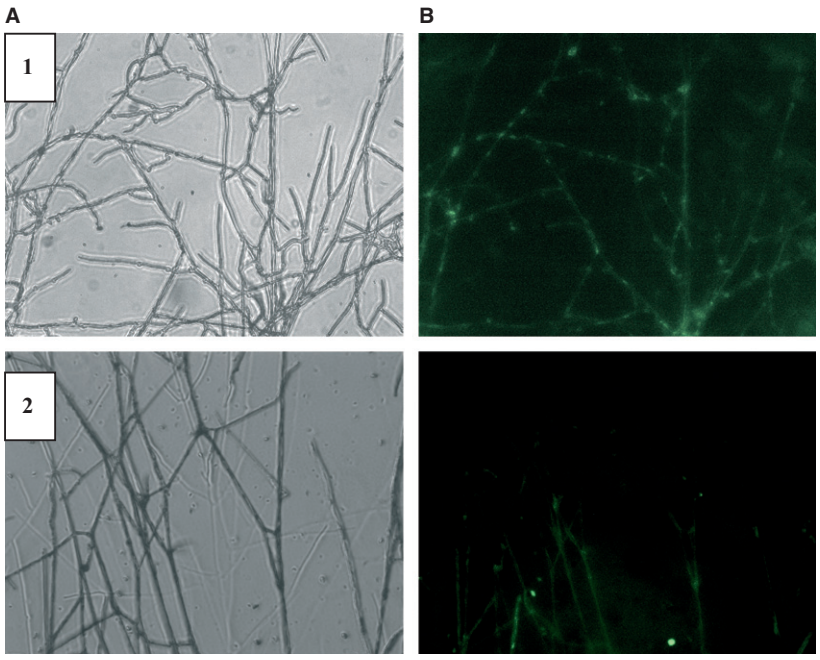


**Fig. 6.** Phase-contrast images (a) and fluorescence micrographs (b) of transformed mycelium containing the plasmids pEGFPa1b and pEGFPCBX (1: transformant 5; 2: transformant 43). The green color in the mycelium is due to GFP.

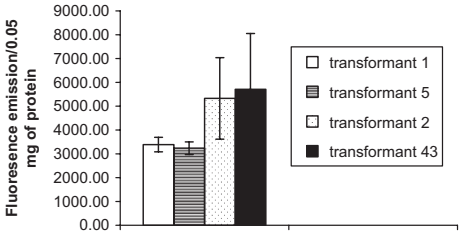


**Fig. 7.** Bar graph showing the fluorescence emission intensity at 500–550 nm of some of the transformants (transformants number 1, 5, 2, and 43), after subtracting the background fluorescence emission of control (nontransformed fungus).

*C. cinereus* (Burns *et al.*, 2005). A number of factors such as inactivation of transforming DNA by preferential methylation (Mooibroek *et al.*, 1990), inactivation of gene expression of AT-rich sequences (Schuren & Wessels, 1998; Scholtmeijer *et al.*, 2001), need of introns for mRNA accumulation (Lugones *et al.*, 1999; Scholtmeijer *et al.*, 2001; Burns *et al.*, 2005) seem to hamper transgene expression of GFP in basidiomycetes. Moreover, in a few manuscripts so far reported on transformation of *P. ostreatus* with the GFP gene, green fluorescence after transformation was unstable (Li *et al.*, 2006), or no quantitative measurement of protein expression was reported (Ding *et al.*, 2011).

## Conclusions

In this manuscript, a transcriptional induction of a laccase promoter was demonstrated in *P. ostreatus* by enhanced GFP expression, based on a PEG-mediated procedure for fungal transformation. The promoter of *poxa1b* was chosen among the different *P. ostreatus* laccase promoters, because it contains the highest number of putative MREs sites and *poxa1b* transcript is the most copper-affected among the *P. ostreatus* laccase transcripts.

Cotransformation with pTM1 vector conferring carboxin resistance and pEGFPea1b vector containing *egfp* gene under the control of *poxa1b* promoter region was carried out and compared to transformation with the unique pEGFPCBX vector containing both carboxin resistance cassette and *poxa1b* promoter-*egfp* gene cassette. The presence of *egfp* gene was demonstrated in most of the carboxin-resistant transformants. Southern hybridization analysis of the transformants 5 (cotransformed with pTM1 and pEGFPea1b vectors) and 43 (transformed with the unique pEGFPCBX vector) showed that the introduced sequence was integrated ectopically into the chromosomal DNA with one or more copy numbers. Transcription of *egfp* in the transformants 5 and 43 was also demonstrated. An intracellular fluorescence emission up to around 5,000 (Units per 0.05 mg of protein) in comparison with the nontransformed mycelium was measured. No significant difference of fluorescence emission was observed comparing *pEGFPea1b and* pEGFPCBX transformants. However, a less transformation efficiency was achieved using the bigger pEGFPCBX vector. By analyzing intracellular fluorescence emission by transformants growth in the presence of copper sulfate, an increase in green fluorescence was revealed up to 20 000 fluorescence unit per 0.05 mg of proteins, providing *in vivo* demon-

stration of susceptibility of *poxa1b* laccase promoter to the metal.

The developed system allowed both *in vivo* demonstration of copper-induction of expression driven by *poxa1b* promoter and its quantitative analysis. This will allow investigation of the role of putative metal response elements present in this promoter.

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Applied and Environmental Microbiology

# Transcriptional and Enzymatic Profiling of Pleurotus ostreatus Laccase Genes in Submerged and Solid-State Fermentation Cultures

**Raúl Castanera, Gúmer Pérez, Alejandra Omarini, Manuel Alfaro, Antonio G. Pisabarro, Vincenza Faraco, Antonella Amore and Lucía Ramírez**

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# Transcriptional and Enzymatic Profiling of *Pleurotus ostreatus* Laccase Genes in Submerged and Solid-State Fermentation Cultures

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**The genome of the white rot basidiomycete *Pleurotus ostreatus* includes 12 phenol oxidase (laccase) genes. In this study, we examined their expression profiles in different fungal strains under different culture conditions (submerged and solid cultures) and in the presence of a wheat straw extract, which was used as an inducer of the laccase gene family. We used a reverse transcription-quantitative PCR (RT-qPCR)-based approach and focused on determining the reaction parameters (in particular, the reference gene set for the normalization and reaction efficiency determinations) used to achieve an accurate estimation of the relative gene expression values. The results suggested that (i) laccase gene transcription is upregulated in the induced submerged fermentation (iSmF) cultures but downregulated in the solid fermentation (SSF) cultures, (ii) the *Lacc2* and *Lacc10* genes are the main sources of laccase activity in the iSmF cultures upon induction with water-soluble wheat straw extracts, and (iii) an additional, as-yet-uncharacterized activity (Unk1) is specifically induced in SSF cultures that complements the activity of Lacc2 and Lacc10. Moreover, both the enzymatic laccase activities and the *Lacc* gene family transcription profiles greatly differ between closely related strains. These differences can be targeted for biotechnological breeding programs for enzyme production in submerged fermentation reactors.**

*Pleurotus ostreatus* (Jacq.: Fr.) Kumm. (*Dikarya*, *Basidiomycota*, *Agaricomycotina*, *Agaricales*) is a white rot basidiomycete that lives as a saprophyte on dead or decaying wood. White rot basidiomycetes degrade their substrate by secreting different enzymes, of which those involved in lignin degradation are of specific interest (13, 30). In this study, we examined the transcriptome and activity profile of the phenol oxidase (laccase; EC 1.10.3.2) family of lignin-degrading enzymes under different culture conditions. Laccases catalyze the oxidation of various aromatic substrates with a subsequent reduction of molecular oxygen to water (41). The broad substrate specificity of laccases permits their use in multiple biotechnological and industrial applications as inexpensive biologically and environmentally friendly tools for the pretreatment of lignocellulose for bioethanol production, pulp bleaching, dye degradation, and xenobiotic transformation and detoxification (20).

The genome of *P. ostreatus* includes 12 laccase genes (browse the sequence at http://www.jgi.doe.gov). Six appear clustered at the subtelomere region of chromosome VI, and the others map to chromosomes IV, VI, VII, VIII, and XI (two genes) (33). However, only six *P. ostreatus* laccase isozymes, POXA1b (Lacc6), POXA1w, POXA2, POXA3a (Lacc2), POXA3b, and POXC (Lacc10), have been biochemically characterized to date (10, 11, 32). Among them, Lacc2, Lacc6, and Lacc10 are the most studied, and their genes have been expressed heterologously in yeast (8, 40). Furthermore, two new laccase isoforms in solid fermentation cultures of *P. ostreatus* have been recently described (16), although they remain uncharacterized.

Information is available on the biochemical characteristics of some *P. ostreatus* laccases, their transcriptional regulation by metals, aromatic compounds, and nitrogen and carbon (39), and the evolution of their activity in liquid cultures (44). However, only one published study has described the expression profile of this gene family by the use of real-time quantitative PCR (qPCR) (12), and that study examined only one strain.

Reverse transcription followed by qPCR (RT-qPCR) represents the most powerful technology to quantitatively amplify trace amounts of mRNA (14, 36). RT-qPCR is considered to be the gold standard for measuring gene expression (42) because of its high sensitivity and specificity, robust reproducibility, and wide dynamic range (36, 37). This technique requires the careful selection and validation of reference genes (internal standards), which are processed in parallel with the target gene (26). Moreover, it is critical to determine the amplification efficiency (35, 43), which is used in mathematical models for the accurate estimation of the expression levels.

In this paper, we present an in-depth study of the laccase gene family expression profile in five *P. ostreatus* strains cultivated under submerged fermentation (SmF) and solid-state fermentation (SSF) conditions and the effect of an aqueous wheat straw extract on this profile. For this study, we focused on the careful selection and validation of reference genes and their numbers. The results showed different laccase transcription profiles in the solid and liquid cultures and revealed that the *Lacc2* and *Lacc10* genes are more highly expressed and are the main source of laccase activity in the submerged cultures whereas, in the solid cultures, Lacc2 and

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Lacc10, together with a third laccase isozyme (Unk1), are the main sources of laccase activity.

## MATERIALS AND METHODS

**Fungal strains, culture conditions, and experimental design.** Five *P. ostreatus* strains were used in this study: the dikaryotic strain N001, the monokaryotic protoclones PC9 and PC15 described previously by our group (21, 23, 24), and the dikaryotic strains FF and SS, which have fast- and slow-growing phenotypes, respectively, and were produced by mating compatible spores from N001 and separating the resulting cultures by their linear growth rates on solid agar media. The FF and SS strains are almost homozygous for each of the two N001 haplotypes of chromosome VIII. This chromosome carries a strong quantitative trait locus (QTL; $R^2 = 20.27$) that controls the linear growth rate on solid culture media (22). The FF strain carries the haplotype present in the PC9 strain, and the SS strain carries that of PC15.

SmF culture growth was performed in triplicate in 135 ml of chemically defined liquid medium (M7GY) comprising (per liter) 2 g of ammonium tartrate, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1 g of $KH_2PO_4$, 0.5 g of KCl, 10 g of glucose, and 1 ml of trace element solution [0.1 g of $Na_2B_4O_7 \cdot H_2O$, 0.07 g of $ZnSO_4$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of $MnSO_4 \cdot 4H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 0.01 g of $(NH_4)_6Mo_7O_2 \cdot 4H_2O$ per liter]. The cultures were grown in orbital shakers at 150 rpm in the dark at 24°C and covered with synthetic cotton filters to enable air exchange.

In the induction (induced SmF [iSmF]) experiments, 50 ml of aqueous wheat straw extract warmed to 24°C was added to the SmF cultures at the beginning of the stationary-growth phase (ninth day of culture). The wheat straw extract was prepared by agitating lyophilized wheat straw in distilled water (10% [wt/vol]) for 5 h at 25°C. The extract was then filtered, sterilized for 20 min at 121°C, and kept frozen until used. The samples from the iSmF cultures were harvested 48 h after induction.

SSF culture growth was performed in triplicate using glass flasks and 10 g of lyophilized wheat straw hydrated to 70% relative humidity. Extracellular enzyme fractions were harvested every 2 days until day 11, when the mycelium was harvested for RNA extraction. By day 11, the five strains had fully colonized the substrate, and the impact of the exponential-growth phase on laccase transcription should have been minimal.

**Nucleic acid extraction and reverse transcription.** The mycelium from the SmF and iSmF cultures was harvested by pressure filtration and was scraped from the surface of the SSF. Mycelia from the three biological replicates were pooled and ground in a sterile mortar using liquid nitrogen and stored at −80°C until use. Total RNA was extracted from 100 mg of frozen tissue by the use of a fungal RNA E.Z.N.A kit (Omega Bio-Tek, Norcross, GA), and its integrity was determined by electrophoresis on 1% (wt/vol) agarose gels. Duplicate samples were used for nucleic acid concentration measurements, which were quantified using a Nanodrop 2000 spectrophotometer (Thermo-Scientific, Wilmington, DE). Total RNA purity was estimated using the 260-nm/280-nm ratio, and only samples with values between 1.9 and 2.1 were used. The RNA was concentrated and purified using an RNeasy MinElute Cleanup kit (Qiagen Iberia S.L., Madrid, Spain) to achieve a concentration of 1 μg liter$^{-1}$. One μg of total RNA was reverse transcribed to cDNA in a 20-μl volume by the use of an iScript cDNA synthesis kit (Bio-Rad Laboratories S.A., Alcobendas, Spain) with the following reaction mixture: 1× cDNA synthesis kit buffer, 1 μl of reverse transcriptase H+, and 1 μg of total RNA; the volume of the mixture was brought to 20 μl using nuclease-free water. The reaction conditions for the cDNA synthesis were 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The cDNAs were stored at −20°C.

**Primer design and verification.** The PCR primers were designed using PrimerQuest software (Integrated DNA Technologies) with the genome sequences of protoclones PC9 version 1.0 and PC15 version 2.0, which can be obtained from the JGI database (http://www.jgi.doe.gov) (Table 1). The primer sequences were selected to be specific for each of the members of the laccase gene family, and this criterion outweighed the position in the sequence of the gene (i.e., intron/exon boundaries). PCR specificity was validated *in silico* by performing a Primer-BLAST search against all PC9 and PC15 model transcripts, and the RT-PCR products were sequenced and compared with the filtered laccase gene models to confirm the specificity of each primer pair.

**RT-qPCR.** Real-time quantitative PCRs were performed using a Bio-Rad CFX96 thermal cycler. SYBR green fluorescent dye was used to detect the product. Each reaction was set to a final volume of 20 μl and contained 1× IQ SYBR green Supermix (Bio-Rad Laboratories S.A., Alcobendas, Spain), 100 nM forward and reverse primers, and 10 ng of cDNA in nuclease-free water. The amplification program was as follows: 5 min at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 65°C and a final melt curve with increments of 0.5°C every 5 s from 65°C to 95°C. The primer specificities were validated by analyzing the melt curves. Raw fluorescence data were monitored, and the baselines were corrected using Bio-Rad CFX Manager. A multivariable, nonlinear regression model was applied to compute optimal Cp (crossing-point) values. The reactions were performed in triplicate in 96-well microtiter plates, and no-template controls were included for each master mix (one for each primer set). RT-negative controls for every sample (RNA template) were run using the reference gene *Lacc3* as the target. Background DNA contamination was estimated and expressed as a percentage of the relative quantities of the reference gene *Lacc3* according to equation 1:

$$\%\ \text{of genomic DNA background} = \frac{E^{-\mathrm{Cp_{DNA}}}}{E^{-\mathrm{Cp_{NA}}}} \times 100 \qquad (1)$$

where $\mathrm{Cp_{DNA}}$ and $\mathrm{Cp_{NA}}$ represent the Cp values obtained in the qPCRs performed using the RNA control and the retrotranscribed RNA with the endogenous DNA background, respectively. The SmF cultures were used as calibrators for the RT-qPCR relative quantifications.

**Quantification strategy and qPCR data normalization.** The mathematical model used for the relative quantifications of the transcripts was based on the PCR efficiencies and the mean Cp deviations between the sample and control groups. The reaction efficiencies were estimated using the LinReg tool for each sample and a Window of Linearity data set from the exponential phase of the fluorescence plotted in log scale (43). The relative quantities (RQ) were calculated using the calibrated efficiency model (equation 2) and were normalized to a robust index composed of data from three experimentally validated reference genes according to the mathematical model proposed by Pfaffl (35) (equation 3). Measures of the expression stability of the candidate genes (M) were calculated using the GeNorm tool (52). The optimal number of control genes for data normalization was determined by analyzing the pairwise variation among the sequential normalization factors (NF), which were calculated by step inclusion of additional reference genes from our panel, as proposed by Vandesompele et al. (52). Finally, the expression ratios were tested by random pairwise reallocation using REST software (38), which provided 95% confidence intervals and *P* values as follows:

$$\mathrm{RQ} = (E\ \text{target})^{\Delta \mathrm{Cp}\ (\text{control sample})} \qquad (2)$$

$$\text{Relative expression ratio} = \frac{(E\ \text{target})^{\Delta \mathrm{Cp}\ (\text{control sample})}}{(E\ \text{target})^{\Delta \mathrm{Ctp_{ref}}\ (\text{control sample})}} \qquad (3)$$

where "*E* target" represents the PCR amplification efficiency of the gene of interest and ΔCp represents the difference between the Cp values for that gene under control and experimental conditions.

**Enzyme analysis.** In the case of the SmF and iSmF cultures, triplicate 2-ml samples were collected from each biological replicate and centrifuged at 1,0000 rpm for 3 min at 4°C to remove debris. In the SSF cultures, three biological replicate experiments (parallel cultures) per strain per time point were used. In this case, the enzymes released to the medium were extracted from the solid culture by adding 40 ml of sodium acetate buffer (50 mM, pH 5.0) and shaking the extraction mixtures at 150 rpm for 4 h at 10°C as described by de Souza-Cruz et al. (5). Following the extraction, the liquid fraction was collected and centrifuged to remove debris, and the subsequent supernatant was used for enzyme determina-

**TABLE 1** PCR primers, amplicon length, and amplification efficiency for the laccase and reference genes

| Gene | Transcript identification no.[a] | | Chromosome | Primer category | Primer sequence | Amplicon length (bp) | Amplification efficiency |
|---|---|---|---|---|---|---|---|
| | PC15 | PC9 | | | | | |
| Laccase genes | | | | | | | |
| *Lacc1* | 1043420 | 90578 | VI | Fw | GGTACATCCTAGCACCCAATG | 80 | 1.74 ± 0.04 |
| | | | | Rv | GACGAGATCAGTTTCCAAGAGG | | |
| *Lacc2* | 1067328 | 116143 | VIII | Fw | CCCTGGCAGATTGGTATCATG | 142 | 1.74 ± 0.00 |
| | | | | Rv | ATGACAGCGTAAGGGACAAG | | |
| *Lacc3*[b] | 1102751 | 123288 | VI | Fw | TCGTTTCCGTCTCGTTTCTC | 134 | 1.69 ± 0.04 |
| | | | | Rv | CTGCGAAGATTTGGATGCTG | | |
| *Lacc4* | 1077328 | 65894 | 6 | Fw | CCCCATCCTTTCCATCTTCAC | 72 | 1.79 ± 0.04 |
| | | | | Rv | GTAGTTATACACCGAGCTTCCG | | |
| *Lacc5* | 1094975 | 90812 | XI | Fw | CGCATTTGCCGCTTTCTT | 136 | 1.67 ± 0.03 |
| | | | | Rv | GGTGACTAGGACTGAGTATCTC | | |
| *Lacc6* | 1113032 | 81104 | VI | Fw | GTACAACTACGAAAACCCCG | 140 | 1.68 ± 0.02 |
| | | | | Rv | CAAGGTCAAGATGCCAGT | | |
| *Lacc7* | 1077468 | 60400 | VI | Fw | GTTGATAGCCTCCAGATCTTCG | 142 | 1.72 ± 0.02 |
| | | | | Rv | GTAGGATGGCGGAGTTGATG | | |
| *Lacc8*[c] | 1106925 | | VII | Fw | CATTGGCTGTGACTCGAA | 137 | 1.59 ± 0.04 |
| | | | | Rv | GGATCAGAGAATAGCGTTGG | | |
| *Lacc9* | 1089733 | 81107 | VI | Fw | CTATCCTTCGGTATGCTGGTG | 145 | 1.81 ± 0.05 |
| | | | | Rv | ATATTGATGTCTGCGCCTCC | | |
| *Lacc10* | 1089723 | 81117 | VI | Fw | CCTACTTCCCCTTTGGCTATC | 122 | 1.79 ± 0.07 |
| | | | | Rv | ATGACGAGCAAAGAGTGACC | | |
| *Lacc11* | 1043488 | 90573 | VI | Fw | CCTGAATGGTCTGATCTCTGC | 93 | 1.82 ± 0.05 |
| | | | | Rv | CCTATGACTTGGGCTCTTCG | | |
| *Lacc12*[b] | 1094965 | 90834 | XI | Fw | GTACTCATTTTCGGCTCCTG | 84 | 1.76 ± 0.06 |
| | | | | Rv | CCACGTAGTCCATCGCAATA | | |
| Reference genes[c] | | | | | | | |
| *cyt-c* | 1113744 | 112752 | VIII | Fw | GCCTCATAAAGTCGGTCCTAAC | 127 | 1.92 ± 0.08 |
| | | | | Rv | CTCAAATAGGGTGTTCTCGTCC | | |
| *actin1* | 1087906 | 114148 | I | Fw | AGTCGGTGCCTTGGTTAT | 129 | 1.72 ± 0.10 |
| | | | | Rv | ATACCGACCATCACACCT | | |
| *actin2* | 1114037 | 90915 | VIII | Fw | GCCGTGATCTTACCGACTA | 134 | 1.57 ± 0.04 |
| | | | | Rv | CTCCTGCTCAAAGTCCAA | | |
| *cyph* | 1058252 | 72928 | VII | Fw | GACATTGCTATCGACTCCCAG | 84 | 1.69 ± 0.10 |
| | | | | Rv | GAAATTCCTTGCAGTCTTGGG | | |
| *GAPDH* | 1090663 | 87982 | IX | Fw | ACCTTGAGATTACGACCC | 139 | 1.83 ± 0.03 |
| | | | | Rv | GTTGTGGCGTGGATTGTC | | |

[a] Transcript identification numbers correspond to the PC15 version 2.0 and PC9 version 1.0 genomes available at www.jgi.doe.gov.
[b] The *Lacc3* and *Lacc12* genes were also used as reference genes.
[c] Only present in the PC15 genome.

tion. In all cases, three technical replicates of each sample were used to determine each datum.

The laccase activity was determined spectrophotometrically by measuring the oxidation of 2,6-dimethoxyphenol (DMP; $\varepsilon_{480} = 49{,}600\ M^{-1}\ cm^{-1}$) following a 60-s incubation at room temperature. For these incubations, 90 to 450 μl of the sample was added to 500 μl of a 10 mM DMP solution in 0.1 M sodium acetate buffer (pH 5.0) and made up to achieve a 1-ml final volume with the acetate buffer. The enzyme activities were expressed in international units (U), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

**Laccase zymograms in native PAGE.** Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under nondenaturing conditions. The resolving and stacking gels contained 9% acrylamide–50 mM Tris-HCl (pH 9.5) and 4% acrylamide–18 mM Tris-HCl (pH 7.5), respectively. The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). Laccase activity was revealed by soaking the gels in a 20 mM ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) solution made using 0.1 M sodium citrate buffer (pH 3.0). Laccase isozymes purified from the dikaryotic strain ATCC MYA-2306 were used as standards (10, 11, 32).

**Biomass determination.** Three biological replicates of the SmF and iSmF cultures were taken on every second day of culture. The mycelium was harvested by filtering these samples with 10-μm-pore-size nylon filters followed by dehydration for 48 h at 50°C, and the samples were subsequently weighed.

**Phenol and reducing sugar determinations.** The phenol content of the wheat straw extract was estimated using Folin-Denis reagent (9) as proposed by Zhang et al. (55), and the reducing sugar content of the extract was determined using the DNS method (31).

**Statistical analysis.** All laccase activity and biomass measurements were subjected to Student's *t* tests to determine the statistical significance of differences.

## RESULTS

**Parameters used for accurate laccase RT-qPCR gene expression quantification. (i) Reference gene selection.** Seven functionally

different genes (*Cyt-c*, *Actin1*, *Actin2*, *Cyph*, *GAPDH*, *Lacc3*, and *Lacc12*) were tested for use as internal reference genes under our experimental conditions (see Fig. S1 in the supplemental material). Their expression stability (*M*) was calculated for three culture conditions (SmF, iSmF, and SSF) using the FF and PC15 strains because of their different phenotypes. The seven genes had *M* values ranging from 0.8 (*Cyt-c*) to 0.15 (*Lacc3* and *Lacc12*; see Fig. S2 in the supplemental material). Lower *M* values indicate a more stable gene expression profile (52), and our results revealed that *Lacc3*, *Lacc12*, and *Cyph* had the lowest expression variability among the seven genes tested.

**(ii) Optimal number of reference genes.** To determine the optimal number of reference genes, we studied the pairwise variation of NF (52) determined for *n* relative to $n + 1$ reference genes ($V_{n/n+1}$; see Fig. S3 in the supplemental material). This approach enabled us to set the ideal number of reference genes to three, because the inclusion of a fourth gene ($V_{3/4}$) led to a small (0.006) variation in *V*. Moreover, we noted that the $NF_n$-$NF_{n+1}$ pairwise variation correlated positively with the number of reference genes included (data not shown). Consequently, *Lacc3*, *Lacc12*, and *Cyph* were chosen as the reference gene set for data normalization in this study.

**(iii) Amplification efficiency.** The PCR amplification efficiency was calculated for the reference genes and for the members of the laccase gene family by the use of the LinReg PCR tool (43). The efficiency values ranged from 1.59 to 1.82 for the *Lacc* gene set (standard deviation [SD] = 0.23) and from 1.57 to 1.92 (SD = 0.44) for the reference gene set (Table 1). The standard deviations of amplicon efficiencies in comparisons of different samples ranged from 0.01 to 0.06 for the *Lacc* genes and from 0.03 to 0.10 for the reference genes.

**(iv) Genome DNA contamination.** All samples were tested for DNA background contamination. The mean DNA content in the samples was 1.94%. All of the samples had contamination values lower than 5%, with the exception of strain N001 in SSF (7.51%) and strain SS in SmF (5.36%; see Table S1 in the supplemental material).

**Transcriptional profile of the laccase gene family. (i) Submerged fermentation cultures.** We determined growth curves (biomass accumulation) under the conditions used for the SmF and iSmF experiments to analyze the behavior of the five strains in liquid cultures. The five strains showed a linear increase in biomass during the first 7 days of culture; by the 9th day of culture, all of the strains had entered the stationary phase of growth (see Fig. S4 in the supplemental material), and they had reached similar levels of biomass content by day 11 in the SmF cultures (see Fig. 2). The addition of the wheat straw extract on day 9 of culture produced significant differences in the accumulated fungal mass by day 11 in the iSmF cultures (see Fig. 2). The increases in biomass differed between strains and ranged between 1.57 and 22.57 g $liter^{-1}$ in the SS and PC9 strains, respectively. We analyzed the composition of the wheat straw extract, and we found that it contained 4.01 ± 0.22 g $liter^{-1}$ reducing sugar and 0.24 ± 0.03 g $liter^{-1}$ gallic acid equivalents (an estimation of the amount of polyphenolic compounds).

The laccase gene family expression profiles were quantified on day 11 using 10 of the 12 genes, because the *Lacc3* and *Lacc12* genes had been used in the reference set. In Fig. 1, we show the effect of the addition of wheat straw extract to the iSmF cultures on the transcriptional profiles of the members of this gene family (see Table S2 in the supplemental material). We observed that 65% of the analyzed *Lacc* genes were differentially expressed compared with the control ($P < 0.05$) after the addition of wheat straw extract, with 46% of the genes being upregulated and 19% downregulated (Table 2). The proportion of differentially regulated genes was higher in the monokaryotic strains than in the dikaryotic strains (84% versus 52%, respectively). Furthermore, this proportion was also higher when the fast-growing strains were compared as a group with the slow-growing strains (75% versus 50%, respectively). The genes showing the greatest upregulation in all the strains were *Lacc2* and *Lacc10* (see Fig. S5 in the supplemental material). The highest upregulation was observed in *Lacc2* in the PC9 strain (7.7-fold upregulation) and in *Lacc10* in the N001 strain (5.2-fold upregulation; Fig. 1).

**(ii) Solid-state fermentation cultures.** To profile the laccase gene expression in SSF cultures, the cultures were incubated for 11 days as described above. By that time point, all of the strains had completely colonized the substrate. The transcriptional profile of the 10 *Lacc* genes in SSF differed greatly from that observed in the SmF and iSmF cultures (Fig. 1; see also Table S3 in the supplemental material). Our results revealed that 42% of the genes were differentially expressed compared with the control conditions (SmF), with only 4% of the genes being upregulated and 38% downregulated (Table 2). Similar to the results seen with the iSmF cultures, the proportion of differentially regulated genes was higher in monokaryons than in dikaryons (53% versus 34%) and in fast-growing versus slow-growing strains (50% versus 30%).

**Enzymatic laccase activity profile in SmF, iSmF, and SSF cultures. (i) Laccase activity in SmF, iSmF, and SSF cultures.** To complement the results observed when analyzing the laccase family transcription profile in iSmF, we monitored the differences in laccase enzymatic activity associated with the addition of wheat straw extract. The laccase activity released into the culture medium was assayed on day 11 in the SmF and iSmF cultures. The activity measured in the control cultures (SmF) ranged between 0 and 0.73 U $liter^{-1}$ for the SS and FF strains, respectively (Fig. 2). However, significant ($P < 0.05$) increases in activity were observed in four of the five fungal strains following the addition of wheat straw extract, and enzymatic activities of more than 40 U $liter^{-1}$ were recovered for three of the strains. Notably, no laccase activity was detected in the PC15 iSmF cultures, despite a large increase in biomass following the addition of the inducer to this strain.

The activity extractable from the SSF cultures was measured at different time points (Fig. 3). This activity was found to be highly variable between the biological replicates. Laccase activity was detected in four of the strains studied, and it was not detected in cultures of the monokaryotic PC15 strain. The N001 and PC9 strains exhibited similar evolutions of laccase activity over time, whereas the dikaryotic FF and SS strains displayed a more erratic evolution of activity.

**(ii) Laccase isozymes in SmF, iSmF, and SSF.** The released (SmF and iSmF cultures) and extractable (SSF cultures) laccase isozymes were examined using PAGE followed by *in situ* detection of enzymatic activity. The zymograms revealed the presence of three isozymes, which corresponded to Lacc2, Lacc10, and an unknown isoform (Unk1) in the SSF (Fig. 4). The Lacc2 and Lacc10 isozymes were also detected in the fast-growing strains (N001, PC9, and FF) in the iSmF and SSF cultures. No laccase isozymes

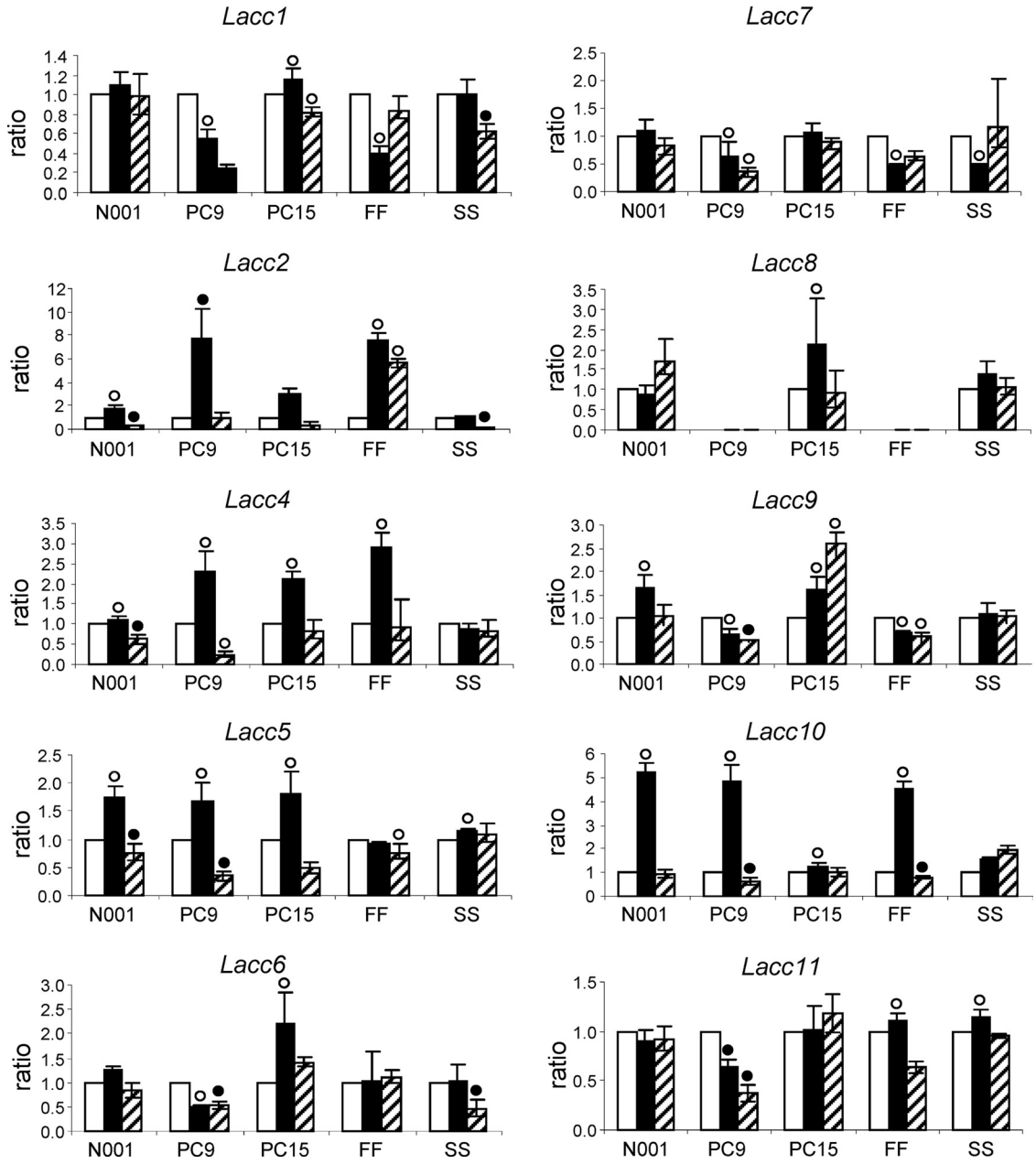


**FIG 1** Relative expression ratios of 10 *Lacc* genes of *P. ostreatus* cultured in iSmF (black bars) and on SSF (striped bars) media relative to the expression in the SmF control culture (white bars), according to equation 3 in the text. The bars represent the standard errors of the means of three independent measurements of the same pooled samples (see the text). The statistical significance of the differences between sample means is indicated with open circles ($P < 0.05$) or solid circles ($P < 0.01$).

were detected in any of the SmF cultures, in the PC15 samples, or in the iSmF sample of the SS strain.

## DISCUSSION

The existence of gene families comprising apparently redundant members raises questions concerning the actual function of these members. Phenol oxidases (laccases; EC 1.10.3.2) form a family of 12 genes in the white rot basidiomycete *P. ostreatus*, and they play an active role in lignin degradation (33, 34). Studying their differential expression enables the examination of the functions of the gene family members. This examination has been performed in certain cases for certain members of the laccase gene family (12, 29, 48); however, to date, not all of the gene family members have been examined. For the present study, using RT-qPCR, we report the differential expression characteristics of laccase genes in different strains and under different growth conditions.

**RT-qPCR conditions for profiling the expression of gene families.** Gene expression varies as a result of biological and experimental variation (53). The principal sources of this experimental variation include RNA extraction, the amount of starting material, random experimental errors, and the reverse transcrip-

**TABLE 2** Numbers and proportions of laccase genes up- and downregulated in monokaryotic, dikaryotic, fast-growing, and slow-growing strains in induced submerged and solid fermentation cultures[a]

| Laccase gene category | Strain group[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | | mk | | dk | | FG | | SG | |
| | *n* | % | *n* | % | *n* | % | *n* | % | *n* | % |
| Differentially expressed | 51 | 53 | 26 | 68 | 25 | 43 | 29 | 52 | 16 | 40 |
| Total[c] | 96 | | 38 | | 58 | | 56 | | 40 | |
| iSmf (differential expression)[d] | 31 | 65 | 16 | 84 | 15 | 52 | 21 | 75 | 10 | 50 |
| Upregulated | 22 | 46 | 11 | 58 | 11 | 38 | 13 | 46 | 9 | 45 |
| Downregulated | 9 | 19 | 5 | 26 | 4 | 14 | 8 | 29 | 1 | 5 |
| SSF (differential expression)[d] | 20 | 42 | 10 | 53 | 10 | 34 | 14 | 50 | 6 | 30 |
| Upregulated | 2 | 4 | 1 | 5 | 1 | 3 | 1 | 4 | 1 | 5 |
| Downregulated | 18 | 38 | 9 | 47 | 9 | 31 | 13 | 46 | 5 | 25 |

[a] *n*, numbers of laccase genes; %, proportions of laccase genes; mk, monokaryotic strains; dk, dikaryotic strains; FG, fast-growing strains; SG, slow-growing strains; iSmF, induced submerged fermentation; SSF, solid-state fermentation.
[b] The members of the strain groups were as follows: mk, strains PC15 and PC9; dk, strains N001, FF, and SS; FG, strains N001, FF, and PC9; SG, strains PC15 and SS.
[c] Total numbers of laccase genes studied in each strain group.
[d] Numbers and proportions of the laccase genes differentially ($P < 0.05$) expressed in iSmF and SSF cultures. For the proportion values (%), note that half of the total number of genes were studied in the iSmF and the other half in the SSF experiments.

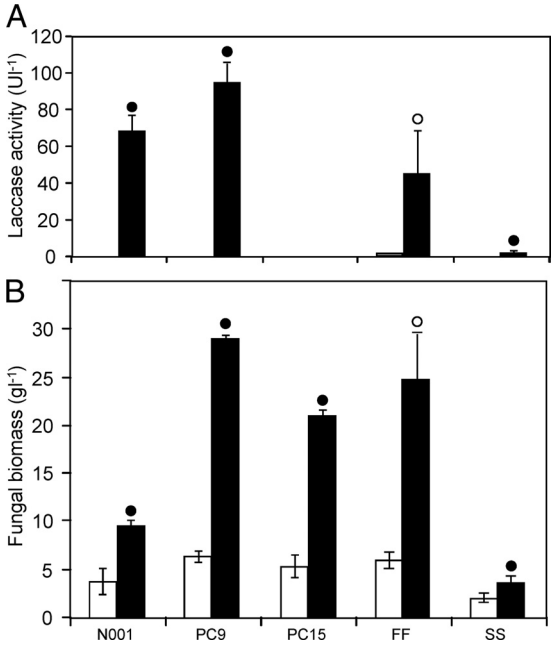


**FIG 2** Extracellular laccase activity determined using DMP (A) or biomass (B) as the substrate in SmF (white bars) and iSmF (black bars) cultures. Bars represent the standard deviations of the means of the results of three biological replicate experiments. The statistical significance of the differences between sample means is indicated with open circles ($P < 0.05$) or solid circles ($P < 0.01$).

tion process (49). To measure gene expression accurately using RT-qPCR, normalization relative to an endogenous control that is processed in parallel with the target genes is required (26). Ideal endogenous reference genes should have equivalent transcript levels in all cells at every developmental stage and should be unaffected by the experimental conditions (52). Housekeeping genes, such as those encoding β-actin, β-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rRNA, are frequently used as reference genes without any experimental validation, although fluctuations in their expression stability could cause unacceptable changes in gene expression estimations (46). In the search for appropriate internal standards for our experiment, we tested five housekeeping genes (*Cyt-c*, *GAPDH*, *Actin1*, *Actin2*, and *Cyph*) that are frequently used as standards in RT-qPCR assays and two *Lacc* genes (*Lacc3* and *Lacc12*), expression of which was stable under our experimental conditions. Our results revealed that expression of *Cyt-c*, *GAPDH*, *Actin1*, and *Actin2* was dependent on the media and culture conditions, with *Cyt-c* showing the most unstable expression. *Cyph*, *Lacc3*, and *Lacc12* expression levels were more stable under our experimental conditions (Fig. 1; see also Fig. S2 in the supplemental material), and *Lacc3* and *Lacc12* were expressed at approximately the same level as the target genes, as recommended for optimal endogenous controls (1). *Lacc12* encodes a fruit-body-specific laccase (25), and its stability under our experimental conditions was due to its basal expression level during vegetative growth. In the case of *Lacc3*, our results suggest that it is expressed constitutively. Other constitutively expressed laccases have been described in studies of *Pleurotus sajor-caju* growing in media containing different levels of carbon, nitrogen, and aromatic compounds (48).

We determined the optimal number of reference genes, as proposed by Vandesompele et al. (52), and we concluded that three stable reference genes were sufficient for qPCR data normalization, as reported by other authors (6). We observed a positive linear correlation between the increase in $NF_n$-$NF_{n+1}$ pairwise variation and the number of reference genes included that differed from the U-shaped correlation reported in other studies (52); this finding suggests that the sequential increase in $V_{n/n+1}$ is due to the inclusion of genes that have relatively unstable expression (27).

In summary, we suggest using the *Lacc3*, *Lacc12*, and *Cyph* genes for reference gene searches in further *P. ostreatus* RT-qPCR studies performed under similar conditions, and we stress the importance of validating the expression stability of the reference genes for each specific set of experiments (2, 52).

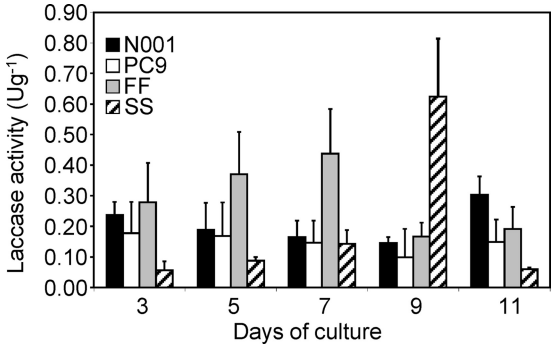


**FIG 3** Laccase activity extractable from SSF cultures harvested at different times. The monokaryotic PC15 strain did not show activity during any of the experiments and is not included here. The enzymatic activity was determined using DMP as the substrate. The bars represent the standard deviations of the means of the results of three biological replicate experiments.

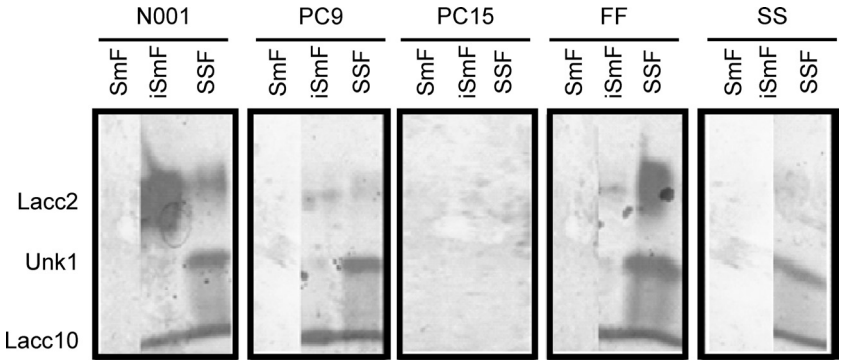


**FIG 4** Zymograms of the *P. ostreatus* laccases detected in SmF, iSmF, and SSF cultures. A 37.5-μl volume of protein extract was loaded in each lane.

PCR amplification efficiency is among the major factors that affect amplification kinetics. This efficiency is influenced significantly by the primers used (2, 18), the PCR components, and sample processing (50). Additionally, cDNA integrity and the RT reaction are the main sources of variability between samples for a given amplicon. The RT itself can interfere with cDNA first-strand PCR amplification via direct interaction with specific primer-template combinations (4), thereby inhibiting polymerase activity and altering PCR efficiency. Cp values are strongly influenced by the amplification efficiency, and a moderate (i.e., 0.1) intersample efficiency difference has been shown to lead to large differences in gene expression (36). Therefore, it is important to use a PCR model that considers amplification efficiency values for each sample when studying the expression stability of the reference gene set.

PCR efficiencies are typically calculated from standard curves based on serial dilutions of a reference cDNA sample (15). This method is highly reproducible for the same transcript and tissue in an experiment, but it has two primary disadvantages: (i) the efficiency must be calculated for each amplicon (disregarding intersample variation), and (ii) it tends to overestimate the real amplification efficiency and produces values greater than 100% in some cases (36). This overestimation is common when the cDNA samples are contaminated with salt, phenol, and chloroform and/or ethanol (43). Moreover, this method is expensive and time-consuming, particularly if many genes and tissues are used. We have used an alternative approach for estimating PCR efficiency that is based on the increase in fluorescence that occurs during the exponential phase of the PCR (43). In theory, this method underestimates efficiencies compared with the dilution method because the data are calculated using the reaction linear phase, which can be restrictive (17). Nevertheless, we found this method to be sensitive and reliable because it produced amplicon efficiency values lower than 100%, with standard deviations between different samples ranging from 0.01 to 0.10.

**Transcriptional profile of the laccase gene family.** The expression levels of members of the laccase gene family were profiled on day 11 of culturing. In the SmF and iSmF cultures, the biomass had reached a stationary accumulation phase at least 48 h before that time point (see Fig. S1 in the supplemental material), and in the SSF cultures, the mycelium had already colonized the entire substrate by that time. For the experiments in which laccase expression was induced, a wheat straw extract was added to the corresponding cultures (iSmF) by the ninth day of culturing.

The laccase gene family transcription profiles in the iSmF and SSF cultures were different from those seen under the control conditions (SmF). A greater number of *Lacc* genes were expressed differentially ($P < 0.05$) in the iSmF cultures (65%) than in the SSF cultures (42%); notably, whereas most of the differentially expressed genes in iSmF were upregulated (46% upregulated versus 19% downregulated), the majority were downregulated in SSF (4% upregulated versus 38% downregulated).

The high percentage of genes with altered transcriptional responses in iSmF reveals a complex regulation mechanism that could be related to the sensitivity of the laccase gene family to the phenolic compounds and sugars present in the inducer extract. The genes showing the highest upregulation among all of the strains were *Lacc2* (4.2-fold average upregulation) and *Lacc10* (3.5-fold average upregulation) (Fig. 1; see also Table S2 in the supplemental material). The promoters of these genes contain a higher number of motifs that are sensitive to components present in the wheat straw extract, such as XRE (xenobiotic response elements) and MRE (metal-responsive elements), than the promoters of other genes in this family (39). The presence of these elements was confirmed in the strains used in this work (data not shown), and consequently, the expression of these genes is expected to be sensitive to these inducers. The only gene for which a small downregulation was observed under these conditions was *Lacc7* (Fig. 1; see also Table S2 in the supplemental material).

The laccase transcriptional profile of the dikaryotic strains appeared more stable (i.e., they showed a lower number of differentially expressed genes) than that of the monokaryotic strains, and slow-growing strains also showed a more stable laccase expression pattern than fast-growing strains (Table 2). Careful evaluation of the underlying cause of these differences revealed that the monokaryotic PC9 strain (fast-growing phenotype) was more sensitive to the induction and culture conditions than the other strains (see Tables S2 and S3 in the supplemental material).

In the SSF cultures, there was an overall downregulation of the laccase gene expression (Table 2) that was notably robust in certain cases (*Lacc2* in strains N001, PC15, and SS; see Table S3 in the supplemental information). The complexity of the culture conditions (pH, moisture content, air transfer, wheat straw composition) hinders efforts to determine the elements responsible for these changes. However, we believe that the water content of the culture could be one of the most relevant factors because it could affect the diffusion of the phenolic compounds into the substrate, thereby affecting laccase gene transcription. Moreover, Economou et al. proposed that an increase in water content contributes

to increased sugar availability (7), which is an additional critical factor for laccase production in most fungi (28). Similarly, Sharma and Arora (47) reported that laccase activity in *Phlebia floridensis* cultured on wheat straw increased with water content and reached a 34-fold increase at the maximum moisture content, and studies performed with *Aspergillus oryzae* cultured on wheat bran have indicated that expression of genes encoding other secreted enzymes was dependent on the water content in the substrate (19).

**Laccase enzymatic activity.** The laccase inducer effect of the wheat straw extract was assayed in SmF cultures following 2 days of induction. Whereas the laccase activity was negligible in the control SmF cultures (Fig. 2), as reported by other authors on the basis of studies of submerged uninduced cultures (39), a significant ($P < 0.05$) increase in extracellular laccase activity was observed in the fast-growing strains following the addition of wheat straw extract. This increase reached a maximum of 94.67 U liter$^{-1}$ (PC9) and occurred in the same samples that showed transcriptional upregulation of *Lacc2* and *Lacc10* (Fig. 2). In contrast, no laccase activity was induced in the slow-growing strains. These results demonstrate that the increase in laccase activity was independent of biomass increase, because the PC15 strain showed a significant increase in biomass (upon the addition of the inducer) but no laccase activity. The induction of laccase activity was evident in the fast-growing strains (N001, PC9, and FF) that are homozygous for the PC9 variant of chromosome VIII and that contain at least one copy of the PC9 alleles for the *Lacc2* and *Lacc10* genes. The transcriptional response of *Lacc2* and *Lacc10* discussed above, together with the strong induction of the extracellular enzymatic activity, suggests an important role for these two enzymes in the lignin-degrading machinery of *P. ostreatus*.

The patterns of evolution of laccase activity seen during growth of SSF cultures (Fig. 3) differed between strains. The PC15 strain did not release any detectable laccase activity into the solid substrate during the entire culturing period, similar to iSmF culture results (Fig. 2). These data suggest that most (if not all) laccase activity secreted by N001 was due to the presence of the PC9 nucleus in this dikaryon. However, we can rule out the possibility of an overall deficiency in laccase production associated with the PC15 nucleus because this activity was observed in the SS strain, which bears the PC15 haplotype for chromosome VIII and a slow-growing phenotype.

The dikaryotic FF and SS strains exhibited different laccase activity patterns over time (Fig. 3) that could have been caused by their different efficiencies in substrate colonization. Each of the two strains is homozygous for one of the two variants of N001 chromosome VIII. This chromosome carries a QTL for dikaryotic growth rate ($R^2 = 20.3$) and the gene *Lacc2*. The differences in the laccase patterns of these dikaryotic strains in SSF cultures seen over time suggest that other factors beyond the laccase family genotype control the production of these enzymes in natural environments. Several of these factors have been genetically mapped as activity QTLs (44). Laccase activity values recorded for the SSF cultures were similar to others reported for *Pleurotus* species growing in similar substrates (3) but lower than those reported for SSF cultures made using agricultural wastes (16, 45).

**Isozyme patterns in SmF and SSF.** The zymograms of the intra- and extracellular laccase fractions revealed three isozymes: Lacc2, Lacc10, and Unk1 (Fig. 4). Unk1 had been recently described in a study of SSF cultures performed using tomato pomace (16), and it appeared to be abundant under the SSF conditions used in our study, which were more similar to the natural environmental conditions under which *P. ostreatus* grows than the conditions used for the submerged cultures. Interestingly, expression of this isoform does not appear to be induced by the wheat straw extract and consequently could be associated with the physical culture conditions, rather than with the presence of phenolic inducers. Thus far, Unk1 has not been associated with any laccase gene, no PCR-specific primers are available for it, and its transcription profile is unknown. These facts could explain the apparent discrepancy between the SSF transcription profile and the enzymatic activity data that were obtained.

Laccase upregulation by aromatic compounds (such as ferulic, p-coumaric, vanillic, and p-hydroxybenzoic acids), which occur widely in cell walls of gramineous plants (54), has been theorized to constitute a protective response to potentially toxic lignin-related compounds (51). Our results suggest that this type of water-extractable inducer, which causes transcriptional upregulation of most of *Lacc* genes in submerged fermentation, can be used to produce enzyme cocktails that can be further used in lignin-degrading industrial processes.

In summary, the results presented in this paper show that (i) laccase gene transcription is upregulated in iSmF cultures but is downregulated in SSF cultures, (ii) *Lacc2* and *Lacc10* genes are primarily responsible for the laccase activity in SmF upon induction with water-soluble wheat straw extracts, and (iii) an additional, as-yet-uncharacterized activity (Unk1) is specifically induced in SSF cultures that complements the activity of Lacc2 and Lacc10. Moreover, the patterns of enzymatic laccase activity and the *Lacc* gene family transcription profiles differ markedly between closely related strains. These differences could be exploited by biotechnological breeding programs optimizing enzyme production in submerged fermentation reactors.

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R.C. and G.P. carried out the qPCR experiments. A.O. carried out the enzymatic determinations and set up the straw extraction protocol. M.A., R.C., A.A., and V.F. carried out the protein analyses. A.G.P. revised and edited the manuscript. L.R. led and coordinated the project. The manuscript was written by R.C. and L.R.

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Short Communication

# Characterization of laccase isoforms produced by *Pleurotus ostreatus* in solid state fermentation of sugarcane bagasse

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ABSTRACT

Laccases are oxidative enzymes linked to biological degradation of lignin. The aim of this work was to evaluate the effect of inducers and different concentrations of nitrogen on production level of total laccase activity and pattern of laccase isoforms, produced in solid state fermentation of sugarcane bagasse by a selected strain of *Pleurotus ostreatus*. The addition of yeast extract 5 g/L, copper sulfate 150 μM and ferulic acid 2 mM provided highest enzymatic activity (167 U/g) and zymograms indicated the presence of six laccase isoforms (POXA1b, POXA3, POXC and three other isoforms). Results of protein identification by mass spectrometry confirmed the presence of POXC and POXA3 as the main isoenzymes, and also identified a glyoxal oxidase and three galactose oxidases. The fact that the isoenzyme POXA1b was not identified in the analyzed samples can be possibly explained by its sensitivity to protease degradation.


## 1. Introduction

Sugarcane bagasse is an important agro-residue generated in high amount (186 million tons/year) by the sugar and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane, and is composed of 19–24% lignin, 27–32% hemicellulose, 32–44% cellulose and 4.5–9% ashes (Soccol et al., 2011). Lignin is a recalcitrant aromatic polymer that can be oxidized by laccases, manganese peroxidases and lignin peroxidases. These enzymes catalyze one-electron oxidation of lignin units, producing aromatic radicals (Giardina et al., 2000). Laccases (E.C. 1.10.3.2, *p*-diphenol:dioxygen oxidoreductases) are blue multicopper oxidases able to oxidize a variety of phenolic compounds, with concomitant reduction of molecular oxygen to water. These enzymes are secreted in multiple isoforms depending on the fungal species and the environmental conditions, and this variety is related to the diversity of their roles: lignin degradation/synthesis, fruiting bodies development, pigment production, cell detoxification (Piscitelli et al., 2011). The biochemical diversity of laccase isoenzymes appears to be due to the multiplicity of laccase genes; however, regulation of their expression can be substantially diverse between fungal species (Palmieri et al., 2003).

*Pleurotus ostreatus* belongs to a class of white-rot fungi that produces laccases, manganese peroxidases but not lignin peroxidases (Giardina et al., 2000). The sequencing and annotation of the *P. ostreatus* PC 15 genome version 2.0 (Joint Genome Institute, 2011) indicates the presence of at least 12 genes of multicopper oxidases. Some of the corresponding enzymes have been purified and characterized, and these include POXA1b (Giardina et al., 1999), POXA1w and POXA2 (Palmieri et al., 1997), POXA3 (Palmieri et al., 2003) and POXC, previously named POX2 (Giardina et al., 1996) where POX means phenol oxidase. Other isoenzymes whose sequences have been determined are POX1 (Giardina et al., 1995), POX3 and POX4 (Pezzella et al., 2009). POXC is the most abundantly produced under all growth conditions examined according to Giardina et al. (1999). The objective of these studies was to evaluate the effect of known inducers of laccase expression (copper sulfate and ferulic acid) and two levels of organic nitrogen concentration in the form of yeast extract, on the production level of total laccase activity and laccase isoforms – identified by zymograms and mass spectrometry – produced in solid state fermentation, utilizing the sugarcane bagasse as substrate and a selected strain of *P. ostreatus* (coded Pl 22 Em).

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## 2. Methods

### 2.1. Solid state fermentation

Erlenmeyer flasks of 125 mL containing 1 g of sugarcane bagasse were autoclaved and humidified with a saline solution (sterilized by filtration, 15 mL/g bagasse) presenting the following constant composition: $MgSO_4{\cdot}7H_2O$ (0.3 g/L), $FeSO_4{\cdot}7H_2O$ (0.005 g/L), $MnSO_4{\cdot}H_2O$ (0.00156 g/L), $ZnSO_4{\cdot}7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), $CoCl_2$ (0.002 g/L), $KH_2PO_4$ (1.5 g/L), pH 5.5, with some differences regarding the following variables: yeast extract (2 or 5 g/L, containing 10.5% w/w total nitrogen, sterilized by autoclaving at 121 °C, 1 atm for 15 min); $CuSO_4$ (0 or 150 μM, sterilized by filtration), ferulic acid (2 mM, sterilized by filtration and added after 48 h of fermentation). These concentrations were chosen on the basis of optimization experiments for laccase production by *P. ostreatus* (data not shown). The strains *P. ostreatus* 22 Em (Pl 22 Em – selected for presenting the highest laccase production in sugarcane bagasse among 45 strains of basidiomycetes) and *P. ostreatus* ATCC MYA-2306 (Pl ATCC – standard dikaryotic strain for laccase production in liquid culture) were reactivated in PDA dishes. After 7 days of growth, 4 disks of 7 mm diameter were transferred to Erlenmeyer flasks of 250 mL containing 50 mL of Czapek liquid medium and the antibiotic streptomycin sulfate (0.1 g/L), added to reduce the risk of bacterial contamination. After 5 days of growth at 28 °C and 125 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a homogenizer (IKA T-10 basic Ultra-Turrax) and resuspended in the residual medium to a lower final volume (10% of the initial volume). 0.2 mL of the homogenized mycelium was transferred to the fermentation flasks, which were manually homogenized and incubated at 28 °C for different times.

### 2.2. Extraction of the enzymes

Enzymes produced by solid fermentation were extracted by solid–liquid extraction using sodium phosphate buffer as solvent ($NaH_2PO_4{\cdot}H_2O$, 50 mM, pH 7.0) containing 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), at the proportion of 1:10 (w/w) (Iandolo et al., 2011). The mixture was homogenized in vortex for 1 min and centrifuged for 7500 rpm, 4 °C, 45 min. The supernatant was separated and submitted to analyses.

### 2.3. Liquid fermentation

Liquid fermentation was conducted in PDY medium (potato dextrose 24 g/L and yeast extract 5 g/L) containing $Cu^{2+}$ 150 μM, added after autoclaving, in Erlenmeyer flasks of 500 mL filled with 250 mL (final volume). The strains Pl 22 Em and Pl ATCC were reactivated in PDA dishes and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Erlenmeyer flasks of 250 mL containing 50 mL of Czapek liquid medium and the antibiotic streptomycin sulfate (0.1 g/L). After 5 days of growth at 28 °C and 125 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a homogenizer (IKA T-10 basic Ultra-Turrax) and resuspended in the residual medium to a lower final volume (10% of the initial volume). 2.5 mL of the homogenized mycelium were transferred to the fermentation flasks, which were incubated at 28 °C and 125 rpm.

### 2.4. Laccase activity and protein concentration assays

The enzymatic activity of laccases was assayed by the oxidation of ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid). The reaction mixture contained 100 μL of ABTS 20 mM (in sodium citrate buffer 0.1 M, pH 3.0), sample (usually 20–50 μL) and sodium citrate buffer ($C_6H_8O_7{\cdot}H_2O$ 0.1 M, pH 3.0) up to 1 mL. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon$ = 36,000 $M^{-1}\,cm^{-1}$). The enzyme activity was expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min. Protein concentration was determined using the BioRad Protein Assay, with bovine serum albumin as a standard.

### 2.5. Enzyme preparations

Enzyme solutions were concentrated by ultrafiltration and dialyzed in sodium phosphate buffer (50 mM, pH 7.0). Samples were centrifuged at 8000 rpm, 4 °C in Amicon tubes (Amicon Ultra centrifugal filters 0.5 mL, Ultracel 30 kDa membrane – Millipore).

### 2.6. Zymograms of native PAGE

Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under non-denaturing conditions. The separating and stacking gels contained, respectively, 9% and 4% acrylamide and 50 mM Tris–HCl (pH 9.5) and 18 mM Tris–HCl (pH 7.5) as buffers. The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). Visualization of the bands was achieved by the addition of ABTS 20 mM to the gel submerged in sodium citrate buffer (0.1 M, pH 3.0) after electrophoresis.

### 2.7. Preparation of enzymes for protein identification

Enzymes from the samples selected for enzyme identification were extracted according to item 2.2 and concentrated by ammonium sulfate precipitation (80% saturation, 4 °C, overnight) followed by centrifugation (8000 rpm, 4 °C, 40 min). Protein precipitate was suspended in 1 mL sodium phosphate buffer (50 mM, pH 6.5) and extensively dialyzed against the same buffer in a 12,000–14,000 Daltons membrane (Delchimica dialysis tubing – visking, code DTV 12000), until the ammonium sulfate was completely removed. Activity and protein concentration in the enzyme suspension were determined according to item 2.4. Semi-denaturating SDS–PAGE was performed by loading the protein suspension in 0.1% SDS buffer not containing 2-mercaptoethanol and without boiling. The separating and stacking gels contained, respectively, 12.5 and 4% acrylamide and 375 mM Tris–HCl plus 0.1% SDS (pH 8.8) and 125 mM Tris–HCl plus 0.1% SDS (pH 6.8) as buffers. The electrode reservoir solution contained 25 mM Tris, 190 mM glycine and 3.5 mM SDS (pH 8.4). Visualization of the bands was achieved by Coomassie brilliant blue staining.

### 2.8. Protein identification

Coomassie blue-stained protein bands were excised from the gels and washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubating the bands with 10 mM dithiothreitol (DTT) for 45 min at 56 °C. Cysteines were alkylated by incubation in 5 mM iodoacetamide for 15 min at room temperature in the dark. The bands were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/μL) in 50 mM ammonium bicarbonate buffer, pH 8.5. Gel particles were incubated at 4 °C for 2 h. The buffer solution was then removed and a new aliquot of buffer solution was added for 18 h at 37 °C. Peptides were then extracted by washing the gel particles with 0.1% (v/v) formic acid in 50% (v/v) acetonitrile at room temperature and lyophilized. Peptide mixtures were analyzed by LC–MS/MS, using a HPLC–Chip LC system (Agilent 1200) connected to a Q-TOF 6520 (Agilent Technologies). Lyophilized samples were resuspended in 10 μL of 0.1% (v/v) formic acid. After loading, the peptide

mixtures were concentrated and washed at 4 μL/min in a 40 nL enrichment column with 0.2% (v/v) formic acid in 2% (v/v) acetonitrile. Fractionation was carried out on a C-18 reverse phase column (75 μm × 43 mm) at a flow rate of 0.4 μL/min with a linear gradient of eluent B (95% v/v acetonitrile and 0.2% v/v formic acid) in eluent A (2% v/v acetonitrile and 0.1% v/v formic acid) from 7% to 80% in 51 min. Mass spectrometry analyses were performed using data dependent acquisition MS scans (mass range 300–2400 $m/z$), followed by MS/MS scans (mass range 100–2000 $m/z$) of the 4 most intense ions of a chromatographic peak. Raw data from LC–MS/MS were converted to m/z data, and searched against the PleosPC15 database available at the Joint Genome Institute's website (http://genome.jgi-psf.org/PleosPC15_1) using the licensed version of Mascot 2.1 (Matrix Science).

## 3. Results and discussion

### 3.1. Evaluation of the effect of copper sulfate and ferulic acid addition on laccase activity production level and laccase isoenzymes pattern

Results in Table 1 showed a peak of activity at the 5th day, highest values being obtained with ferulic acid: 167 U/g with 5 g/L YE and 86.8 U/g with 2 g/LYE. Other values reported in literature for laccase production by *P. ostreatus* in solid state fermentation are 65.42 U/g with copper as inducer (Mishra and Kumar, 2007) and 36 U/g without inducers (Iandolo et al., 2011). According to the visual analysis of the native PAGE zymogram (Fig. 1), the isoenzyme POXC was the most abundantly produced under all conditions evaluated, which is in accordance with Giardina et al. (1996, 1999). The expression of the isoenzyme POXA3 (Palmieri et al., 2003) was induced by copper sulfate and even more by the combination of copper sulfate and ferulic acid, when comparing the three samples obtained at the 8th day. However, samples of the 10th day presented a more intense POXA3 band, indicating an increase of this isoenzyme production. The band corresponding to the isoenzyme POXA1b (Giardina et al., 1999) could be visualized only in those samples containing ferulic acid. Besides the three known isoforms POXA1b, POXA3 and POXC, three more bands were visualized. However, an isoenzyme with electrophoretic mobility similar to that of the isoform 3 has already been identified by Lettera et al. (2010) as LACC12. Moreover, isoforms with similar electrophoretic mobility to that of the isoforms 1 and 2 have already been detected during solid state fermentation of *P. ostreatus* ATCC on tomato pomace (Iandolo et al., 2011).

It has been previously demonstrated that the addition of copper sulfate 150 μM to a *P. ostreatus* liquid culture medium causes a 30-fold increase in total laccase activity and induces the isoenzymes POXC and POX A1b in *P. ostreatus* at the level of gene transcription (Palmieri et al., 2000). Analysis of the *poxc* and *poxa1b* promoter regions revealed the presence of multiple putative metal responsive elements (Faraco et al., 2002). In fact, in this experiment, the total laccase activity obtained in the presence of copper was 38-fold higher in comparison with the basal medium when analyzing the activities obtained at the 5th day at the same concentration of yeast extract (Table 1). Different studies have shown that laccase production is regulated by metal ions such as $Cu^{2+}$ and $Fe^{3+}$ by gene expression induction or through translational or post-translational regulation (Fonseca et al., 2010). Baldrian and Gabriel (2002) concluded that $Cu^{2+}$ not only induces laccase by the expression of laccase genes in *P. ostreatus*, but it also positively affects activity and stability of the enzyme. Aromatic compounds such as ferulic acid have shown to stimulate laccase production in many different strains (Elisashvili and Kachlishvili, 2009). Haars and Hüttermann (1983) demonstrated that the surface of the fungal hyphae contains specific receptors for small phenolic compounds that stimulate *de novo* synthesis of laccase.

**Table 1**
Maximum level of laccase activity produced by the strain *P. ostreatus* 22 Em on sugarcane bagasse in different conditions of yeast extract (YE) concentrations and inducers – $Cu^{2+}$ and ferulic acid (Fer).

| YE (g/L) | Inducer | Time (days) | U/g dry substrate |
|---|---|---|---|
| 2 | 0 | 5 | 1.06 |
| | | 8 | 14.6 |
| | | 10 | 11.6 |
| 2 | $Cu^{2+}$ 150 μM | 5 | 40.5 |
| | | 10 | 22.3 |
| | | 12 | 18.0 |
| 2 | $Cu^{2+}$ 150 μM + Fer 2 mM | 5 | 86.8 |
| | | 10 | 32.2 |
| | | 12 | 17.6 |
| 5 | $Cu^{2+}$ 150 μM | 5 | 51.7 |
| | | 10 | 21.4 |
| | | 12 | 10.7 |
| 5 | $Cu^{2+}$ 150 μM + Fer 2 mM | 5 | 167 |
| | | 10 | 57.1 |
| | | 12 | 37.6 |

According to the zymogram reported in Fig. 2, the intensity of the band related to POXC was not affected by different concentrations of yeast extract and the presence of both inducers, and its intensity decreased only after 15 days (sample 5Fer15). The expression of the isoenzyme POXA3 was induced by ferulic acid, in accordance with the previous results, for both concentrations of organic nitrogen, and the expression kinetics was dependent on the concentration of yeast extract. For the isoenzyme POXA1b, the higher concentration of organic nitrogen had an apparent negative effect. It is important to remark that this isoenzyme is very sensitive to protease degradation (Palmieri et al., 2000), so this effect could be possibly explained by the relation between the presence of proteases and yeast extract concentration. Ferulic acid did not significantly induce the expression of POXA1b from the 10th day on, in comparison with copper sulfate alone, when the concentration of yeast extract was 2 g/L, however for the concentration of 5 g/L this effect was observed from the 12th day on. The new band 1 was more clearly visualized when the higher concentration of yeast extract was used (5 g/L), and increased with fermentation time in the presence of ferulic acid. However, in the absence of ferulic acid, the most intense band was obtained at the 10th day of fermentation. The isoforms 2 and 3 were also more intense in the presence of higher concentration of yeast extract, and ferulic acid showed an observable positive effect on their expression only at this condition. Also the reference strain *P. ostreatus* ATCC produced three new bands on solid state fermentation of sugarcane bagasse, indicating that this pattern is more related to the fermentation conditions than to the strain in this case.

### 3.2. Comparison of P. ostreatus 22 Em with the standard strain P. ostreatus ATCC in liquid culture

Cultivation in liquid medium was performed in order to verify if the strain *P. ostreatus* 22 Em would reveal a pattern of laccase isoforms different of that produced in solid state fermentation. Fig. 3 represents the zymogram of laccases produced in liquid culture by the strains *P. ostreatus* 22 Em and *P. ostreatus* ATCC. The new bands revealed during solid state fermentation were not observed, corroborating the conclusion of the previous experiments that the expression of the laccase isoforms 1, 2 and 3 depends on the form of conducting the fermentation – on solid or liquid state, and also on the culture medium composition. Also the isoenzyme POXA1b was not detected in the medium fermented by *P. ostreatus* 22 Em. It is worth noting that *P. ostreatus* 22 Em produced an isoenzyme (isoform 4) very close to the band corresponding to

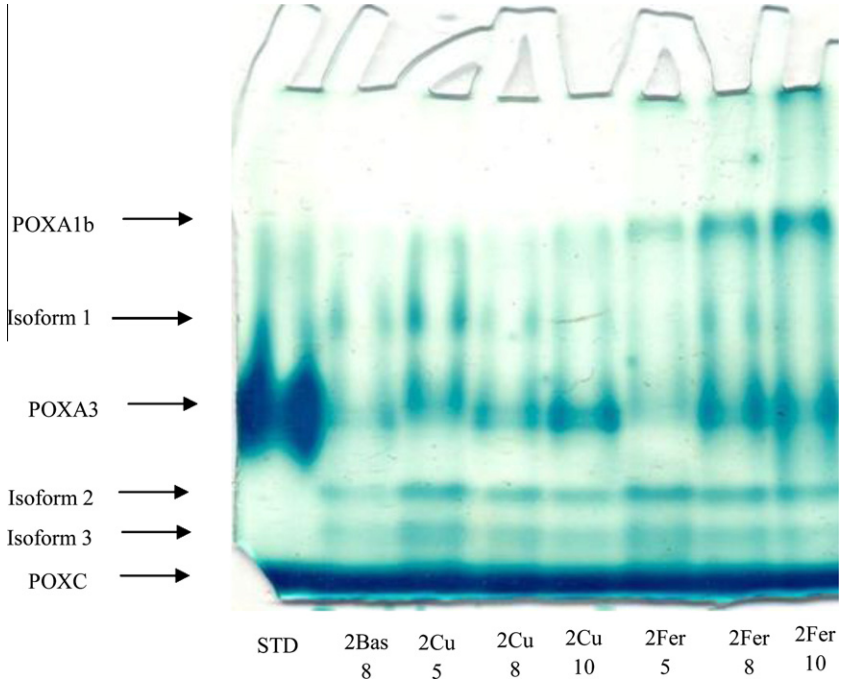


**Fig. 1.** Zymogram of laccases produced under different conditions of solid state fermentation by the strain *P. ostreatus* 22 Em, with 2 g/L yeast extract. STD – standards (POXA1b, POXA3 and POXC secreted by Pl ATCC); Bas – Basal medium (without inducers); Cu – with $Cu^{2+}$ 150 μM; Fer – with $Cu^{2+}$ 150 μM and ferulic acid 2 mM; numbers below represent the time of fermentation (5, 8 and 10 days). All enzymes were loaded with an activity of 0.0075 U.

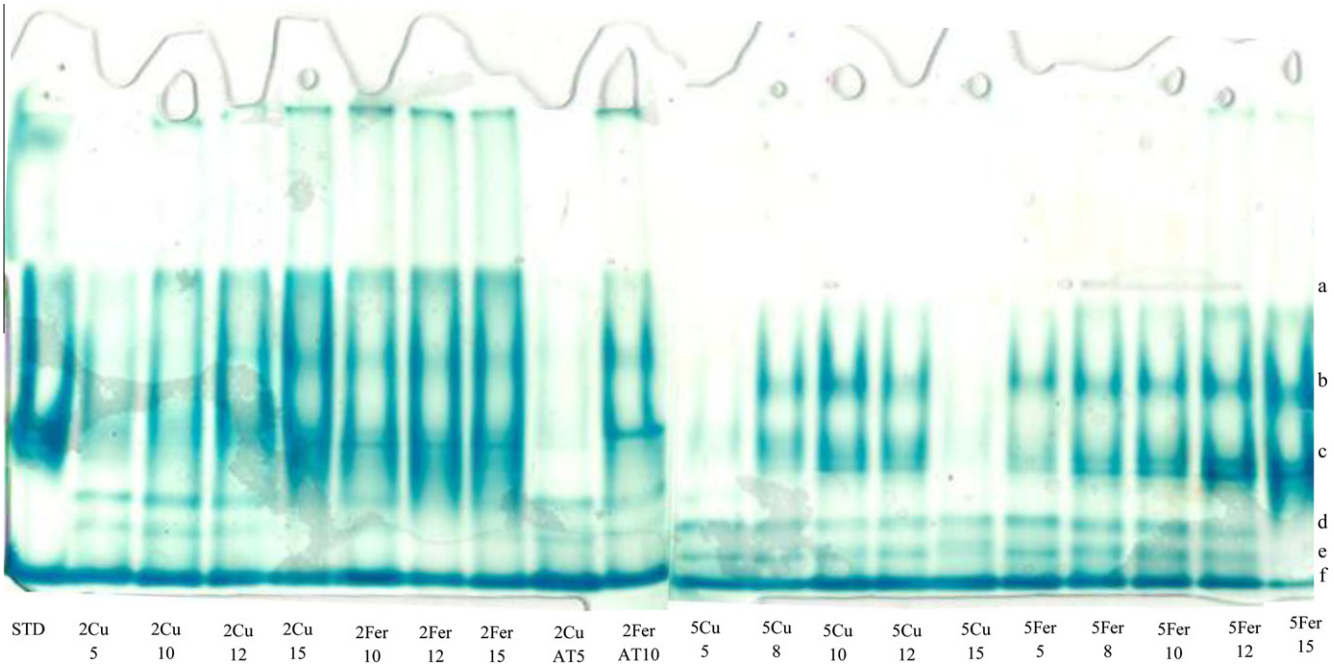


**Fig. 2.** Zymogram of laccases produced under different conditions of solid state fermentation by the strain *P. ostreatus* 22 Em. STD – standards ([a]POXA1b, [c]POXA3 and [f]POXC); [b]Isoform 1; [d]Isoform 2; [e]Isoform 3; 2Cu – with yeast extract 2 g/L and $Cu^{2+}$ 150 μM; 2Fer – with yeast extract 2 g/L, $Cu^{2+}$ 150 μM and ferulic acid 2 mM; 5Cu – with yeast extract 5 g/L and $Cu^{2+}$ 150 μM; 5Fer – with yeast extract 5 g/L, $Cu^{2+}$ 150 μM and ferulic acid 2 mM; AT represents that the strain *P. ostreatus* ATCC was used for comparison; numbers below represent the time of fermentation (5, 8, 10, 12 and 15 days). All enzymes were loaded with an activity of 0.015 U.

the isoenzyme POXC, with similar electrophoretic mobility to one reported by Lettera et al. (2010) in different growth conditions.

### 3.3. Protein identification

A sample containing the six isoforms of laccases (condition: 5 g/L yeast extract, with $CuSO_4$ 150 μM and ferulic acid 2 mM, after 10 days) was selected to get an enzyme preparation to perform identification by mass spectrometry. Proteomic analysis confidently assessed the presence of the isoenzymes POXA3 and POXC. Interestingly a glyoxal oxidase and three galactose oxidases, which are enzymes linked to lignin degradation, were also identified (Table 2). No POXA1b isoenzyme could be identified; its absence can be possibly explained by its sensitivity to extracellular protease degradation, as already reported for *P. ostreatus* (Palmieri et al., 2000).

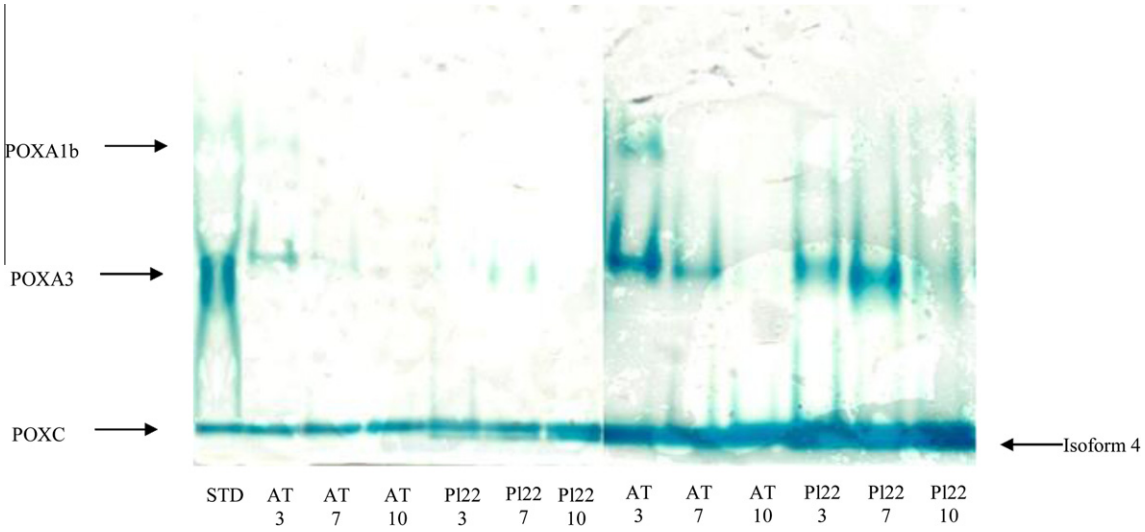


**Fig. 3.** Zymogram of laccases (same gel at two times of staining) produced in liquid culture (PDY medium with $Cu^{2+}$ 150 μM) by the strains *P. ostreatus* 22 Em and *P. ostreatus* ATCC. STD – standards (POXA1b, POXA3 and POXC); AT – strain *P. ostreatus* ATCC; Pl22 – strain *P. ostreatus* 22 Em; numbers below represent the time of fermentation (3, 7 and 10 days). All enzymes were loaded with an activity of 0.015 U.

**Table 2**
Protein identification by searching PleosPC15 genome database with MS/MS ion search Mascot software (Matrix Science), oxidation on Met, cyclization of Gln at N-terminus of the peptides to Pyro-Glu, as variable modifications.

| Protein name (Accession Number) | Number of peptides | Sequence coverage (%) |
|---|---|---|
| Glyoxal oxidase (52532) | 11 | 26 |
| Laccase POXA3 (32778) | 8 | 15 |
| Galactose oxidase (24058) | 5 | 9 |
| Galactose oxidase (28647) | 6 | 14 |
| Galactose oxidase (174951) | 6 | 7 |
| Laccase POXC (36257) | 2 | 2 |

*Note:* Only proteins identified with at least two peptides were considered as significant. Peptides with individual ion scores >20 were considered.

## 4. Conclusions

The highest laccase activity (167 U/g) was achieved at the 5th day of solid state fermentation with yeast extract 5 g/L, $CuSO_4$ 150 μM and ferulic acid 2 mM. Six laccase isoforms (POXA1b, POXA3, POXC and three other isoforms) were observed in the native PAGE zymograms. Ferulic acid induced the expression of POXA3 and POXA1b, and the expression of POXC was not affected by nitrogen concentration and inducers. Higher nitrogen concentration together with ferulic acid induced the expression of isoforms 1, 2 and 3. Mass spectrometry confirmed the presence of POXC and POXA3, and also identified a glyoxal oxidase and three galactose oxidases.

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# Last Advances in Synthesis of Added Value Compounds and Materials by Laccase-mediated Biocatalysis

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**Abstract:** Laccases represent versatile catalysts being able to oxidize a wide range of aromatic substrates and are susceptible of several industrial applications based on both oxidative degradation reactions and synthetic chemistry. The range of laccase based synthetic reactions is extremely wide. Laccases are able to catalyze transformation of antibiotics based on both β-lactams functionalization and phtalides functionalization. These enzymes can also catalyze derivatization of amino acids to obtain metabolically stable amino acid analogues, maximizing biological response while minimizing toxicity, thus representing an useful system for drug development. Biomolecules having antioxidative and anticancer activity can also be produced by laccase-mediated reactions of flavonoids oxidative coupling and phenoxazinones synthesis. Application of laccases to production of new derivatives of the hormones resveratrol, 17ß-estradiol, totarol and isoeugenol and oligomerization products of substituted imidazoles was also reported, with applications for pharmacological purposes due to hormonal activity of the products. The enzymatic preparation of aromatic polymeric materials by the action of laccases represents a viable and non-toxic alternative to the usual formaldehyde-based chemical production of these compounds and it has been reported for several substrates such as 2,6-dimethylphenol, 4-hydroxybenzoic acid derivatives, 3,5-dimethoxy-4-hydroxybenzoic acid and 3,5-dimethyl-4-hydroxybenzoic acid, aniline and acrylamide. Moreover, laccase-mediated biografting of phenols or certain other types of low-molecular weight compounds provides a method for tailoring the surface of lignocellulosics or for adhesion enhancement in binderless wood boards under mild conditions and usually without harmful solvents. Laccase-mediated modification of lignocellulosic materials is accomplished through two main routes: coupling of low-molecular weight compounds onto lignocellulosic materials and laccase mediated cross-linking of lignin molecules *in situ*. Depending on the choice of laccase substrate, properties such as improved strength properties, increased antimicrobial resistance, or hydrophilicity/ hydrophobicity can be imparted to lignocellulosic materials.

**Keywords:** Amino acids, Antibiotics, Antioxidant, Antitumoral drug, Biocatalysts, Green chemistry, Polymerization, Grafting.

## 1. INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductases, EC 1.10. 3.2), representing the largest subgroup of blue multicopper oxidases (MCO) [1], is one of the oldest reported enzymes and it has been arousing great interest in the scientific community as one of the ''greenest'' enzymes of the 21st century [2]. After the first discovery of a laccase in the sap of the Japanese lacquer tree *Rhus vernicifera* [3], several laccases produced by both plants and fungi have been characterized. Laccases and laccase-like proteins have been also described in some kinds of bacteria [4, 5] and in insects [6, 7]. In plants, laccases participate in biosynthesis of lignin in the wood and cellular walls of herbaceous species [8, 9]. Fungal laccases currently account for the most important group of MCOs with respect to number and extent of characterization and they generally are monomeric globular proteins of approximately 60–70 kDa containinig four Cu atoms per molecule [10, 11]. A variety of physiological roles are carried out by fungal laccases, including morphogenesis, fungal plant pathogen/host interaction, stress defense and lignin degradation [12, 13, 14].

Laccases are able to oxidize a wide range of aromatic substrates such as phenols, polyphenols, anilines, aryl diamines, methoxysubstituted phenols, hydroxyindols, benzenethiols, metal compounds and many others. Moreover, small molecules, known as redox mediators, can act as intermediate substrates for laccases, enabling laccase to indirectly oxidize other molecules with high redox potentials and thus further enlarging the repertory of laccase catalyzed oxidative reactions (Fig. **1A**) [15]. This extremely wide substrate specificity makes laccases hugely attractive biocatalysts for a range of industrial applications. These include pulp delignification and biobleaching [16], treatment of textile effluents [17], dye-bleaching in the textile industries [18], enzymatic removal of phenolic compounds in beverages [19], detection of molecules in biosensor devices [20], power production in biofuel cells [21]. Besides these applications based on degradation reactions, laccases find also application in synthetic chemistry, for polymerization of aromatic monomers and production of polymeric materials having application in wood composites, fiber bonding, laminates, coatings, and adhesives, for synthesis of dyes and products of pharmaceutical importance [22- 24]. Laccases have been extensively reviewed from both a general [1, 7, 10, 25] and focused outlook [22- 24, 26-28].

The aim of this review is to provide an overview of the most relevant aspects of synthesis of added value compounds and materials by laccase-mediated biocatalysis, including synthesis and derivatization of biomolecules -such as antibiotics, amino acids and

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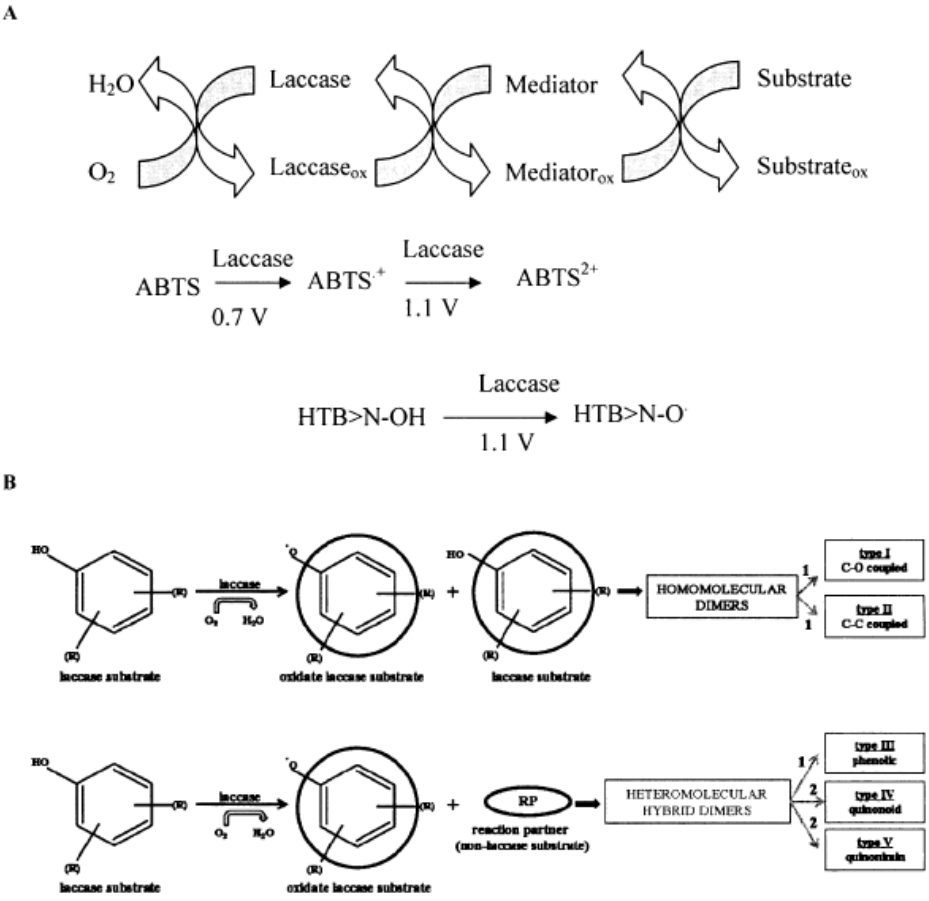



**Fig. (1) A:** Reactions catalyzed by laccase-mediator (ABTS -2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)- or HBT -1-hydroxybenzotriazole) systems and redox potentials of the oxidation reactions of ABTS and HBT by laccase; **B:** Aromatic substrate laccase oxidation and fates of radicals. R= substituent. Reaction type= *1*: oxidative coupling, oxidative condensation or phenolic oxidative coupling; *2*: oxidation coupled with nuclear amination.

other biomolecules having antioxidative, anticancer and hormonal activities-, production of polymeric materials and modification of fibres.

## 2. LACCASE CATALYZED REACTION AND FATES OF RADICALS

In laccases, the four Cu atoms are distributed into three sites, defined according to their spectroscopic properties [14]. Typical metal content of laccases includes one type-1 copper (Cu1), one type-2 (Cu2) and two type-3 copper (Cu3) atoms, with Cu2 and Cu3 atoms arranged in a trinuclear cluster (TNC).

The four Cu atoms are essential for catalytic activity of laccases and they work as a battery accumulating the electrons coming from 4 mono-electron oxidations of 4 reducing aromatic substrate molecules before reducing molecular oxygen to water molecule and restoring the active form of the enzyme [14]. The Cu1 is the primary electron acceptor site, where the four single-electron oxidations of a reducing substrate occur. Reduction of Cu1 is rate-limiting *in the overall* catalytic process [10]. Laccase catalyzed oxidation of phenols, anilines, and benzenethiols correlates with the redox potential difference between laccases Cu1 and the substrate [29]. Laccases are commonly classified as low-medium and high redox potential laccases (HRPLs) according to their redox potential at the Cu1 site, ranging from +430 mV in bacterial and plant laccases to +790 mV in some fungal laccases. The latter ones are by far the most important from a biotechnological point of view [14]. HRPLs are typically secreted by ligninolytic basidiomycetes, the so-called white-rot fungi [2].

The electrons uptaken by laccases through aromatic substrate oxidation are transferred from the Cu1 through the highly conserved His–Cys–His tripeptide to the TNC, where $O_2$ is reduced to water [30]. The reaction of the fully reduced enzyme with $O_2$ proceeds *via* two sequential two-electron steps, generating the peroxy intermediate (PI) and the native intermediate (NI). The first step is rate determining, while the second one, involving the 2 electron reductive cleavage of the O–O bond, is faster. The three Cu2/Cu3 atoms, arranged in a triangular manner, facilitate dioxygen binding, leading to reduction of molecular oxygen using the four electrons transferred from Cu1 [10].

Phenols- typical laccase substrates- are oxidized to phenoxyl free radicals by direct action of laccases. The radical produced by the mono-electron oxidation of the aromatic substrate can then have different fates belonging to three main typologies (Fig. **1B**) [23]: i) homomolecular coupling by intermolecular nucleophilic attack of

radical by itself generating homomolecular dimers, ii) non-phenolic coupling by reaction of radical with a non laccase substrate generating heteromolecular dimers, or iii) rearrangement of chemical structure.

The produced homomolecular dimers can be C–O coupled (type I dimers) or C–C coupled (types II dimers). These reactions are referred to as "oxidative coupling" (type I), "oxidative condensation" and "phenolic oxidative coupling" (type II). After longer reaction times, oligomers and polymers can be generated from the dimers.

Laccases are also able to produce heteromolecular hybrid molecules by coupling an aromatic hydroxylate laccase substrate and a non-laccase substrate (variable reaction partner). The reaction can result in (1) phenolic structures (type III dimers), (2) quinonoid structures (type IV dimers), or (3) quinonimin structures (type V dimers). If the non-laccase substrate contains a free amino group, the quinonoid or the quinonimin structures can be generated by oxidation coupled with nuclear amination. The reaction products types III, IV, and V can be C–O, C–N, or C–S coupled heteromolecular dimers or trimers (Fig. **1B**).

In addition to these processes, reactions involving transformations without coupling have been described. These include phenolic oxidation of the substrate resulting in oxidized laccase substrates [31], oxidative cleavage resulting in lower molecular weight products [32] and oxidative decarboxylation resulting in decarboxylated laccase substrates [33, 34].

Further laccase catalyzed reactions include: quinones production reported for some substrates, such as syringaldazine [35] and 1,2,4,5-tetramethoxybenzene [36]; loss of groups (carboxylic, methoxyl, halogens) (Fig. **2A**) [37,38]; Diels Alder reactions (Fig. **2B**) [39] and Michael addition (Fig. **2C**) [40,41].

## 3. SYNTHESIS AND DERIVATIZATION OF BIOMOLECULES

### 3.1. Antibiotics

Laccases are able to catalyze transformation of antibiotics, attracting great interest due to the need to discover new pharmaceuticals to overcome the increasing microbial resistance to the already used antimicrobial agents. Various examples of novel antibiotics developed by laccase-mediated reactions have been so far reported [34, 42-47].

As far as β-lactams functionalization is concerned, Mikolasch and coworkers [44] reported synthesis of eight novel kinds of peni-

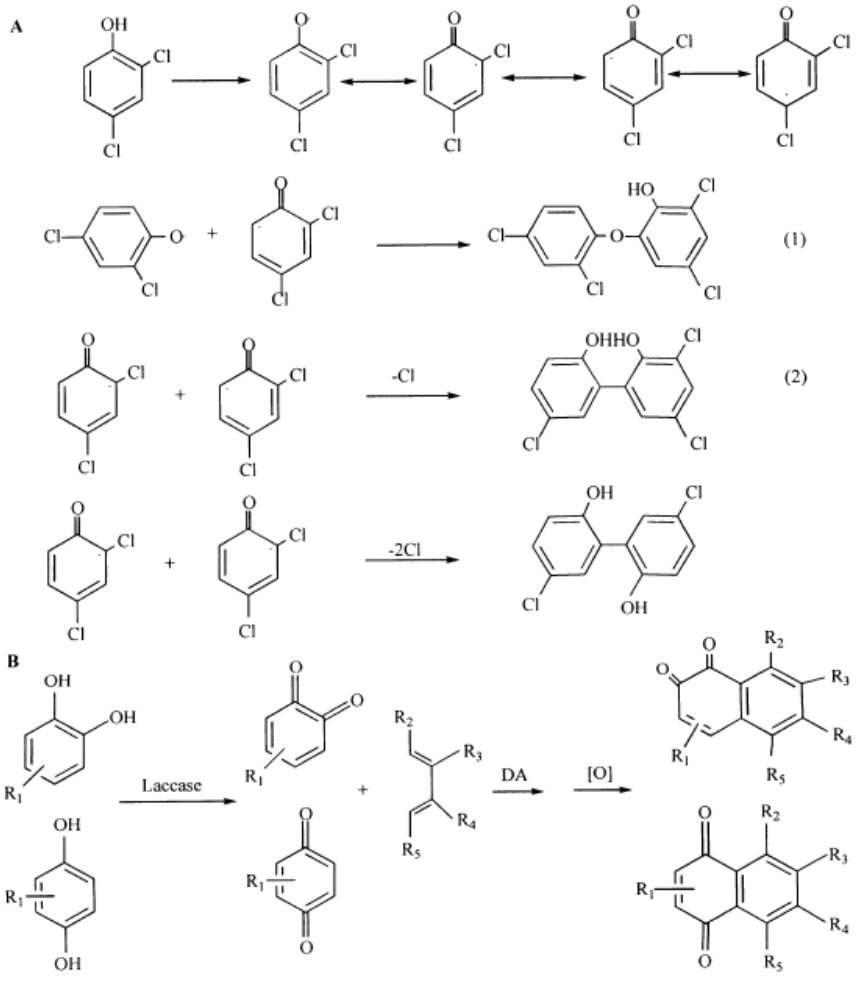


**Fig. (2). Contd....**

C

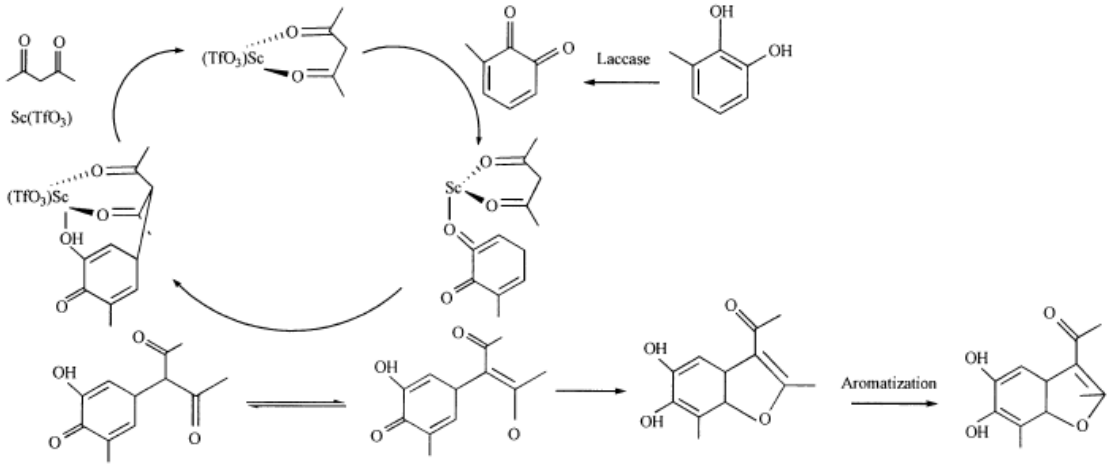
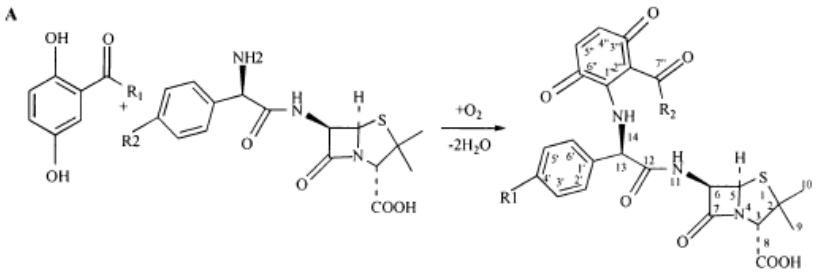



**Fig. (2) A:** Possible coupling reactions between free radicals generated during the laccase mediated transformation of 2,4-dichlorophenol involving the release of one substituent (1) and (2) two substituents in a single coupling [37]; **B:** Laccase initiated cascade synthesis of substitute naphthoquinones via aqueous Diels–Alder reaction (3a) [39]; **C:** Proposed mechanism of laccase/Sc(OTf)3 catalytic system for the synthesis of 3-acetyl-5,6-dihydroxy-2,7-dimethylbenzofuran [40].

A




| | R1 |
|---|---|
| 1a | $NH(CH_2)_2OH$ |
| 1b | $NH_2$ |
| 1c | $OCH_3$ |
| 1d | $OCH_2CH_3$ |

| | R2 |
|---|---|
| 2a | OH |
| 2b | H |

| | R1 | R2 |
|---|---|---|
| 3a | 8" 9" 10" $NH(CH_2)_2OH$ | OH |
| 3b | 8" $NH_2$ | OH |
| 3c | 8" $OCH_3$ | OH |
| 3d | 8" 9" $OCH_2CH_3$ | OH |
| 3e | 8" 9" 10" $NH(CH_2)_2OH$ | H |
| 3f | 8" $NH_2$ | H |
| 3g | 8" $OCH_3$ | H |
| 3h | 8" 9" $OCH_2CH_3$ | H |

B

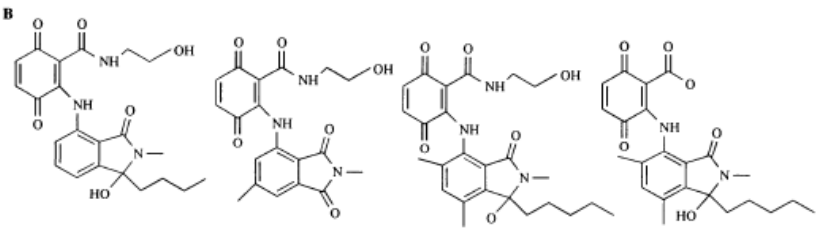


**Fig. (3A).** 2,5-Dihydroxybenzoic Acid Derivatives (1a to d), β -Lactam Antibiotics (2a, b) and the Products 3a to h, obtained by Mikolash *et al.* [44]; **B**: Coupling Products of N-analogous corollosporines with derivatives of 2,5-dihydroxybenzoic acid produced by reaction with Laccase [46].

cillin (Fig. **3A**) by the laccase catalyzed reaction between ampicillin or amoxicillin and four different derivatives of 2,5-dihydroxybenzoic acid. The new penicillins were obtained as products of amination of 2,5-dihydroxybenzoic acid derivatives with a yield of around 98% after 3 h of incubation with *Trametes* sp. laccase. Analogous laccase catalyzed amination of the four 2,5-

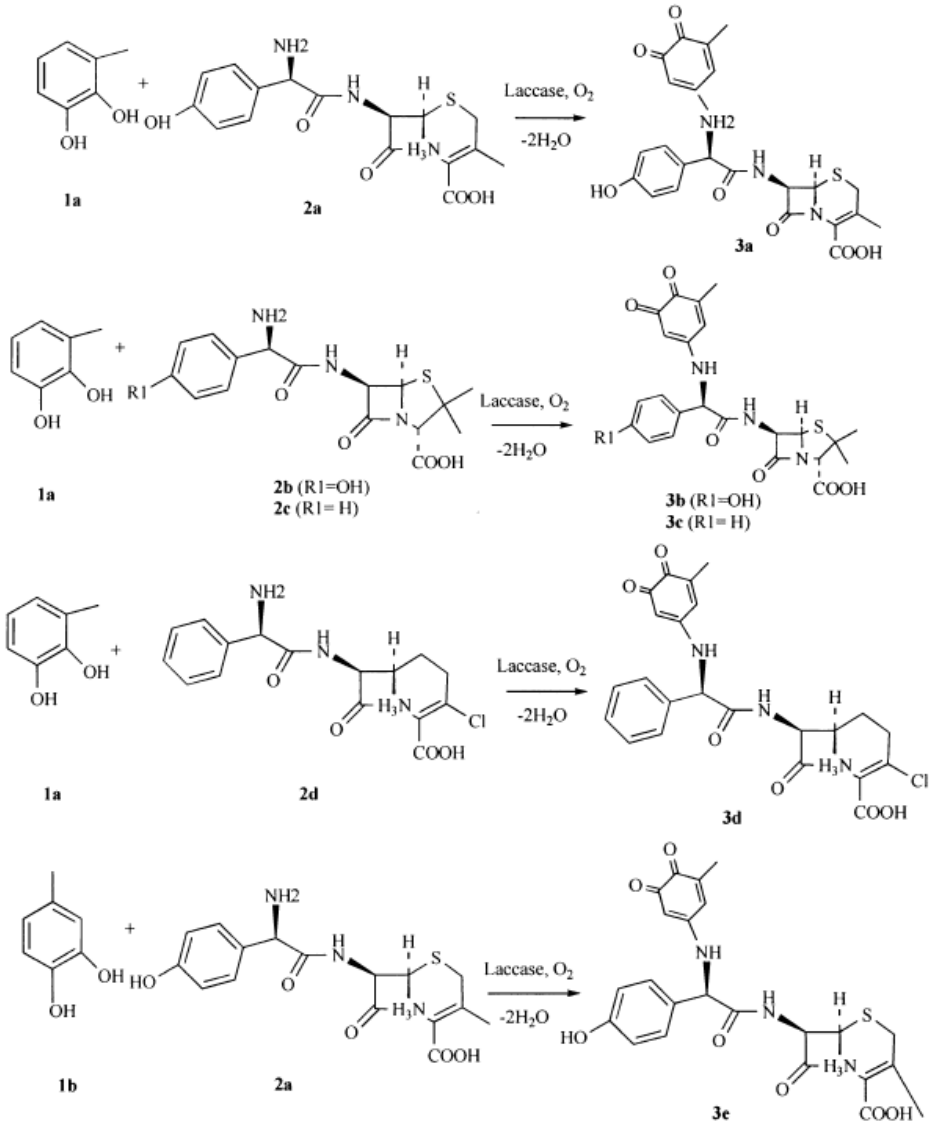
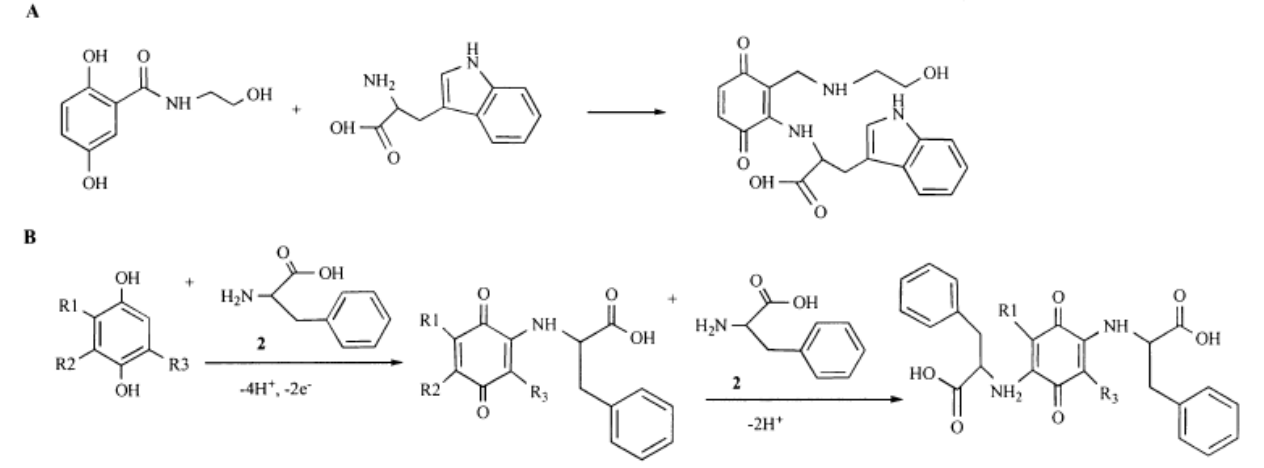



**Fig. (4).** Products (3a to 3e) of amination of catechols (1a, 1b) with b -Lactam Antibiotics (2a to 2d) catalyzed by laccase [47].

dihydroxybenzoic acid derivatives was carried out also using aminocephalosporins cefadroxil, cefalexin, cefaclor, and the structurally related carbacephem loracarbef, thus generating sixteen novel cephalosporins [45]. The comparison between reaction yields obtained by using laccases from *Trametes* sp. and *Myceliophthora thermophila* showed a complete conversion by means of the former one, whilst laccase from *M. thermophila* gave also diaminated products and some not identified byproducts decreasing product yields to 50%. All the new products were able to inhibit growth of several gram positive strains, including multidrug resistant *Staphylococcus* and *Enterococcus* strains, and of the gram negative *Escherichia coli* and to exhibit protective action on mice against lethal effects of an infection with *Staphylococcus aureus* [44,45].

Similar reactions of amination catalyzed by laccase from *Trametes* sp. allowed also phtalides functionalization through the reaction between the antimicrobial substances *N*-analogous corollosporines and dihydroxylated arenes, derivates of 2,5-dihydroxybenzoic acid and 4-methylcatechol. Four out of the thirty-four transformation products (Fig. **3B**) were selected for scaling up and characterization, showing able to inhibit growth of several gram positive *Staphylococcus* strains with higher antimicrobial activity than the parent compounds, in contrast with the above studies where no increase was reported [46]. It is worth noting that all the above mentioned new antibiotics showed negligible cytotoxicity and acute toxicity.

In the context of β-lactams functionalization, Mikolasch and coworkers [47] reported also amination of catechols with four amino-β-lactams, i.e. cefadroxil, amoxicillin, ampicillin and the structurally related carbacephem loracarbef using laccase from *Trametes* sp. (Fig. **4**). Among the tested reagents catechol, 3-methylcatechol and 4-methylcatechol, only 3-methylcatechol gave the desired monoaminated products as unique product after 1.5 h of incubation. On the contrary, longer incubation times with 3-methylcatechol or other catechols produced low yield of monoami-




| 1 | R1 | R2 | R3 | | 3 | R1 | R2 | R3 | | 4 | R1 | R3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| a | H | H | H | | a | H | H | H | | a | H | H |
| b | $CH_3$ | H | H | | b | $CH_3$ | H | H | | b | $CH_3$ | H |
| c | $CH_3$ | $CH_3$ | H | | c | $CH_3$ | $CH_3$ | H | | | | |
| d | $CH_3$ | $CH_3$ | $CH_3$ | | d | $CH_3$ | $CH_3$ | $CH_3$ | | | | |

**Fig. (5). A:** 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide (substrate 1), L-tryptophan (educt 2) and the product 2-[2-(2-hydroxy-ethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1H-indol-3-yl)-propionic acid (3) formed due to a laccase catalyzed reaction [48]; **B:** 1,4-hydroquinone, methyl-1,4-hydroquinone, 2,3-dimethyl-1,4-hydroquinone and trimethyl-1,4-hydroquinone (substrates 1a–d), respectively with L-phenylalanine (educt 2) and the products (3a–d), (4a, b) as results of laccase-catalyzed reaction [54].

nated products, because of formation of undesirable by-products through side reactions. All the monoaminated products coming from reaction of 3-methylcatechol were shown exhibiting antimicrobial activity. However, only one out of these products was shown not cytotoxic whilst all the other exhibited low but not negligible cytotoxicity.

### 3.2. Amino Acids

Interest in application of amino acid derivatives as active ingredients of pharmaceuticals is increasing. Laccases are susceptible of performing the derivatization of amino acids to obtain metabolically stable amino acid analogues, maximizing biological response while minimizing toxicity, thus representing an useful system for drug development.

As a matter of fact, Manda and co. [48] reported the derivatization of L-tryptophan through reaction with the substrate 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide catalyzed by a laccase from *Pycnoporus cinnabarinus* (Fig. **5A**). The laccase-catalyzed C–N-coupling on the amino group of the aliphatic side chain gave the quinoid compound 2-[2-(2-hydroxyethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1*H*-indol-3-yl)-propionic acid as the main product with a yield of 70% after 90 min incubation. The derivative was obtained by nucleophilic attack of the radical cation formed from the *p*-dihydroxylated phenolic compound by laccase. Radicals generated by laccases through one-electron oxidations of substrate can be generally transformed into quinones, which can undergo nucleophilic attack by substances having amino groups, generating C–N-coupled quinonoid products [49]. Such laccase-induced aminobenzoquinones were also described with 4-aminobenzoic acid as amino partner [50]. The study of Manda and co. [48] is an example of enzymatic derivatization of a natural amino acid with a *p*-hydroquinone to form a quinonoid C–N-dimer as product. The aminobenzoquinones are synthesized mostly by chemical methods, showing the main disadvantage of involving harsh conditions [51-53]. On the contrary, application of enzymatic catalysis to the production of aminobenzoquinones provides several advantages such as high stability and specificity of catalyst, the use of mild reaction conditions and environmental friendly reaction route.

Another example of laccases application to derivatization of amino acids was reported by Hahn and coworkers [54], using laccases from *P. cinnabarinus* and *M. thermophila* to catalyze reactions of L-phenylalanine with different *para*-hydroquinones (Fig. **5B**).

Reactions with 1,4-hydroquinone, methyl-1,4-hydroquinone, 2,3-dimethyl-1,4-hydroquinone and trimethyl-1,4-hydroquinone were investigated, demonstrating that derivatization of the amino acid depends on the number and position of substituents in the substrates. The amination of unsubstituted and monomethylated 1,4-hydroquinone leads to two coupling products, heteromolecular dimer and trimer. These products result from nucleophilic attack of the amino acid to quinones formed from the radicals produced by laccase oxidation. This nuclear amination provides a C–N coupled heteromolecular product, consisting of the quinone and the amino acid L-phenylalanine, as described for other aminations [44, 50, 55, 56]. Reaction of hydroquinones with amines can give two aminations dependent on the substitution grade. After coupling of the first amine to the hydroquinone, a further amine molecule can react with the *para*-position to the first amine group, if there is no other substituent. The *ortho*-position for the diamination is not possible, due to the steric bulk of the first amino substituent, whereas the preferential substitution is in *p*-position [57], as likewise for other diaminations [55, 58, 59]. On the other hand, the reaction of 2,3-dimethyl-1,4-hydroquinone or trimethyl-1,4-hydroquinone with L-

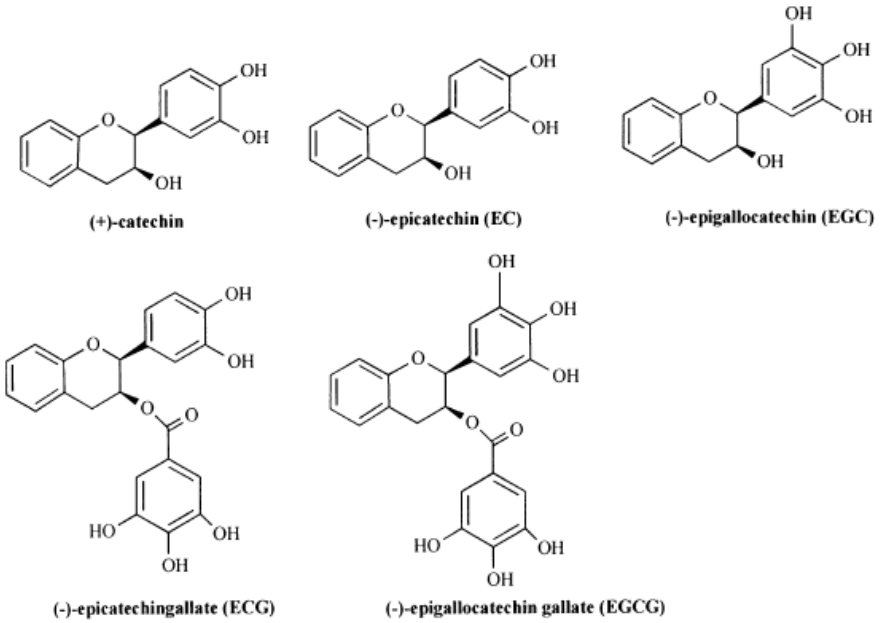



**Fig. (6).** Structures of the flavonoids(+)-catechin, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)- epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG).

phenylalanine provides the heteromolecular dimer as the unique final product resulting from the first nucleophilic addition, since a second amination is hindered due to the methyl groups at C2 and C3 at the aromatic ring of 2,3-dimethyl-1,4-hydroquinone and additionally at C5 of trimethyl-1,4-hydroquinone. These derivatizations of amino acid with quinones can represent the basis for development of new pharmaceuticals, considering that 1,4-quinones are components of important bioactive compounds, such as antibiotics and chemotherapeutics [60-63].

### 3.3. Other Biomolecules

Besides antibiotics and amino acids, a wide a range of biomolecules can be generated by reactions catalyzed by laccases. The range of biological action is quite wide including antioxidant, anti-inflammatory, hormonal, analgesic, sedative to anti-proliferative, anti-neoplastic effects as below described.

## ANTIOXIDATIVE AND ANTICANCER ACTIVITY

### Flavonoids Oxidative Coupling

Flavonoids are a broad class of low molecular weight secondary plant polyphenolics, benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Fig. **6**) [64].

Their biological and pharmacological effects, including antioxidant, anti-mutagenic, anti-carcinogenic, anti-viral and anti-inflammatory properties, have been demonstrated in numerous human, animal and *in vitro* studies [65]. Catechin (Fig. **6**), an ingredient of green tea and wine and member of the flavonoids, is aerobically subjected to oxidative coupling. Hosny and Rosazza [66] showed that the oxidation of (+)-catechin by laccase with hydroquinone as a shuttle oxidant generates catechin-hydroquinone adducts having antioxidant properties. It is worth noting that even if these antioxidants were obtained by a radical process, they retain enhanced antioxidant activity, even better than the antioxidant capacity of catechins. Es-Safi and co. [67] reported the synthesis of catechin derivatives by *Trametes versicolor* laccase, leading to the production of three different flavonoids. It was proven that the first oxidation does not occur on the phenolic functions but on the hydroxy group at C3, resulting in the major formation of C2 oxidized products. The new derivatives were shown to possess an improved antioxidant activity, with a relatively higher free radical scavenging activity than that (+)-catechin, indicating that the introduction of a substituent yielded compounds with improved antiradical efficacy in solution. In the polymerization of catechin using laccase derived from *Myceliophthora* sp. as a catalyst, the synthesized poly(catechin) showed excellent antioxidant properties, exhibiting greatly improved radical scavenging activity, protective effects against low-density lipoprotein (LDL) oxidation, and inhibition of xanthine oxidase activity, compared with the catechin monomer [68, 69].

Epigallocatechin gallate (EGCG) (Fig. **6**) is a major ingredient of green tea possessing powerful antioxidant and cancer-chemopreventive properties due to the actions of radical scavenging, enzyme inhibition, and metal chelation. The polymer obtained by laccase-catalyzed oxidative coupling of EGCG showed much higher superoxide anion scavenging activity than the EGCG monomer and enzymatically synthesized poly(catechin) [70].

Rutin (Fig. **7A**) is one of the most commonly found flavonol glycosides identified as vitamin P with quercetin and hesperidin. When the oxidative polymerization of rutin using a *Myceliophthora* sp. laccase as a catalyst was examined to produce a flavonoid polymer, it was demonstrated that antioxidant activity of rutin is greatly amplified by laccase-catalyzed oxidative coupling [71]. Antioxidant properties of the synthetic polymer were shown greatly improved compared with the rutin monomer: it was a more potent protector from LDL oxidation and from oxidative injury induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) than the monomer.

Laccase activity in plants results in the formation of brown pigments, referred to as tannins (Fig. **7B**).

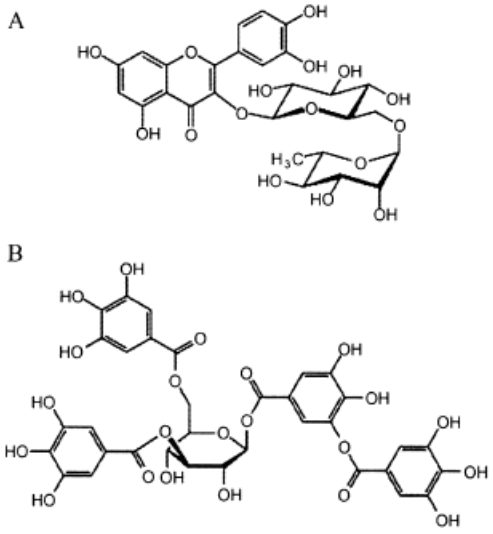


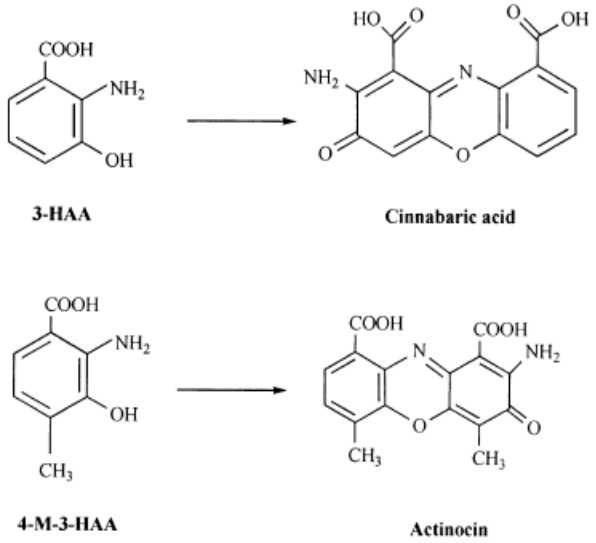
**Fig. (7). A:** Structure of rutin; **B:** Structure of tannic acid, as a typical tannin.

Laccase-dependent pigment production is also reported in numerous fungi and bacteria. The laccase of the yeast *Cryptococcus neoformans* forms melanin-like pigmentation outside the cell wall in the presence of exogenous substrates, contributing to its virulence in humans. Fowler *et al.* [72] showed that *C. neoformans* and *Bacillus subtilis* are able to create melanin-like pigments from a variety of flavonoid molecules. Since flavonoids are ubiquitous plant molecules and often-considered antimicrobial agents, they postulated that they are the natural targets of laccase activity and result in the formation of a defensive melanin-like coat. Their results suggest a new mechanism by which flavonoid-melanin formation may occur, using not only A- and C-ring linkages, but also monomer links through the B-ring of the flavonoid structure. They also showed that resveratrol and other non- and mono-hydroxylated polyphenol substrates have the ability to restrict pigment formation and may be potent inhibitors of laccase activity.

## Phenoxazinones Synthesis

Synthesis of the phenoxazinone derivative, cinnabarinic acid (CA) forming a part of mechanism to protect mammalian tissue from oxidative damage, was achieved by a laccase mediated transformation consisting of oxidative condensation of two molecules of 3-hydroxyanthranilic acid (3-HAA) (Fig. **8A**) [73]. During cultivation of the basidiomycete fungus *P. cinnabarinus*, the presence of 3-HAA and a red pigment identified as the phenoxazinone derivative CA was detected. Carrying out *in vitro* oxidation of 3-HAA by purified *P. cinnabarinus* laccase, this enzyme was responsible for the conversion of 3-HAA to CA. A common feature of the three members of the genus *Pycnoporus* (Kars.), *P cinnabarinus*, *P. sanguineus* and *P. coccineus*, is the production of three main typologies of pigments containing the phenoxazinone ring structure, -CA, cinnabarin and tramesanguin-, differing in the oxidation state of their substituents. These differences could result from oxidative coupling of different o-aminophenol precursors, having either the alcohol, aldehyde or carboxyl group as substituents, whose production could be strain specific or depending on the culture conditions.

Osiadacz and coworkers [74] investigated the ability of the immobilized laccase from the fungus *T. versicolor* to form derivatives based on the structure of phenoxazinone chromophore, *i.e.* cinnabarinic acid and actinocin (2-amino-4,6-dimethyl-3-phenoxazinone-1,9-carboxylic acid, Fig. **8**), using two different substrates: 3-HAA and its 4-methylderivative (4-M-3-HAA). A product exhibiting physicochemical features identical to those of synthetic cinnabarinic acid was obtained by oxidation of 3-HAA [74], prompting the authors to use 4-M-3-HAA for actinocin formation (Fig. **8B**). Ability of the immobilized laccase to synthesize actinocin, a phenoxazinone derivative closely related to cinnabarinic acid, by oxidation of 4-methyl-3-hydroxyanthranilic acid up to a yield of 74%, was thus demonstrated. Laccase-mediated conversion of 4-M-3-HAA yielded a product with physicochemical features which were shown identical to those found in synthetic actinocin.



**3-HAA** **Cinnabaric acid**

**4-M-3-HAA** **Actinocin**

**Fig. (8).** Laccase mediated synthesis of cinnabaric acid (A) [73] and actinocin (B) [74].

Actinocin represents the chromophore moiety of the actinomycin antibiotics group [75], and is able to block transcription of tumor cell DNA, thus contributing to fight against cancer [74, 76]. The laccase catalyzed formation of actinocin by conversion of aminophenol derivatives represents a valuable alternative to the chemical way for the construction of novel, actinomycin-like cytotoxic derivatives comprising a phenoxazinone chromophore, *i.e.* DNA topoisomerase inhibitors and/or DNA sequence reading drugs. If the substrates contain both an aminophenolic moiety and a non-aromatic part susceptible to chemical oxidation, laccases can be used as regioselective reagents. The method described by Osiadacz and coworkers [74] is quicker and more efficient than the synthetic one (*i.e.* oxidation with air oxygen in 1% $(NH_4)_2CO_3$). The proposed procedure could be hopefully applied to the chemoenzymatic synthesis of actinocin derivatives.

With the aim of developing enzymatic synthesis of a new class of bioactive water-soluble chromophores, Bruyneel and coworkers [77] investigated the regioselective sulfonation of 2-hydroxyaniline, demonstrating that insertion of a sulfonate group into a carbon–metal bond provides an efficient and selective process for the synthesis of 3-hydroxyorthanilic acid (Fig. **9**).

When this sulfonated compound is subjected to laccase oxidation, the major product of biotransformation is 2-amino-3-oxo-3*H*-phenoxazin-1,9-disulfonic acid, identified as the sulfonate analogue of cinnabarinic acid. Phenoxazinone chromophores attract great interest for the development of fluorescent probes for the detection

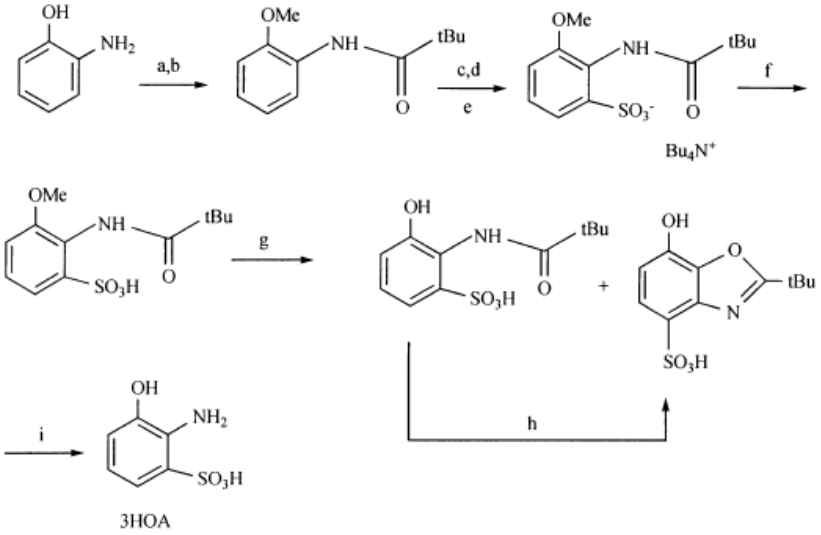



**Fig. (9).** Optimised synthesis of 3-hydroxyorthanilic acid reported by Bruyneel *et al.* [77].

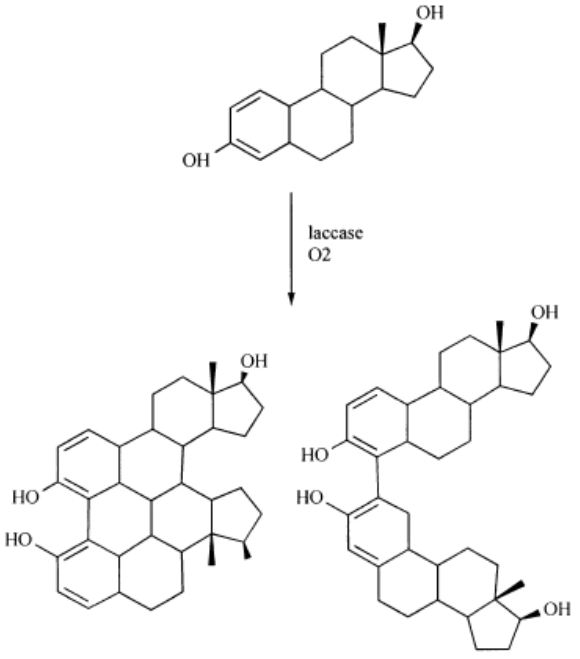



**Fig. (10).** Laccase-catalyzed dimerization of 17b-estradiol [84].

of hydroxyl radicals which are largely responsible for DNA damage by endogenous effects but also by several indirect effects. The poor solubility of the available phenoxazinone derivatives represents a drawback for their application. A route to make these molecules soluble in aqueous media is to add a highly polar substituent such as a sulfonate group. The usual chemical synthesis of phenoxazine derivatives involves the condensation of nitroso compounds at elevated temperatures through highly toxic and energy-consuming processes. The biomimetic synthesis of the sulfonated phenoxazinone derivative based on the use of laccase developed by Bruyneel and coworkers [77] represents a more environmental friendly alternative to the usual chemical synthesis.

The study of new synthetic routes to aminoquinones is of great interest because some antineoplast drugs in use, like mitomycin, or under development, like nakijiquinone-derivatives [78] or herbamycin-derivatives [79], contain an aminoquinone moiety. Several simple aminoquinones possess activity against a number of cancer cell-lines [80, 81] as well as antiallergic or 5-lipoxygenase inhibiting activity [82, 83].

### Hormonal Activity

Two different research groups [84,85] have exploited some years ago the laccase capabilities to produce new dimeric derivatives of the hormone 17ß-estradiol (Fig. **10**), suggesting the participation of the enzyme in both the formation and the coupling of the radical intermediates. A significant and unexpected solvent influence on the coupling of phenols catalyzed by laccases was demonstrated for the first time, thus confirming the general validity of the "medium engineering" approach to influence enzymatic performance [84].

Nicotra and co. [86] reported the application of laccases to the biotransformation of resveratrol (Fig. **11**) and synthesis of trans-dehydrodimers catalyzed by laccases from *M. thermophyla* and from *Trametes pubescens*.

Production of novel dimeric derivatives was also achieved by laccase oxidation of totarol/isoeugenol (Fig. **11**) [87]. Ncanana and coworkers [87] used laccases from *T. pubescens*, achieving a good yield of C-C and C-O linked dimeric by laccase-catalyzed oxidation of totarol (Fig. **12**), under very mild reaction conditions. Reaction conditions were optimized by investigating the effects of buffer composition, pH and salt concentration. It was shown that salt concentration does not affect the bioconversion, differently from the pH and the temperature of the reaction, 4.5 and 30°C being the better value, respectively.

Shiba and coworkers [88] used laccases from *R. vernicifera* and *P. coccineus* to catalyze the oxidation of isoeugenol and coniferyl alcohol. Kinetics of the oxidation reactions was studied and the potential of applying these reactions to synthesis of 2-(4-hydroxy-

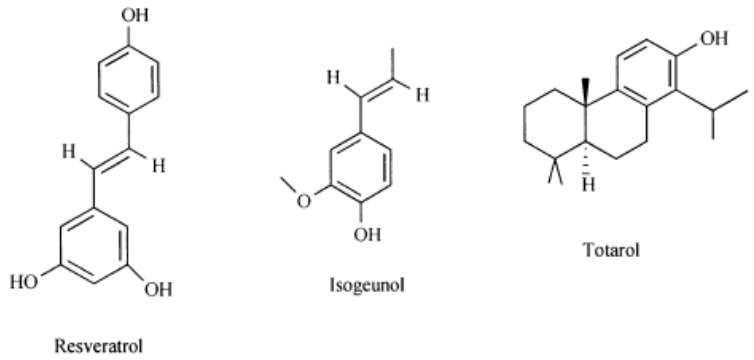



**Fig. (11).** Strutures of resveratrol, totarol and isogeunol.

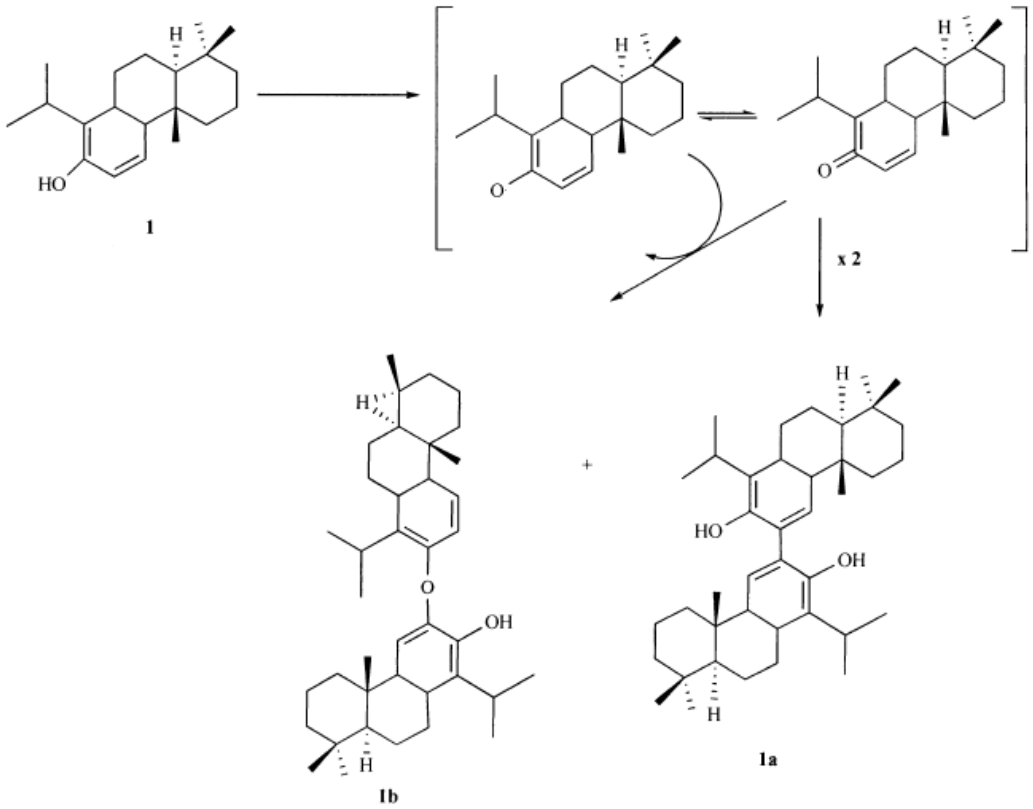



**Fig. (12).** Synthesis and structures of the dimeric products 1a and 1b obtained by the laccase-catalyzed oxidation of totarol (1) [87].

phenyl) coumaran derivatives from the corresponding 1-(4-hydroxyphenyl)–1-propene compounds was explored.

Moreover, an even more complex mixture of products was obtained by oxidation of substituted imidazoles [89]. These novel substituted imidazoles or oligomerization products are applicable for pharmacological purposes due to their hormonal activity. In another study, derivatization of the natural compound 3-(3,4-dihydroxyphenyl)-propionic acid can be achieved by laccase-catalyzed *N*-coupling of aromatic and aliphatic amines. The derivatives of this antiviral compound may have interesting pharmaceutical uses. More recently, nuclear amination of *p*-hydroquinones with primary aromatic amines catalyzed by laccases in the presence of $O_2$ resulting in the formation of the corresponding monoaminated or diaminated quinines was reported [48, 55].

## 4. SYNTHESIS AND DERIVATIZATION OF POLYMERIC MATERIALS

### 4.1. Polymerization

In the production of polymers, enzymes provide an environmental friendly alternative, due to their mild reaction conditions and highly specific non-destructive transformations targeted to surfaces [90]. Furthermore, the high specificity of enzymes can be exploited in the synthesis of polymers otherwise impossible to be produced through conventional synthesis, such as optically active polymers with potential as catalysts in asymmetric syntheses, and as chiral absorbents for the separation of racemic mixtures. Enzymatic polymerization through laccase catalyzed reactions has been recently reviewed [22-24].

## Polyphenolic Polymers

The enzymatic preparation of polymeric polyphenols by the action of laccases represents a viable and non-toxic alternative to the usual formaldehyde-based chemical production of these compounds [91]. Advantages for enzymatic synthesis of polyphenols are summarized as follows : (i) the polymerization of phenols proceeds under mild reaction conditions without use of toxic reagents; (ii) phenol monomers having various substituents are polymerized to give a new class of functional polyaromatics; (iii) the structure and solubility of the polymer can be controlled by changing the reaction conditions; (iv) the procedures of the polymerization as well as the polymer isolation are very convenient.

Although polymerization reactions of phenolic compounds by oxidases are useful for polymerization, detoxification, and decrease of bioavailability of environmental pollutants, their application in organic and polymer chemistry remains a relative new field in fungal and white biotechnology [23].

Poly(2,6-dimethyl-1,4-oxyphenylene)-"poly(phenylene oxide)", PPO-, is widely used as high-performance engineering plastic, since the polymer has excellent chemical and physical properties, and mechanically tough property. PPO was first prepared from 2,6-dimethylphenol monomer using a copper/amine catalyst system. The polymerization was also performed in the presence of *P. coccineus* laccase (Fig. **13**) [92-93]. Laccase catalysis induced a new type of oxidative polymerization of 4-hydroxybenzoic acid derivatives, 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid) and 3,5-dimethyl-4-hydroxybenzoic acid, involving elimination of carbon dioxide and hydrogen from the monomer to give PPO derivatives with molecular weight up to $1.8 \times 10^4$.

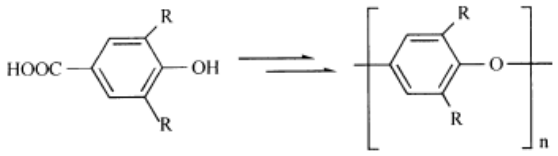



**Fig. (13).** Synthesis of poly(2,6-dimethyl-1,4-oxyphenylene)-"poly(phenylene oxide).

Aktas and coworkers [94, 95] described an enzymatic oxidative process to polymerize catechol with a *T. versicolor* laccase. In the laccase-catalyzed polymerization of catechol, an insoluble precipitate, easily removable from wastewater streams, is formed, with the concomitant production of poly(catechol), an useful polymer whose structure contains functional groups that can be reversibly reduced or oxidized (Fig. **14**), namely a redox polymer [96].

1-naphtol polymerization catalyzed by the same laccase was also reported, along with the production of the inert phenolic polymer poly(1-naphtol) [97]. Since one of the more challenging tasks in the enzymatic industrial processes for the most economical use is their experimental design and optimization, a response surface methodology (RSM) was successfully applied to enzymatic biotransformation of 1-naphthol [98]. The oxidative polymerization of pyrogallol to poly(pyrogallol) has also been reported [99]. Fukuda and coworkers [100] described the transformation of bisphenol A (BPA), an endocrine disrupting chemical, by a laccase from the basidiomycete *Trametes villosa* in high-molecular-weight (MW) and low MW compounds, identifying one of the low MW compounds as 4-isopropenylphenol. The isolation and identification of the high MW reaction products was reported [101], one of which identified as a BPA dimer, 5,59-bis- [1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,29-diol. The authors suggested that the laccase reaction includes, as one of its steps, the polymerization of BPA to form oligomers (removed by filtration), followed by either the addition of phenol moieties or the degradation of the oligomer to release 4-isopropenylphenol. Further structural and quantitative analysis of high MW compounds was reported by Fukuda and coworkers [100], along with demonstration of removal of BPA's estrogenic activity by the laccase.

Urushi, a natural resinous sap produced by the *R. vernicifera* tree, can be regarded as the only example of a practical natural paint that utilizes *in vitro* enzymatic catalysis for hardening [102]. It is an insoluble polymeric material formed by the cross-linking of urushiol monomer, whose structure is a catechol derivative, with unsaturated hydrocarbon chain consisting of monoenes, dienes, and trienes at the 3-, or 4-position of catechol. Cross-linking of the urushiol is supposed to be accomplished mainly by a laccase-catalyzed oxidative coupling of the phenol moiety of the urushiol and a subsequent autoxidation of unsaturated alkyl chains in air (Fig. **15**).

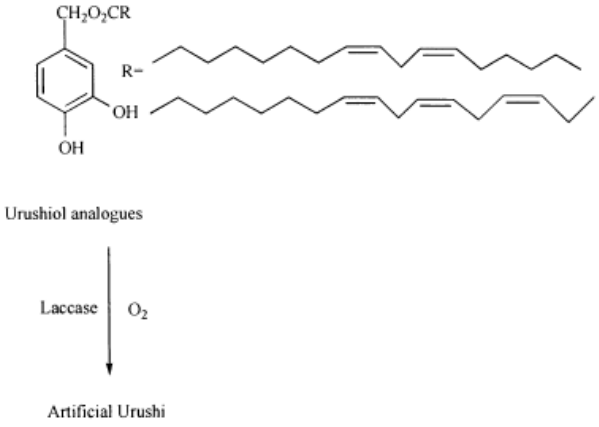



**Fig. (15).** Urushiol transformation.

"Artificial urushi" may be defined as the cross-linked film prepared by *in vitro* laccase catalyzed oxidation of urushiol monomers. Kobayashi and coworkers [103] reported the enzymatic synthesis of artificial urushi by a *P. coccineus* laccase after designing new urushiol analogues, in which the unsaturated group is connected with the phenolic group through an ester linkage. The artificial urushi prepared by laccase under mild conditions and without the use of organic solvents yielded cross-linked film with a high hardness and gloss surface, comparable to that of natural urushi coating [103, 104].

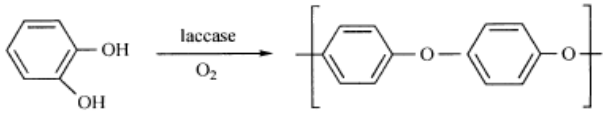



**Fig. (14).** Poly(catechol) synthesis.

**Polyaniline and Polaycrylamides**

Polyaniline is one of the most important conducting polymers, which may be used as active component of organic lightweight batteries, microelectronics, optical display, for anticorrosive protection, in bioanalysis, etc. [105], due to its good electrical and optical properties, as well as high environmental stability. Polyaniline is commonly synthesized by oxidizing aniline monomer under strongly acidic conditions using ammonium persulfate as an initiator of oxidative polymerization [106]. Besides the environmental concerns, a further drawback to be overcome in polyaniline synthesis is its poor solubility in common solvents. An alternative approach for the synthesis of water-soluble conducting polyaniline has been recently developed by means of horseradish peroxidase [107]. Moreover, Karamyshev and coworkers [108] reported the synthesis of water-soluble conducting polyaniline catalyzed by a *Coriolus hirsutus* laccase in the presence of sulfonated polystyrene (SPS) as a template. Remarkable advantages compared to the commonly used horseradish peroxidase were obtained.

It has also been reported that laccase induces radical polymerization of acrylamide with or without mediator [109]. Enzymatic polymerization of acrylamide was carried out in water using laccase from *Pycnoporus coccineus* at relatively low temperature (50 °C) whilst in the presence of 2,4-pentanedione, the vinyl polymerization catalyzed by laccase was efficiently achieved at room temperature.

Since many phenols and their derivatives are considered hazardous pollutants, emphasis has been put on the use of laccases for wastewater treatment and removal of aromatic compounds *via* polymerization. This strategy is able to reduce both treatment costs and energy use, and furthermore may be beneficial also for producing useful side products, i.e. valuable redox polymers [97].

### 4.2. Laccase-mediated Coupling and Grafting Reactions

Grafting usually refers to coupling of small molecules to natural or synthetic polymers, traditionally carried out with the aid of a metal catalyst at high temperatures. There is now increasing interest in the search for enzymes as an alternative to metal catalysts for mediation of coupling reactions. The enzymatic modification of synthetic materials has immense potential in both the functionalization of bulk materials, such as polyacrylonitrile, polyamide or polyester, and the production of polymers for special applications (e.g. for the production of medical devices and electronics) [90].

The ability of laccases to generate reactive species (phenoxy radicals), thereby enhancing the reactivity of molecules/polymers, forms the basis of their application in polymer chemistry [110]. The generated reactive species provide ideal sites for cross-linking (coupling) of desired functional molecules leading to grafting and consequent formation of new materials or materials with novel properties. The nature of grafting products depends on the position of the radical, as the oxidized molecule tries to stabilize itself through resonance. Laccase-mediated biografting of phenols or certain other types of low-molecular weight compounds provides a method for tailoring the surface of lignocellulosics or for adhesion enhancement in binderless wood boards under mild conditions and usually without harmful solvents [16]. The presence of mediators has further expanded the applications of these enzymes in modifying "inert" polymers. Laccase-mediated modification of lignocellulosic materials is accomplished through two main routes: coupling of low-molecular weight compounds onto lignocellulosic materials and laccase mediated cross-linking of lignin molecules *in situ*. Depending on the choice of laccase substrate, properties such as improved strength properties, increased antimicrobial resistance, or hydrophilicity/ hydrophobicity can be imparted to lignocellulosic materials.

Detailed lists of enzymatic polymer modification through laccase catalysed reactions can be found in recent reviews [28, 110].

**Modification of Proteins**

The potential of making value-added products in food industry through laccase-assisted coupling of proteins and carbohydrates has been also demonstrated [111]. The laccase-catalyzed oxidation can proceed in proteins and carbohydrates *via* phenolic moieties present in the polymers or through certain amino acid residues of proteins. A *T. hirsuta* laccase was proved to increase the dough strength and improve the bread-making quality of white wheat flour breads, increasing bread softness and volume. Laccase-assisted cross-linking of low molecular weight phenolic model compounds of flour may be responsible for the observed improvement in laccase-treated flour.

Kim and co. [112] reported the synthesis of protein–flavonoid conjugates and following anchoring of conjugates onto cationised fibers by laccase (*M. thermophila*, Novozym® 51003). By the anchoring of α-casein–catechin conjugates onto flax fibres, the surface became hydrophilic, with a lower contact angle, and the final products obtained showed a generation of new colour with acceptable durability in terms of washing resistance.

Schröder and co. [113] showed that laccase-assisted modification of flax fibers with ferulic acid or hydroquinone monomers improved their antibacterial activity toward *B. subtilis* and *E. coli*. Further, it was reported that antibacterial properties could be imparted also to wool fibres by a laccase (*Trametes sp.*)-mediated grafting of lauryl gallate [114]. The one-step process enzymatic coating of wool with lauryl gallate also provided antioxidant and water repellent properties. The same group [115] also showed the grafting of wool with the bi-functional phenolic compound nordihydroguaiaretic acid (NDGA), through a laccase catalyzed reaction in an aqueous–ethanol mixture. The enzymatic grafting of the bifunctional phenolic compound on the fibers, combined to possible formation of inter/intramolecular bridges and cross-links in wool proteins, provided a significant improvement of the physical and mechanical properties of the fabrics. NDGA grafting on wool provided antioxidant activity and it was efficient in protecting wool against the undesirable photoyellowing caused by UV-radiation. Shrink resistance, crease recovery angle and tensile strength of the modified samples also increased compared to the untreated ones.

Recently, the *M. thermophila* laccase was exploited to assist the binding of chitosan and catechin onto a previous enzymatically oxidized linen surface [116], demonstrating that this enzyme is able to oxidize phenols naturally existing in flax fibres, and that the *o*-quinones formed promote the attachment of chitosan or/and catechin. Antibacterial activity of linen samples grafted with both chitosan and catechin was tested against *S. aureus* and *E. coli*. The latter showed susceptibility only to chitosan treated samples, whereas *S. aureus* was equally sensitive to chitosan and catechin actions.

Shin and co. [117] showed that laccase is also able to colour wool fabric previously padded with hydroquinone. Using dihydroxynaphthalenes as substrates, leather can be coloured by laccase action [118]. The coloured products are lightfast and bound to the natural collagen by a covalent tanning manner. Additionally, laccase has been used for polymerization processes to form melanin like coloured pigments [119]. Polyphenols are the basic building block of pigments coloring leather skin and hair and can be enzy-

matically polymerized to create versions of natural black, brown, and orange melanins.

**Modification of Lignin and Wood**

The potential of laccase-assisted biografting of phenolic acids for improving strength properties of kraft paper made from high kappa pulps has been discussed in several papers by Chandra and coworkers [120-124]. The main focus of their studies has been the grafting of phenolic acids, including 4-hydroxyphenylacetic acid (PAA) [120], 4-hydroxybenzoic acid (4-HBA) [121], and gallic acid [123] to high-lignin content softwood kraft fibers mediated by a *T. villosa* laccase. The results indicate that the strength increase was a combined effect of improvements of hydrogen bonding between fibers and the creation of phenoxy radical cross-links within the sheet [124]. Recently, Witayakran and Ragauskas [125] investigated the possibility of grafting various amino acids onto high-lignin content pulps. The treatment of pulp with a *T. villosa* laccase and histidine provided the best result in increasing carboxyl group content and paper properties. The ability to use laccase to selectively graft amino acids to lignin-rich pulp fibers provides a new and unique fiber modification technology which will have many future opportunities. The ability of laccase and laccase mediator systems in functionalizing pulp fibers was evaluated [126]. In most cases, treatment of flax and sisal pulp with laccases (from *P. cinnabarinus* and *T. villosa*) with or without a mediator in the presence of different phenolic compounds improved strength-related properties (particularly wet tensile strength) in the resulting paper [127-129].

Bioactive phenolic compounds were grafted onto the surface of unbleached kraft liner fibers using a laccase from *T. pubescens* with the aim of obtaining predictable antimicrobial active fiber surfaces against a wide variety of microbes [130]. The obtained results showed that the handsheet paper obtained by laccase antibacterial surface process (LASP) showed a greater efficacy against Gram positive and Gram negative bacteria, if compared with handsheet paper treated only with monomeric phenol derivatives. Antimicrobial activity was proved to be function of grafted structure, time of the treatment and concentration of phenol derivatives. Several phenol compounds were tested, such as acids, essential oils components and dopamine. LASP in the presence of caffeic acid or p-hydroxybenzoic acid produced paper handsheets with strong bactericidal effect on the Gram-positive *S. aureus* even at low phenol monomer concentration (4 mM), whereas a higher concentration of the monomer in the reaction mixture was required to kill the Gram-negative *E. coli*. Among the tested essential oils compounds, isoeugenol was the most effective one: isoeugenol/LASP, besides killing *S. aureus*, showed a bacteriostatic effect on the more resistant spore forming Gram-positive *B. subtilis*. Widsten and coworkers [131] provided mechanistic evidence of the binding of catechin (as an antimicrobial) well as of gallic acid (as a model of hydrolysable tannins) onto putative lignin monomers. Catechin was bond to the phenolic molecules mainly through 5-5 linkages while binding to monomers with sinapyl units was mainly through 4-*O*-5 linkages. The treatments in the presence of the commercial *M. thermophila* laccase significantly improved the antibacterial resistance of veneers and paper made from tannin-treated pulp against *S. aureus*, while a more modest protective effect was observed against *E. coli*. Coupling reactions have also been performed on laminate precursors with the aim of boosting the disinfecting properties of phenol resin used for the impregnated kraft paper used in certain environments such as hospitals and kitchens, where high hygienic requirements exist [132]. Bridging molecules such as 4-hydroxy-3-methoxybenzylurea compatible with melamine resin have been attached to fiber lignin by means of laccase catalysis.

In an effort to use green chemistry technology to increase wood surface hydrophobicity, laccase mediated grafting of pulps has been successfully used for this purpose [114]. Recently, a research group evaluated the ability of a *T. hirsuta* laccase to catalyze the coupling of fluorophenols and alkylamines in order to increase the hydrophobicity of wood veneers [133-135]. It was provided for the first time the evidence for the possibility of establishing covalent bonds *in vitro*, between complex lignin models and different alkylamines as well as fluorophenols. The proposed mechanism showed that the fluorophenols were bound to sinapyl units *via* 4-*O*-5 linkages while coupling to guaicyl units occurred through 5-5 linkages. The alkylamines were bound onto simple phenolics and lignin models through the -$NH_2$ thereby establishing –C-N bonds. The advantage of laccase-mediated covalent binding of molecules onto wood surface is that the grafted molecules are not readily displaced and released into the environment.

Coupling of *in situ* laccase-created radicals in wood chips have been investigated as a way of producing binderless (artificial adhesive-free) wood such as medium density fiberboards (MDFs) and particle boards (Pbs), commonly used for interior building and furnishing [16]. Traditionally, urea–formaldehyde, melamine–urea–formaldehyde and phenol–formaldehyde are used as adhesives in the manufacture of these boards while an additional binder is required for high-density fiberboards (HDFs). Both economical and health benefits could obviously be obtained from binderless production processes. In the for novel adhesion solutions and strength additives for lignocellulosic composite and paper products, a common theme is the attempt to increase the bonding between the fibers or other constituent particles by their chemical modification. An overwhelming majority of the studies in this area are based on oxidative modification of lignin in the particles themselves and/or using added cross-linking material such as technical lignins derived from the paper industry. The activation of lignin for bonding can be carried out by oxidation with phenol-oxidizing enzymes. Widtsen and Kandelbauer [16] recently reviewed such results indicating that the formation of covalent interfiber bonds by radical coupling during pressing is a major contributor to the improved adhesion effect obtained [136-138]. Although laccase-catalyzed bonding requires higher pressing temperatures and longer pressing times, fiberboards made from laccase-treated fibers have a high (up to industrial standard) wet strength compared to boards made from untreated fibers. However, more work is required to improve dimensional stability which is still below the industrial standard. Particularly, the auto-adhesion of beechwood (*Fagus sylvatica*) fibers was enhanced by a pretreatment of the fibers with a *T. villosa* laccase (Novozymes A/S) [138]. The mechanism of enzymatic catalyzed bonding is linked to the generation of stable radicals in lignin by oxidation. Fiber boards made from laccase-treated fibers have a high wet strength compared to boards made from untreated fibers. The surface of laccase-treated fibers showed a markedly increased hydrophobicity, as well as a change in the chemical composition, indicating that lignin extractives precipitated on the fiber surfaces. Thus, increased surface compatibility and entanglement of surface molecules due to the deposition of lignin extractives on the surface of fibers incubated with laccase have also been suggested to improve adhesion [136, 138]. Another approach to substituting synthetic resins in wood composite production is a two-component system in which laccase-treated technical lignin such as kraft lignin or ligno-

sulfonate functions as the adhesive. This method has been applied to the production of PB [139], and the technology has been also patented [140]. The drawback of using the water-soluble lignosulfonates is that the products display poor dimensional stability [139]. A variant processes has also been described in which a laccase-oxidized lignin-based adhesive with 1% methylene diphenyl diisocyanate resulted in particle boards with doubled tensile strength and reduced swelling in water [141]. Patents relating to binderless wood composite boards manufactured from laccase-treated fibers also include that by Qvintus-Leino and coworkers. [142].

### Modification of Cellulose

Laccase-mediated coupling and grafting reactions have also been used for colouring various textiles [143, 144]. In the first attempt [143], cotton cellulose was dyed "*in situ*" with a polymeric dye generated by oxidative coupling of colourless 2,5-diaminobenzenesulfonic acid and 1-hydroxyphenol (catechol) with laccase treated fibers, obtaining up to 70% of dye fixation. However, it was reported that all fabrics showed high wash fastness but low light and friction resistance. Schröder and coworkers [113] used a RSM approach to determine the best conditions for laccase-induced coating of flax fibers by a *T. hirsuta* laccase. A screening was carried out with different phenols for their potential as monomers for enzyme-catalyzed polymerization: all the methoxyphenols showed different coloration with weak fastness properties. An important improvement in the wet rubbing fastness of the dyed sample was observed by covalent fixation of the "in situ" generated polymeric pigment [145]. Aromatic amine moieties of 2,5-diaminobenzenesulfonic acid introduced onto tosylated cotton were coupled and copolymerized with a phenolic compound catechol into coloured product covalently fixed on the fabric upon oxidation with laccase. The controlled amination of cellulose in a first step and the subsequent colouration allowed for up to 95% pigment fixation on the fabric. Recently, Kim and coworkers [146] evaluated the synergistic effect between polyoxometalates (POMs), namely $K_5[SiW_{11}V^{V}O_{40}]\cdot 11H_2O$ and $H_5[PMo_{10}V^{V}{}_2O_{40}]\cdot 13H_2O$, and a laccase from *M. thermophila* in the oxidative polymerization of catechol. Such a laccase-mediator system allowed the formation of a relatively high molecular weight polycatechol. The synthesized polymers were applied as dyes for the dyeing of flax fabrics. This synthetic process allowed a dyeing polymer, provided upon flax coloration, better colour fixation and colour resistance when compared to that obtained by conventional synthesis with laccase solely or with addition of organic mediator (1-hydroxybenzotriazole). Enzymatic coating of functionalized cellulose fibers with catechol was performed in the presence of *Trametes hirsuta* laccase using a dyeing procedure with C.I. Reactive Black 5 (RB5) followed by reduction with sodium hydrosulfite [147]. Cellulase enzymes were used on coated and control samples to obtain the analytes linked with the soluble sugars in solution, to prove the reaction concepts described in this paper. Hydrolyzed coated-cellulose showed lower concentration of reducing sugars (1188 mg/L) than control samples (2011 mg/L). The structures of these compounds showed functionalized glucose and cellobiose units coupled to poly(catechol) molecules. Afterwards, the same authors investigated (i) the simultaneous enzymatic polymerization and coloration of fabrics and (ii) the polymerization of flavonoids with laccase, followed by a further coloration of the flax fabrics [148]. They demonstrated that he increase of temperature, the presence of salt and the use of unbleached fabrics allowed the final color to be improved. Colorized flax fabrics with oxidized quercetin solution showed a color fixation two times higher than the fabrics colorized with oxidized morin. Finally, the polymerization of flavonoids and their binding to fibers were verified, confirming this environmentally friendly process as useful for the coloration of flax fabrics.

## 5. CONCLUSION AND PERSPECTIVES

This review highlights the usefulness of laccases in recent synthetic applications. Laccase or laccase mediator systems do provide alternative and environmental friendly oxidation methods that can be used to replace a host of traditional chemical oxidants for a wide range of substrates. Taking the pressing societies demand for green chemistry solutions and the variety of opportunities surrounding laccases into account, it is likely to believe that laccases will remain the subject of intensive investigations in the different sectors for many years. The application of laccase in organic synthesis will surely grow in the future as a function of our understanding of the enzyme structure and catalytic mechanism and our ability to discover or create new tailor-made laccases, bringing them to the market at feasible cost.

To make these applications feasible at an industrial level, some imperative targets, such as increase of levels and reduction of cost of laccases production, as well as improvement of their properties through development of tailor-made laccases for each specific purpose, are being pursued.

Although large amounts of laccases are secreted by filamentous fungi, an efficient and cost-effective production system at bioreactor scale is still lacking [149], representing one of the main limitations to the exploitation of laccases as biocatalysts. Therefore, many efforts are being focused on improvement of the production levels by recombinant expression. Several laccases have been cloned and overexpressed and some of them are now commercially available [150]. Directed evolution of laccases proved to be a reliable method to develop improved catalysts for specific applications [151, 152]. Recently, an alternative and promising strategy has been developed combining classical breeding of different fungal strains and laccase induction through fungal spent media, with the aim to reduce laccase production costs [153]. As a further route to improving cost of laccase application, several methods of laccase immobilization have been developed, in order to reduce the amount of enzyme required for the use in synthesis process [154]. In most cases, immobilization leads to increased enzyme stability and improved resistance to changes in pH and temperature. However, in some cases, the interaction of products with the immobilization matrix can take place decreasing yield of their obtaining. This makes necessary the development of suitable and efficient production systems at the bioreactor scale. The use of a membrane reactor to retain the enzyme in a defined reaction volume was reported for coupling dihydrocaffeic acid with 4-aminobenzoic acid [155].

## CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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