NEW WILD-TYPE AND ENGINEERED BIOCATALYSTS AND BIOSYSTEMS FOR CONVERSION OF THE LIGNOCELLULOSIC BIOMASS *ARUNDO DONAX* INTO FUEL ETHANOL

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Alla mia famiglia

The genes are master programmers [...].
They are judged according to the success of their programs in coping with all the hazards that life throws at their survival machines.
[cit. Richard Dawkins]

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Abstract

The hydrolysis of lignocellulosic biomasses to obtain fermentable sugars is complex process which requires multiple and synergistic enzymes such as cellulases, hemicellulases (xylanases) and accessory enzymes (e.g. arabinofuranosidases and pectinases). The improvement of the hydrolysis yield and the reduction of the needed amounts of the enzymes involved in the process and thus their costs represent the main challenges of second generation ethanol production. To meet these necessities, different strategies can be adopted such as the isolation and identification of new microorganisms producing high levels of (hemi)cellulase activities and the development of new tailor-made enzymes with high specific activity and high resistance at the conditions used in the hydrolysis process.

In this project, compost obtained from agro-industrial wastes was investigated as a source of new bacteria producing new accessory enzymes. The *Paenibacillus xylanolyticus* 2-6L was isolated from agro-industrial wastes based compost and its pectinolytic activity was characterized. A pectate lyase (Paenxyl Pel) was identified, purified and characterized. Paenxyl Pel exhibits an optimum temperature of 60-70°C and optimal pH value of 9.0 for its pectinase activity on pectin from citrus fruit. Paenxyl Pel shows high thermoresistance and pH resistance and it follows a Michaelis–Menten kinetic towards pectin from citrus fruit, pectin from sugar beet pulp, high ester pectin extracted from citrus peel (>50% esterified) and polygalacturonic acid (PLA). These results suggest that pectate lyase from *P. xylanoliticus* shows an unusual activity combining traits of pectate lyase and pectin lyase.

The development of tailor made arabinofuranosidase was carried out by using directed evolution of PoAbf, the α-L-arabinofuranosidase produced by fungus *Pleurotus ostreatus*. The best evolved variant of the first generation library, rPoAbf F435Y/Y446F, was recombinantly expressed in *Pichia pastoris*, purified and characterized. It exhibited a K_{cat} of 7.3*103 ± 0.3 min^{-1}, around 3-fold higher than that of the wild-type and a K_M (0.54 ± 0.06 mM), 30% lower than that of the wild-type towards towards p-nitrophenyl-α-L-arabinofuranoside. The mutant showed also improved catalytic properties towards pNP-β-D-glucopyranoside (K_{cat} of 50.85 ± 0.21 min^{-1} *versus* 11.0 ± 0.6 min^{-1}) and it was shown able to hydrolyze larch arabinogalactan that is not recognized by the wild-type. The mutant was also more active than the wild-type towards arabinoxylan and was able to hydrolyze arabinan that was not transformed by the wild-type. Even if the mutant rPoAbf F435Y/Y446F was selected for its higher activity, it showed also a higher resistance to temperature and pH than the wild-type and it was shown to be a versatile enzyme.

The biocatalysts rPoAbf wild-type and its evolved variant and rCelStrep from *Streptomyces sp*. G12 recombinantly expressed in *Escherichia coli,* were tested in the hydrolysis of lignocellulosic biomasses by using these enzymes in combination with other enzymatic activities involved in the hydrolysis of lignocellulose. The data suggest that the investigated biocatalysts act synergistically with bacterial and fungal enzymes. Moreover, rPoAbf and its evolved variant improved xylan and arabinan conversion of tested lignocellulosic biomasses highlighting the importance of accessory enzymes to obtain high xylan hydrolysis.

Riassunto

Combustibili da fonti rinnovabili

Il progressivo esaurimento delle risorse fossili e le emissioni di gas a effetto serra derivanti dal loro utilizzo hanno suscitato, nel corso degli anni, l'interesse per la ricerca di fonti di energia rinnovabili. Nell'ottica di una bioeconomia sostenibile, la conversione di biomasse in carburanti, tra cui il bioetanolo, si prospetta come una valida alternativa ai carburanti fossili, offrendo maggiore sicurezza energetica, riducendo le emissioni di CO_2 e contrastando l'innalzamento della temperatura del pianeta a seguito dell'effetto serra (Balat, 2011).

In ambito Europeo, la Direttiva 2009/28/CE, promuove la produzione e l'uso dei biocombustibili, stabilendo che questi dovranno sostituire del 10% i carburanti fossili (benzina e diesel) entro il 2020. Tra i biocarburanti di nuova generazione, il bioetanolo suscita maggiore interesse. Il bioetanolo è ottenuto dalla fermentazione alcolica degli zuccheri e dei polisaccaridi preventivamente idrolizzati (e.g. amido, cellulosa). Il bioetanolo, e gli altri biocarburanti, sono classificati in 3 generazioni sulla base della biomassa dalla quale vengono prodotti: i) i biocarburanti di prima generazione sono prodotti dalla fermentazione di zuccheri ottenuti da materie prime agroalimentari; ii) i biocarburanti di seconda generazione sono generati da materie prime che non hanno impatto sulla filiera alimentare, ovvero da biomasse lignocellulosiche; iii) i biocarburanti di terza generazione sono ottenuti dalle alghe.

Il processo di produzione del bioetanolo di seconda generazione

Circa il 60% del bioetanolo nel mondo è prodotto a partire dalla canna da zucchero mentre il 40% è prodotto da altre colture quali grano e mais (Dufey, 2006). I paese *leader* nella produzione di bioetanolo di I generazione sono USA e Brasile che insieme producono il 90% del bioetanolo del mondo. Tuttavia, il bioetanolo di I generazione suscita diverse problematiche, come la quantità di materia prima disponibile e la competizione tra uso di colture agricole per l'alimentazione e il loro uso per produzione di biocarburanti. Per il superamento dei suddetti limiti, particolare attenzione si sta rivolgendo alla produzione di bioetanolo di II generazione. In particolare, con la produzione di bioetanolo di II generazione si elimina il problema della competizione per i terreni agricoli, in quanto materie prime sono rappresentate da residui delle colture agricole e forestali, da frazioni cartacea ed organica dei rifiuti urbani, da scarti delle lavorazioni industriale e da colture dedicate che andrebbero ad occupare terreni marginali. Dei principali componenti della biomassa lignocellulosica (cellulosa, emicellulosa e lignina), soltanto la cellulosa e l'emicellulosa sono costituite da zuccheri fermentabili, pertanto solo queste possono essere utilizzate per la produzione dell'etanolo. Uno dei punti critici che caratterizza perciò la produzione di bioetanolo di II generazione è la separazione fisica dei polisaccaridi dalla lignina. Per questo motivo, l'attuale processo adottato per la produzione di bioetanolo di seconda generazione prevede una prima fase di pretrattamento, volta a rimuovere la lignina. Questo può essere effettuato mediante trattamenti chimico–fisici (il più comune è la *steam explosion)*, chimici (attraverso l'impiego di acidi), o meccanici. Una volta liberate dalla lignina, la cellulosa e l'emicellulosa sono quindi sottoposte ad un processo di idrolisi che potrà realizzarsi o per via chimica o enzimatica, da cui si ottengono i monosaccaridi che potranno così essere fermentati in bioetanolo.

L'idrolisi enzimatica, che ha minore impatto ambientale rispetto all'idrolisi

acida, prevede l'azione cooperativa di diversi enzimi: cellulasi, emicellulasi ed enzimi accessori che agiscono sulle catene laterali di xilani e mannani. I costi di produzione degli enzimi coinvolti in questo processo risultano ancora molto elevati, incidendo fortemente sui costi totali del processo di conversione della biomassa in bioetanolo.

Le principali problematiche relative alla produzione competitiva di bioetanolo di II generazione sono pertanto rappresentate dall'incremento dell'efficienza/resa dell'idrolisi delle componenti polisaccaridiche della biomassa e dalla riduzione dei costi di produzione degli enzimi.

Strategie per migliorare il processo di produzione del bioetanolo di II generazione

Una delle strategie adottate per il miglioramento della competitività del processo di idrolisi enzimatica è la ricerca nuovi enzimi accessori, cruciali per la completa idrolisi delle componenti polisaccaridiche (pectinasi, feruloil esterasi, arabinofuranosidasi, mannanasi) a partire da microrganismi isolati da ambienti naturali, fonti di biocatalizzatori per la conversione di cellulosa ed emicellulosa. Un'ulteriore strategia riguarda il miglioramento delle proprietà dei biocatalizzatori mediante mutagenesi. Infine, data la complessità delle biomasse lignocellulosiche, lo sviluppo di nuove formulazioni (cocktail) di enzimi *ad hoc* per la conversione di tutte le componenti polisaccaridiche, può rappresentare una strategia per il miglioramento della resa di idrolisi e la riduzione dei costi del processo.

Identificazione di nuovi enzimi accessori per la conversione delle biomasse lignocellulosiche

Nell'ambito del progetto 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, responsabile scientifico Vincenza Faraco), questo lavoro di dottorato ha previsto l'identificazione di nuovi enzimi ad attività pectinasica a partire da due microrganismi, 1-5H6 e 2-6L3, isolati da compost ottenuto da scarti agro-industriali dal gruppo della Professoressa Pepe del dipartimento di Agraria dell'Università di Napoli "Federico II". I due microorganismi sono stati identificati come *Paenibacillus xylanilyticus* mediante l'analisi molecolare, condotta attraverso l'amplificazione della regione 16S del DNA ribosomiale (rDNA). Di entrambi i microrganismi sono stati seguiti gli andamenti di produzione dell'attività pectinasica, attraverso dosaggio enzimatico in mezzo liquido con il substrato pectina da agrumi.

P. xylanilyticus 2-6L3 è risultato il ceppo più produttivo con un massimo di produzione di attività pari a 1.16 UmL^{-1} tra la ventisima e ventiquattresima ora di crescita. Al fine di identificare le proteine responsabili dell'attività pectinasica, è stata messa a punto la procedura di zimografia accoppiata ad analisi di proteomica, in collaborazione con il gruppo della Professoressa Birolo del dipartimento di Scienze Chimiche dell'Università di Napoli "Federico II", che ha condotto all'identificazione di tre putative proteine coinvolte nell'idrolisi della pectina. In dettaglio sono state identificate una α-N-arabinofuranosidasi da *Bacillus pumilus* SAFR-032 (NCBI: gi 157693268); una β-galactosidasi da *Bacillus pumilus* ATCC 7061 (NCBI: gi 194016271), e una pectato liasi da *Bacillus sp.* (NCBI: gi 4589753). Questi risultati hanno suggerito che le pectinasi prodotte dal microrganismo selezionato non sono ancora state depositate in banca dati.

Al fine di caratterizzare la pectinasi prodotta da *P. xylanilyticus* 2-6L3 (Paenxyl Pel), è stato messo a punto un protocollo di purificazione consistente in una precipitazione in solfato d'ammonio, seguita da cromatografia ad interazione idrofobica e concentrazione mediante ultrafiltrazione. Il peso molecolare della proteina in condizioni denaturanti è stato stimato intorno ai 37,000 Da, un dato simile a quello di altre pectinasi purificate da ceppi del genere *Bacillus*. L'enzima ha una temperatura ottimale di reazione in un *range*, compreso tra 50 e 70°C ad un pH ottimale di 9. L'enzima mostra un' emivita di a 48h a 60°C e di 24h a 70 °C; a 40°C e 50°C l'enzima mantiene l'80% di attività dopo 96 ore di incubazione. Inoltre ha una resistenza al pH di circa 15 giorni a pH 8.0.

Si è dimostrato che la pectinasi da *P. xylanilyticus* segue una cinetica di Michaelis–Menten nell'idrolisi di diversi substrati come riportato in tabella 1. Sulla base dei risultati ottenuti, è possibile evidenziare che l'enzima PaenxylPel presenta una duplice attività enzimatica come pectato liasi e pectin liasi, agendo su substrati altamente metilati e non metilati.

Substrato	K_M (gL^{-1})	V_{max}($gL^{-1}min^{-1}$)
pectina da agrumi	0.10±0.03	1,953±0.08
pectina da polpa di barbabietole	0.36±0.06	0.79±0.07
pectina altamente m**etilata estratta dalla buccia degli agrumi(>50% esterificazione)**	1.07±0.05	0.22±0.05
acido poligalatturonico (PLA)	0.20±0.05	0.74±0.05

Tabella 1: Valori delle costanti cintetiche di Michaelis–Menten dell'enzima PaenxylPel verso diversi substrati

Evoluzione guidata dell'arabinofuranosidasi PoAbf prodotta dal fungo *Pleurotus ostreatus*

Sempre nell'ambito del progetto ENERBIOCHEM, le attività svolte durante questo dottorato hanno riguardato l'evoluzione guidata -mediante mutazione casuale- dell' arabinofuranosidasi (PoAbf) in *Saccharomyces cerevisiae* al fine di crearne varianti dotate di migliorate proprietà catalitiche e resistenza alle condizioni operative di interesse per la sua applicazione industriale. L'evoluzione guidata riproduce, in vitro, il naturale processo di evoluzione molecolare ed è in grado di generare una pletora potenzialmente infinita di varianti proteiche con nuove funzioni e proprietà. La facilità di manipolazione di *S. cerevisiae*, insieme all'alta frequenza di ricombinazione, e alla disponibilità nel nostro laboratorio di un sistema di evoluzione guidata basato sul suo utilizzo e applicato con successo ad altri enzimi (Festa *et al*., 2008, Miele *et al.*, 2010 a e b), ci hanno indotto ad utilizzare il suddetto sistema nella realizzazione di questo tipo di esperimenti. Per ottenere le varianti cDNA codificante PoAbf sono stati realizzati esperimenti di polimerizzazione a catena a bassa fedeltà (*Error-prone* PCR) utilizzando pSAL4-*poabf* come stampo. Per la collezione di prima generazione sono stati allestiti due set di esperimenti utilizzando quantità di DNA tali da avere rispettivamente un alto (HMF) e un medio tasso (MMF) di mutazioni. Le due collezioni di cDNA mutati ottenute sono state clonate nel vettore di espressione pSAL4 mediante esperimenti di ricombinazione omologa *in vivo.* Le colonie ottenute a seguito della trasformazione del lievito sono state analizzate su terreno selettivo *SD-medium* che consente la produzione dell'enzima ricombinante grazie all'utilizzo del solfato di rame come

induttore. Si sono ottenuti circa 6000 ceppi producenti proteine variamente mutate: 3300 per medio tasso di mutazione (MMF) e 3100 con alto tasso di mutazione (HMF). È stato quindi effettuato un primo screening su mezzo solido contenente il substrato cromogenico *4-methylumbelliferyl-α-L-arabinofuranoside* (MUA). Da questo primo screening sono stati selezionati 1100 cloni MMF e 1000 cloni HMF. Inoltre, per la successiva selezione dei mutanti in coltura liquida, è stata messa a punto una metodologia di *high-throughput screening*, mediante allestimento di colture liquide dei mutanti in piastre multipozzetto. Come primo criterio di selezione si è scelto di cercare i mutanti dotati di maggiore attività sul substrato *p-nitrofenil-α-L-arabinofuranoside* (pNPA) rispetto all'enzima *wild-type*. L'*high-throughput screening* è stato eseguito utilizzando un sistema automatizzato (*Biomek® Workstation Laboratory Automation 3000, Beckman Coulter*). Il saggio di attività arabinofuranosidasica è stato effettuato secondo il protocollo Yanay e Sato (2000), con opportune modifiche, al primo giorno di crescita. I rapporti trai volumi di tutti i componenti della reazione sono stati variati fino ad individuare le condizioni ottimali di saggio. Questo secondo *screening* ha permesso di selezionare 6 mutanti MMF e 18 mutanti HMF con attività più alta del *wild-type* di 7 volte per ulteriore screening in beuta. A tal fine sono state effettuate colture liquide in beuta dei 24 mutanti ed è stata valutata la produzione dell'enzima rPoAbf mediante saggio di attività secondo il protocollo Yanay e Sato (2000) a 24, 48, 72 e 96 ore di crescita. Questi esperimenti hanno consentito di selezionare 1 mutante MMF e 6 mutanti HMF con attività arabinofuranosidasica 2-3 volte maggiore rispetto all'enzima *wild-type*. Questi 7 mutanti sono stati sottoposti ad analisi di sequenza per identificare le mutazioni presenti. L'analisi di sequenza ha mostrato che 3 dei cloni selezionati presentano le stesse sostituzioni amminoacidiche per cui sono stati selezionati 4 mutanti (1 MMF e 3 HMF) per le successive analisi. È risultato che due mutazioni, F435Y e Y446F sono conservate in tutti i mutanti il che farebbe ipotizzarne un ruolo importante. L'enzima PoAbf recante le mutazioni F435Y/Y446F, ottenuto mediante mutagenesi sito diretta dell'enzima *wild-type*, è stato espresso in forma ricombinante in *Pichia pastoris* per la over-produzione e caratterizzazione. Le caratteristiche della variante sono state analizzate paragonandole a quelle dell'enzima *wild-type*. L'attività specifica dell'enzima ricombinante rPoAbf F435Y/Y446F, adoperando il pNPA come substrato, è risultata essere maggiore di 2 volte rispetto a quella dell'enzima *wild-type* sia a 20°C (36.8±1.8 *versus* 16.7±0.0 mU/O.D.$_{600nm}$) che a 28 °C (8.0±0.3 *versus* 4.8±0.4 mU/O.D. $_{600nm}$).

Il mutante segue la cinetica di Michaelis-Menten con una K_{cat} 7273.75± 331.09 min^{-1} circa 3 volte più alta di quella del *wild-type* (K_{cat} 2249.25±207.77 min^{-1}), ed una K_M (0.54±0.06 mM) più bassa di quella del *wild-type* (0.76±0.04 mM). Il mutante mostra un *range* di valori di pH ottimali (3-6) diversamente dall'enzima *wild-type* che mostra un valore di pH ottimale pari a 5. D'altra parte come osservato per il *wild-type* la temperatura ottimale del mutante è di 40°C. È stata inoltre valutata la capacità dell'enzima PoAbf F435Y/Y446F di idrolizzare altri substrati sintetici: pNP-β-D-xilopiranoside, pNP-α-D-glucopiranoside, pNP-β-D-glucopiranoside e oNP-β-D-galattopiranoside; tra questi l'enzima mostra attività solo verso il pNP-β-D-glucopiranoside, con una cinetica di Michaelis-Menten, con K_M e K_{cat} simile a quelle dell'enzima *wild-type* (K_M 5.25±0.07 mM *versus* 4.07±0.15 mM e K_{cat} 50.85±0.21 min^{-1} *versus* 11.0±0.6 min^{-1}. È stata valutata, inoltre, la capacità dell'enzima mutato di idrolizzare substrati naturali, i risultati sono mostrati in Tabella 2.

Substrato	Quantità di zuccheri rilasciati (μg/mL)	
	rPoAbf *wild-type*	**rPoAbf F435Y/Y446F**
Larch rabinogalactan	-	205.4±13.8
CM-linear arabinan	240±8	288.7±4.6
Arabinotrioso	112.7±2.9	111±4
Arabinoesoso	107.4±8	132.06±18.61

Tabella 2: Quantità di zuccheri rilasciati dagli enzimi rPoAbf *wild-type* e rPoAbf F435Y/Y446F

Inoltre l'enzima rPoAbfF435Y/Y446F mostra un' attività endo-1,4-β-xilanasica quando incubato in presenza di *AZO-wheat arabinoxylan* più alta rispetto a quella dell'enzima *wild-type* (0.81±0.03 UmL^{-1} *versus* 0.63±0.03 UmL^{-1}). Le analisi sulla resistenza a pH e temperature ha mostrato che il doppio mutante possiede un più alta resistenza alle temperature testate (40, 50 and 60 °C), in particolare una $T_{1/2}$ di 23 ore invece di 17 h a 50°C e una $T_{1/2}$ di 24 giorni invece di 14 giorni a 40°C. Per quanto riguarda il pH, l'enzima rPoAbfF435Y/Y446F mostra una resistenza simile o più alta dell'enzima *wild-type*, in particolare a pH 3 ha una $T_{1/2}$ di 35 giorni rispetto ai 21 dell'enzima *wild-type*, mentre a pH 8 ha una $T_{1/2}$ di 50 giorni rispetto ai 28 dell'enzima *wild-type* e a pH 7 ha una $T_{1/2}$ di 50 giorni rispetto ai 40 dell'enzima *wild-type*. È stata inoltre valutata la capacità dell'enzima ricombinante PoAbfF435Y/Y446F, di idrolizzare substrati insolubili. L'enzima PoAbfF435Y/Y446F è in grado di idrolizzare l'AZCL-Arabinoxilano con un'attività pari a 3.0 ± 0.2 mU/mL, circa 2 volte maggiore dell'enzima *wild-type*; inoltre il mutante è in grado di idrolizzare l'AZCL-Arabinano (1.28 ± 0.05 mU/mL) che non viene idrolizzato dall'enzima *wild-type*. Il suddetto mutante è stato quindi adoperato come stampo per la costruzione della *library* di evoluzione guidata di seconda generazione, mediante mutazione casuale. Per la collezione di seconda generazione è stato allestito un unico *set* di esperimenti utilizzando quantità di DNA tali da avere un medio tasso (MMF) di mutazioni. A seguito della *Error Prone* PCR, i cDNA mutati ottenuti sono stati clonati nel vettore di espressione pSAL4 mediante esperimenti di ricombinazione omologa *in vivo* come precedentemente effettuato per la *library* di mutanti di prima generazione. Si sono ottenuti circa 6000 varianti, di cui è stato quindi effettuato un primo *screening* su mezzo solido contenente il substrato cromogenico MUA. Da questo primo *screening* sono stati selezionati circa 3000 cloni attivi. Per la successiva fase di selezione in liquido è stata adoperata la metodologia di *high-throughput screening* messa a punto per la *library* di mutanti di prima generazione. Anche in questo caso, come criterio di selezione si è scelto di cercare i mutanti dotati di maggiore attività sul substrato pNPA rispetto all'enzima rPoAbfF435Y/Y446F adoperato come stampo per la costruzione di questa collezione. Questo *screening* ha permesso di selezionare 76 mutanti con attività almeno 3 volte più alta dell'enzima rPoAbfF435Y/Y446F. Tra i 76 mutanti ne sono stati selezionati 19, con attività 4-5 volte più alta dell'enzima rPoAbfF435Y/Y446F di riferimento, per effettuare la valutazione della produzione dell'enzima rPoAbf in mezzo liquido. Questi esperimenti hanno consentito di selezionare 6 mutanti con attività arabinofuranosidasica 2 volte maggiore rispetto all'enzima rPoAbfF435Y/Y446F che sono stati sottoposti ad analisi di sequenza per identificare le mutazioni presenti. Sono stati, quindi, selezionati di 2 mutanti recanti, oltre alle sostituzioni amminoacidiche del mutante adoperato come stampo,

la mutazione K533N in un caso e F434L nell'altro. È stata effettuata la sovra-produzione del doppio mutante dell'arabinofuranosidasi PoAbf recante le sostituzioni amminoacidiche F435Y/Y446F in *S.cerevisiae* mediante allestimento di colture in fermentatore e del triplo mutante recante le sostituzioni amminoacidiche F435Y/Y446F/K533N al fine di accumulare i quantitativi di enzima necessari per la caratterizzazione degli enzimi in questo ospite. Sono stati effettuati diverse prove di purificazione dell'enzima che non hanno consentito di ottenere un campione omogeneo. Ulteriori prove sono quindi necessarie finalizzate all'ottenimento di enzimi sufficientemente puri per la loro successiva caratterizzazione.

L'evoluzione guidata è risultata essere una buona strategia per ottenere mutanti con proprietà catalitiche migliorate rispetto all'enzima di partenza. Inoltre, sebbene il mutante rPoAbfF435Y/Y446F fosse stato selezionato per la sua attività maggiore rispetto all'enzima *wild-type*, ha mostrato anche una più alta resistenza a temperatura e pH dell'enzima *wild-type*.

Biotrasformazione delle biomasse lignocellulosiche cellulosa in zuccheri fermentabili

In collaborazione con il gruppo del Dott. La Cara del Consiglio Nazionale delle Ricerche-Istituto di biochimica delle proteine (CNR-IBP) di Napoli sono state effettuate diverse prove di bioconversione di biomasse lignocellulosiche *corn cobs, Arundo donax e brewer's spent grains* (BSG). Le prove di biotrasformazione effettuate sono state allestite utilizzando sia *mix* di enzimi commerciali, sia i nuovi biocatalizzatori sviluppati nel laboratorio dove questo progetto di dottorato è stato svolto (rPoAbf *wild-type*, rCelStrep), che l'enzima rPoAbfF435Y/Y446F prodotto in questo progetto di dottorato. La capacità dell'enzima rPoAbf di idrolizzare le biomasse pretrattate, è stata valutata in combinazione con *mix* enzimatiche commerciali e con rCelStrep, cellulasi prodotta da un ceppo del genere *Streptomyces*, prodotta in *Escherichia coli*. I risultati ottenuti hanno dimostrato la capacità delle arabinofuranosidasi rPoAbf *wild-type* e rPoAbfF435Y/Y446F di migliorare l'efficienza dell'idrolisi delle biomasse lignocellulosiche adoperate negli esperimenti. Le migliori rese di conversione si sono ottenute adoperando il mutante dell'enzima PoAbf in combinazione con gli enzimi commerciali. Ulteriori prove di bioconversione sono state effettuate durante lo stage presso il laboratorio *Biomass Conversion Research Laboratory* (BCRL) del Dott. Balan della *Michigan State University*. Durante questo periodo la biomassa *A. donax* è stata sottoposta ad un pretrattamento mediante tecnologia *Ammonia Fiber Expansion* (AFEX) e sono state effettuate prove di biotrasformazione delle biomasse *A. donax* e *corn stover*, quest'ultimo adoperato come biomassa di riferimento essendo la tecnologia AFEX già stata ottimizzata per questa biomassa (Gao *et al*., 2011). Per la biomassa *A. donax* sono state testate tre condizioni di pretrattamento AFEX ed è stata determinata la migliore condizione per le successive prove di biotrasformazione valutando, la quantità di glucosio e xilosio rilasciati nella fase di idrolisi. Selezionata la migliore condizione di pretrattamento, sono state messe a punto reazioni di biotrasformazione con enzimi purificati forniti dal gruppo del Dott. Balan seguendo il protocollo riportato da Gao *et al*., 2011. Sono state effettuate prove di idrolisi sostituendo l'arabinofuranosidasi purificata con l'arabinofuranosidasi rPoAbf o il suo mutante rPoAbfF435Y/Y446F e sostituendo la endoglucanasi purificata con rCelstrep. I risultati ottenuti dimostrano la capacità delle

arabinofuranosidasi rPoAbf e rPoAbfF435Y/Y446F di migliorare l'efficienza dell'idrolisi incrementando le rese di xilosio e arabinosio. I risultati ottenuti a seguito dell'aggiunta dei nuovi biocatalizzatori alle *mix* enzimatiche commerciali dimostrano che, per quanto riguarda *A. donax*, le migliori rese di conversione si ottengono quando il mutante di rPoAbf è aggiunto alle *mix* commerciali. Per quanto riguarda il *corn stover,* le migliori rese di idrolisi di ottengono con l'aggiunto di rPoAbf *wild-type*. I dati ottenuti dimostrano che le emicellulasi sono necessarie per l'ottenimento di altre rese di xilosio e arabinosio.

Conclusioni

In sintesi questo progetto di dottorato ha previsto:

- lo sviluppo di un nuovo biocatalizzatore, una pectinasi batterica da *P.xylanolyticus*, di cui sono state studiate le proprietà catalitiche. L'enzima ha mostrato di agire come pectato liasi e pectin liasi agendo su pectine con diverso grado di esterificazione;
- lo sviluppo di due collezioni di mutanti di evoluzione guidata dell' α-L-arabinofuranosidasi da fungo e la messa a punto di un *high-throughput screening* per la selezioni di varianti con migliori proprietà;
- lo sviluppo di un nuovo biocatalizzatore ottenuto dalla *library* di prima generazione di mutanti dell'arabinofuranosidasi, di cui sono state studiate le proprietà catalitiche rispetto all'enzima *wild-type;*
- la valutazione delle abilità idrolitiche dei nuovi biocatalizzatori nella conversione di biomasse lignocellulosiche.

List of Abbreviations

AFEX: Ammonium Fiber Expansion
BSG: Brewer's spent grains
CBH: Cellobiohydrolases
CBP: Consolidated BioProcessing
EG: Endoglucanase
EX: Endo-b-Xylanase
HMF: High mutation frequence
LX: Xylanase
MMF: Medium mutation frequence
MUA: 4-methylumbelliferyl-α-L-arabinofuranoside
PCR: Polymerase Chain Reaction
pNPA: Para-Nitro Phenyl Arabinofuranoside
PoAbf: α-L-arabinofuranosidase from *Pleurotus ostreatus*
rPoAbf: Recombinant α-L-arabinofuranosidase from *Pleurotus ostreatus*
rRNA: Ribosomal Ribonucleic Acid
SDS-PAGE: Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis

Chapter 1
Introduction

1.1 BIOETHANOL AS BIOFUEL

There has been increasing worldwide interest towards research of new sustainable and limitless energy resources due to the finite nature of fossil fuels and its inevitable depletion, alarm over environmental impact, especially related to greenhouse gas emissions (GHGs), global warming and energy security (Balat, 2011; MacCracken, 2008).

Demand for energy in the Mediterranean Basin has more than doubled in the last 30 years, due to population growth and economic development (Faraco & Hadar, 2011). The growing demand for energy exceeds the available sources, including fossil fuels, whilst the available renewable sources of energy can meet only 7% of energy demand (Parliamentary Assembly of the Mediterranean, 2008). World fuel consumption is predicted to increase from 87 MMbbl/d in 2010 to 119 MMbbl/d in 2040 according to the EIA (International Energy Outlook, 2014).

To meet these necessities, the European Parliament promoted the production and use of biofuels in substitution of petroleum-based fuels, establishing a target of 10% reduction of conventional fuels by 2020 with the formulation of "Climate - Energy package 20-20-20" (DIRECTIVE 2009/28/CE).

As described by Meher *et al*., (2006), an alternative fuel must be economically competitive, environmentally acceptable, and readily available (Meher *et al*., 2006). Numerous potential alternative fuels have been proposed, such as methanol, bioethanol, natural gas, biodiesel, hydrogen and solar fuels (Table 3) (Balat, 2011); and they represent an attractive substitution of petroleum-based fuels. Biofuel usually refers to solid (bio-char), liquid (ethanol, biodiesel and vegetable oil) and gaseous (biogas, biosyngas and biohydrogen) fuels derived from biomass (Demirbas, 2008).

BIOFUEL	FOSSIL FUEL
Biobuthanol	Gasoline/butane
Ethanol	Gasoline
Biodiesel	Diesel
Biogas	Natural gas

Table 3: Biofuel alternatives to fossil fuels

Biomass is an attractive renewable resource for production of a wide range of high added value products. Biofuels offer several advantages over fossil fuels (Table 4).

	ADVANTAGES
Economic impact	Agricultural development Reducing dependence on foreign oil
Energy security	Reducing use of fossil fuels Renewability
Environmental impact	Reducing greenhouse gas emissions Reducing air pollution Biodegradability

Table 4: Main advantages of biofuels

The main advantages of biofuels is that their use does not increase CO_2 levels so they can contribute to mitigate GHG emissions (Balat, 2011). Moreover, biofuels

are renewable energy source and reduce dependence on foreign oil (Prasad *et al*., 2007).

The two most promising biofuels are bioethanol and biodiesel. Bioethanol exhibits several advantages, such as high octane number, low cetane number and high heat of vaporization (Balat *et al*., 2008). Unlike fossil fuels, it is a renewable energy source produced by the action of microorganisms on the fermentable sugars present in the raw material and it is used as transportation fuel in a few countries of the world. A variety of biomass feedstock has been explored for ethanol production and they can be classified into three types: i) lignocellulosic materials such as woody biomass, herbaceous perennials and various wastes; ii) starch-rich crops such as maize and grain sorghum; iii) sucrose-rich crops such as sugarcane and sugar beet (Balat *et al*., 2008). Based on feedstock bioethanol and the other biofuels, are classified into three generations:

- First generation biofuels, also called conventional biofuels. They are made from feedstock used for human food too;
- Second generation biofuels, produced from sustainable feedstock that is not edible and does not compete with food production;
- Third generation biofuels, produced using algae (Dragone *et al*., 2010).

1.2 FROM FIRST TO SECOND GENERATION BIOETHANOL

First generation bioethanol is produced from traditional agricultural crops, mainly represented by corn and sugarcane. In particular, Brazil utilizes sugarcane for bioethanol production, USA use starch from corn and Europe utilizes wheat. The world production of ethanol is reported in Table 5. Brazil and USA are the world leaders of bioethanol production, together accounting for about 90% of the world bioethanol production (Renewable Fuels Association (RFA), 2013). It is worth of note that Brazil is the only country that uses ethanol, produced exclusively from sugarcane, as a full substitute for gasoline (Soccol *et al*., 2011; Dufey, 2006).

Continent / Years	Africa	Asia	Australia	Europe	North and Central America	South America
2007	-	627,2	26.4	570.3	6,749.5	5,111.9
2008	-	657.7	26.4	733.6	9,237.27	6,551.4
2009	-	1,069.4	56.80	1,039.52	11,906.99	6,661.1
2010	43.59	785.91	66.04	1,176.88	13,720.99	7,121.8
2011	38.31	889.7	87.2	1,167.64	14,401.34	5,771.9
2012	42	952	71	1,139	13,768	5,800

Table 5: **Global Ethanol Production (Millions of Gallons)**
Data source: Ethanol industry outlook RFA, 2013. F.O. Licht

First generation ethanol production systems pose a concern about competition with food and feed supplies because the production of food and biofuels is dependent on the same resources: land, water, and energy. The U.N. Food and Agriculture Organization, reports that using food grains for biofuels production is increasing food deficit (Pimentel *et al*., 2008). Moreover, bioethanol production from corn has high environmental impact due to use of nitrogen fertilizer and insecticides, high carbon dioxide production, release of greenhouse gases (Pimentel *et al*., 2008). As regards Europe, the current production of first generation ethanol is less than its

consumption and the potential of its future yield is not sufficient to achieve the prefixed European goals, leading to an increase of ethanol importations.

These limitations can be overcome by the use of non-edible biomass sources such as agricultural and forest crop residues, grasses, agro-industrial wastes and organic fraction of municipal solid wastes (OFMSW) (Prasad *et al.*, 2007). Second generation bioethanol production from non-food renewable feedstock known as lignocellulosic biomass is attracting keen interest. Lignocellulosic biomasses are the most abundant renewable resources on Earth, so their use for second generation ethanol production minimizes the conflict between land use for food (and feed) and energy production. Moreover, these raw materials are not expensive and they present a more even geographical distribution than the conventional agricultural feedstock. Second generation bioethanol would represent the key response to the need of increasing renewable energy production particularly in the Mediterranean Basin (Faraco & Hadar, 2011). It is worth noting that only small amounts of cellulose, hemicellulose and lignin composing agricultural residues are currently exploited, the majority being considered wastes. Moreover, second generation ethanol production and use show lower green-house gas emissions (<86%) than the first generation fuels, reducing environmental impact, particularly on climate change.

1.3 COMPOSITION OF LIGNOCELLULOSE FOR SECOND GENERATION BIOETHANOL PRODUCTION

Lignocellulose consists of three types of polymers, cellulose, hemicellulose (both referred as carbohydrate polymers) and lignin (aromatic polymer), bonded by both non-covalent and covalent cross linkages (Figure 1) (Pérez *et al*., 2002; Sánchez, 2009). Cellulose is a highly crystalline linear polymer that is composed of D-glucose units linked by β-1,4-glycosidic bonds (Pérez *et al*., 2002). Hemicellulose is also a polysaccharide, accounting for 25-35% of dry wood (Balat *et al*., 2008). It is a very heterogeneous and ramified polymer, consisting of a mixture of different monosaccharides, such as hexoses (glucose, mannose, and galactose), pentoses (xylan, arabinose), and glucoronic acid. Lignin is present in the cellular wall to give structural support, mechanical resistance, impermeability and defense against microbial attack and oxidative stress. It is an amorphous heteropolymer formed from phenylpropane units joined together by non-hydrolyzable linkages (Sánchez, 2009). The composition of lignocellulosic biomasses depends on plant species and upon growth conditions (Prasad *et al.*, 2007).

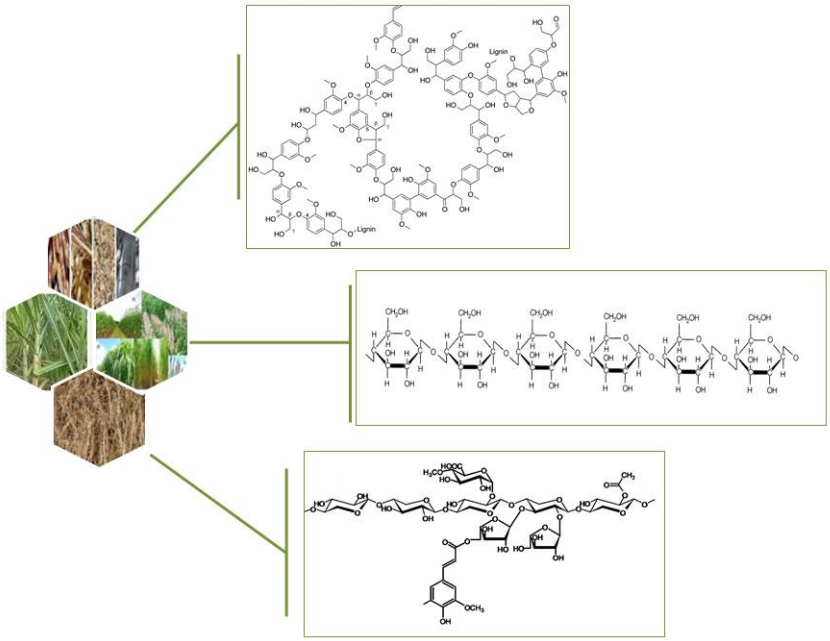

Figure 1: Structure of lignocellulose material

1.4 THE SECOND GENERATION BIOETHANOL PROCESS: COMPONENTS AND PHASES

In general, the process of ethanol production from lignocellulosic materials includes the following key steps (Figure 2): i) pretreatment of lignocellulosic biomass to break lignin barrier; ii) chemical or enzymatic hydrolysis of cellulose and hemicellulose fractions to generate fermentable sugars (saccharification); iii) fermentation of mixed hexose and pentose sugars to ethanol, which include SHF (Separate Hydrolysis and Fermentation) or SSF (Simultaneous Saccharification and Fermentation) or CBP (Consolidated Bioprocessing),iv) recovery and v) distillation (Balat & Balat, 2009).

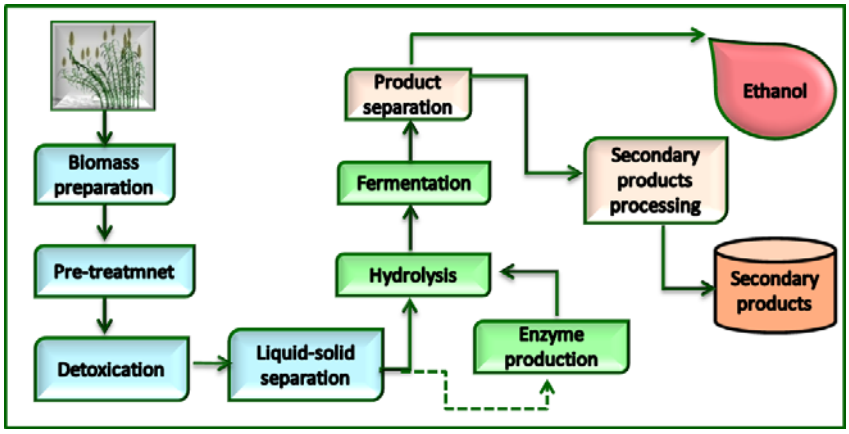

Figure 2: Schematic representation of process steps involved in the production of ethanol from lignocellulosic biomass

The following paper "**The second generation ethanol production" (Paper I),** reviews main steps of second generation ethanol process and enzymes.

1.4.1 PAPER I: THE SECOND GENERATION ETHANOL PRODUCTION

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The second generation ethanol production

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1. Abstract

The shortage of fossil energy sources, the alarm over greenhouse gas emissions (GHGs) and global warming have caused an increasing interest in alternative renewable sources of energy, boosting the worldwide trend to produce and use biofuels in substitution of petroleum–based fuels. Bioethanol represents one of the most promising biofuels. The potential yield of first generation bioethanol, produced from traditional agricultural crops, is not sufficient in many parts of the world. Moreover, first generation ethanol production systems pose a concern about competition with food and feed supplies. To overcome these bottlenecks, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting keen interest. The present manuscript describes the main steps of the second generation bioethanol production process.

2. Introduction

The shortage of energy fossil sources, the alarm over greenhouse gas emissions (GHGs) and global warming [1] have prompted an increasing worldwide interest in alternative renewable sources of energy. The European Parliament with the DIRECTIVE 2009/28/CE — also known as "Climate–Energy package 20–20–20" — has promoted the

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production and use of biofuels in substitution of petroleum–based fuels, establishing a 10% reduction of conventional fuels as the target for 2020.

Bioethanol represents one of the most promising biofuels, exhibiting several advantages, such as high octane number, low cetane number and high heat of vaporization [2]. A variety of biomass feedstock have been explored for ethanol production and can be classified into three types:

a) lignocellulosic materials such as woody biomass, herbaceous perennials and various wastes;
b) starch–rich crops such as maize and grain sorghum;
c) sucrose–rich crops such as sugarcane and sugar beet [3].

First generation bioethanol is produced from traditional agricultural crops, mainly represented by corn, sugarcane and sugar beet. Brazil and USA are the leaders of ethanol production, together accounting for about 90% of the world production. During 2012, the USA has produced 13.3 billion gallons of ethanol [4]. More than 95% of the ethanol produced in the USA for transportation is currently obtained from corn starch and represents 10% of the fuel supply and 25% of all the motor fuel produced [5]. Nowadays, Brazil is the only country that uses ethanol, produced exclusively from sugarcane, as a full substitute for gasoline [6].

The current production of first generation ethanol in the European Union is less than its consumption. Given that the potential yield is not sufficient to accomplish the prefixed European goals, an increase in ethanol importations can be foreseen. Moreover, first generation ethanol production systems pose a concern about the competition with food and feed supplies.

To overcome these bottlenecks, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting keen interest. Lignocellulosic biomasses are the most abundant renewable resources on Earth. Thus, their utilization for second generation ethanol production would minimize the conflict between land use for food (and feed) and energy production. Moreover, these raw materials are less expensive and present a more even geographical distribution than the conventional agricultural feedstock. A large

fraction of lignocelluloses is represented by residual biomass such as agro–industrial wastes, agricultural and forest crop residues and the organic and paper fractions of municipal solid waste (MSW) that would represent the key response to the need of increasing renewable energy production particularly in the Mediterranean basin [7]. It is worth noting that only small amounts of cellulose, hemicellulose and lignin composing agricultural residues are currently exploited, as the majority is considered waste. Moreover, second generation ethanol production and use has lower green–house gas emissions (<86%) than the first generation fuels, reducing environmental impact, particularly on climate change.

Lignocellulose consists of three types of polymers — cellulose, hemicellulose and lignin — bonded by both non–covalent and covalent cross linkages [8, 9]. Cellulose is a highly crystalline linear polymer composed of D–glucose units linked by β–1,4 glycosidic bonds [8]. Hemicellulose is likewise a polysaccharide, accounting for 25–35% of dry wood [3]. It is a very heterogeneous and ramified polymer, consisting of a mixture of various monosaccharides, such as xylose and arabinose (both 5–carbon sugars) and glucose, mannose and galactose (all 6–carbon sugars), and glucoronic acid. Lignin is present in the cellular wall to give structural support, mechanical resistance, impermeability and defence against microbial attack and oxidative stress. It is an amorphous heteropolymer formed by phenylpropane units joined together by non hydrolysable linkages [9].

Bioethanol production from lignocellulosic materials takes place in three phases: the first step consists in breaking the lignin barrier (pretreatment); further step involves the hydrolysis of cellulose and hemicellulose to generate fermentable sugars (saccharification) followed by the fermentation of mixed hexose and pentose sugars to produce ethanol. In this manuscript, the main routes to perform these steps and their components are described.

3. Pretreatment of lignocellulosic materials for ethanol production

The main goals and characteristics of the pretreatment of lignocellulosic materials are to:

a) remove the barrier of lignin,
b) expose plant cell wall polysaccharides,
c) prevent a great degradation or loss of carbohydrate,
d) minimize the concentration of by–products that are inhibitory to the following hydrolysis and fermentation processes, and (4) be economic and environmentally friendly. Numerous strategies of pretreatment have been developed belonging to different categories, i.e. physical (Pyrolysis, Microwave), physicochemical (Steam Explosion, Ammonia Fiber Explosion), chemical (Alkaline Hydrolysis, Acid Hydrolysis, Ozonolysis, Organosolv) and biological.

3.1. *Physical processes*

Pyrolysis

The pyrolysis pretreatment involves the decomposition of cellulose into H_2, CO, and residual char by using temperatures higher than 300 °C; the process is enhanced when carried out in the presence of oxygen. The wide variety of pyrolysis products points to the need of separating and purifying them prior to use [10]. The recovered solution, after separation from the residual char is mainly composed of cellulose, which can be saccharified for the fermentation of ethanol. Different kind of food crops like corn, sugarcane and soybean were pretreated through pyrolysis to generate ethanol [11]. Ethanol can be produced on a large scale using bio–oil hydrolysate produced by fast pyrolysis of loblolly pine particles. The mainly issue is the presence of inhibitor compounds. These can be removed by economically friendly methods (activated carbon, air stripping and microbial), followed by microbial fermentation, reaching 0.4 g ethanol/g glucose produced with a 79% yield [12]. Pyrolytic sugars from poplar were separated from phenols by solvent extraction and were hydrolyzed into glucose using H_2SO_4 as catalyst. The fermentation of poplar hydrolyzed to produce ethanol was carried out by *Saccharomyces cerevisiae*. The yield obtained was 0.473 g ethanol/g glucose [13].

Microwave

The waves, with frequencies between 0.3 GHz and 300 GHz, are absorbed by water, fats and sugars and their energy is transferred to organic molecules generating heat. This leads to the degradation of lignin, making cellulose and hemicellulose more accessible to enzymatic hydrolysis [14]. Lignocellulose–rich sweet sorghum biogases were pretreated and hydrolysed at the same time using microwave irradiation. An ethanol yield based on total sugar of 480 g kg^{-1} was obtained after 24 h of fermentation using a mixed culture of microorganisms [15]. Microwave pretreatment of oil palm empty fruit bunch beer, combined with alkaline conditions, was reported by Nomanbhay *et al.* [16]. A loss of 74% lignin, 24.5% holocellulose and a yield of total reducing sugars of 41% was obtained at the best operative conditions of 3% (w/v) NaOH at 180 W for 12 min [16].

3.2. Physicochemical processes

Steam Explosion

The steam explosion is one of the most currently used methods for the pretreatment of lignocellulosic biomasses. The high temperature (180 to 240 °C) combined to pressure from 1 to 3.5 MPa, followed by an explosive decompression allows one to obtain the breakdown of the rigid lignin structure fibers. The result is a better accessibility of cellulose for enzymatic hydrolysis and fermentation [17]. The optimal steam explosion conditions for sugarcane bagasse are 215 °C for 5 min at maximum operating pressure [18]. After pretreatment and enzymatic saccharification, an overall glucose yield of 86.8% of the content in raw material was achieved. The steam explosion of canola straw increases the glucose recovery in the saccharification process, reaching an increment of 153.22% compared to the control samples, as shown by Garmakhany *et al.* [19].

Ammonia Fiber Explosion (AFEX)

The process consists of treatment with liquid anhydrous ammonia at high temperature (60–100 °C) and pressure (250–300 psi) for 5 min, fol-

lowed by a rapid decrease of the pressure. This causes the disruption of the lignocellulosic structure and the de–crystallization of cellulose, but the lignin remains unchanged; the lignocellulosic biomasses characterized by a higher content of lignin are not efficiently pretreated with this method [20]. The AFEX pretreatment of lignocellulosic materials derived from forages and agricultural residues was performed by Belkacemi *et al.* [21]. The saccharification of the pretreated materials, without any detoxification, allows one to obtain a 60 to 80% of theoretical yield of sugars. Subsequent ethanol fermentation of the hydrolysate by *Pachysolen tannophilus* ATCC 32691 resulted in a theoretical yield of 40–60% after 24 h. AFEX followed by enzymatic hydrolysis has been applied to coastal Bermuda grass by Myoung *et al.* [22]. Pretreatment at 100 °C for 30 min produced 94.8% of theoretical sugar yield, corresponding to the maximum sugar yield achieved with the AFEX treatment.

3.3. Chemical processes

Alkaline Hydrolysis

Alkaline hydrolysis (mainly lime ($CaO/Ca(OH)_2$), NaOH, Na_2CO_3) is effective in removing lignin and improving the subsequent enzymatic hydrolysis of the pretreated biomass. Its advantage with respect to other pretreatment technologies is the use of lower temperatures and pressures; however, it requires more time, hours or days rather than minutes or seconds. Compared with the other kinds of pretreatment, alkaline hydrolysis represents a slow process, requires neutralization, and the recovery of added alkali [23]. The optimization of alkaline pretreatment of coffee pulp for ethanol fermentation was performed by Menezes *et al.* [24]. The pretreatment using 4% (w/v) NaOH for 25 min gave the best results with a recovery of cellulose pulp of 69.18% and the production of 13.66 g/L of ethanol with a yield of 0.4 g ethanol/g glucose after the fermentation step. Alvarez *et al.* [25] used alkaline hydrolysis to pretreat different kinds of pine residues. They showed that the hydrolysis yield mainly depends on temperature and alkali concentration. Among the tested operative conditions, 2.5% NaOH for 90 min at 120 °C resulted in the best yield of glucose (41.33% w/w) after enzymatic hydrolysis.

The process could theoretically produce a maximum yield of 90.19% of ethanol/substrate (glucose) and about 80 L of bioethanol per dry ton of woody biomass.

Acid Hydrolysis

Acid hydrolysis represents a widely used pretreatment method because the use of concentrated or diluted acids (usually between 0.2% and 2.5% w/w) at temperatures between 130–210 °C allows one to obtain high yields of sugars [26]. The hemicellulose, easier to be hydrolyzed than cellulose, is attacked by acid solutions [20]. Depending on the reaction conditions, such as temperature, acid concentration and hydrolysis time, the amount of sugar decomposition products, e.g. furfural and 5–hydromethylfurfural (5–HMF), that inhibit the subsequent ethanol fermentation, changes [27]. The hydrolysate obtained by the sulphuric acid pretreatment of *Curcuma longa* waste was directly fermented without a detoxification step as shown by Nguyen *et al.* [28]. The acid hydrolysis was carried out at 122.68 °C with 4.91% sulphuric acid for 50 min, obtaining an ethanol yield of 30.57%. Some acid compounds can be used as pretreatment catalysts, but also as a nitrogen source in the fermentation process for bioethanol production. Rice straw was pretreated with 0.65% nitric acid at 158.8 °C for 5.86 min as reported by Kim *et al.* [29]. After neutralization, the pretreated rice straw was used in the fermentation run without adding any nitrogen sources, reaching an ethanol yield from 10.92 g/L to 14.50 g/L by using *Pichia stipitis*.

Ozonolysis

Ozone attacks the aromatic rings of the lignin structure without damaging hemicellulose and cellulose. The process is affected by ozone concentration, biomass type and moisture content [14]. The ozonolysis of sugarcane bagasse in a fixed bed reactor at room temperature was performed by Travaini *et al.* [30] to evaluate the best samples moisture and ozone concentrations for sugar saccharification. The ozonolysis, under the best experimental conditions (40% sample moisture and 3.44% ozone), increased fermentable carbohydrate release considerably during enzymatic hydrolysis. Glucose and

xylose yields increased from 6.64% and 2.05%, for raw bagasse, to 41.79% and 52.44% for pretreated bagasse. The ozonolysis process is relatively expensive due to large requirements of ozone, but has some advantages: high dry matter concentrations (45–60%), very low production of inhibitory products, and reactions performed at atmospheric conditions [31]. The negative issues are the ineffective removal of lignin and the low yields of reducing sugars. However, combination of ozonolysis with other pretreatment processes has shown promising results. Ozonolysis was studied separately and in combination with wet disk milling (WDM) for the pretreatment of sugarcane bagasse and straw, with the aim of improving their enzymatic saccharification. The use of WDM followed by ozonolysis resulted in glucose yields of 81.1% for bagasse and 92.4% for straw, with shorter WDM times [32].

Organosolv

The use of organic solvent or mixtures of solvents in combination with water causes hydrolysis of the internal bonds in lignin and also of those between lignin and hemicellulose. Ethanol, methanol, acetone, and ethylene glycol are the solvents commonly used in the process. Besides the use of solvents, high temperatures (200 °C) are required, but depending on the type of biomass and the catalyst used, lower temperatures can be sufficient. It could be possible to use organic and inorganic acids as catalysts [14]. Geng *et al.* [33] showed that the addition of an acid catalyst in the organosolv pretreatment of horticultural waste, followed by H_2O_2 post pretreatment, was feasible. The enzymatic hydrolysis of the pretreated waste resulted in a hydrolysate containing 26.9 g/L reducing sugar, while the following fermentation by using *Saccharomyces cerevisiae* produced 11.69 g/L ethanol. Different organosolv operative conditions for the pretreatment of rice straw were tested by Amiri *et al.* [34], the best conditions resulting in 75% (v/v) aqueous ethanol containing 1% w/w sulphuric acid at 180 °C for 30 min. The enzymatic hydrolysis of the pretreated straw allowed one to obtain a glucose yield of 46.2%, which was then fermented to 22.5 g ethanol by *Clostridium acetobutylicum* NRRL B–591.

3.4. *Biological processes*

Biological pretreatment can represent the most environmentally friendly method for the delignification of lignocellulosic biomasses, since it does not require energy and chemical compounds. Although biological pretreatment is considered a promising technology because of its environmental advantages, the process has not been largely applied because it requires a long time (about 3–5 months), results in very high feedstock loss during pretreatment and because the rate of following hydrolysis is very low [35]. The best effective microorganisms that can be employed in the biological delignification are the brown and white rot fungi, which synthesize ligninolytic enzymes. Brown rot attacks cellulose while white and soft rots attack both cellulose and lignin [14]. The biological pretreatment of the rubber wood (*Hevea brasiliensis*) with the white rot fungus *Ceriporiopsis subvermispora* was investigated by Nazarpour *et al.* [36]. After 90 days of fermentation, the lignin and hemicelluloses loss was 45.06% and 42.08%, respectively, while the loss of cellulose was very low (9.50%). This treatment increased the sugar yield to about 27.67% during the subsequently hydrolysis. The hydrolysate obtained from wheat straw pretreated with the white–rot fungus *Irpex lacteus* was fermented by the yeast *Pachysolen tannophilus*. The ethanol yield obtained was between 23 and 35% greater than the yields typically obtained with a conventional process, in which wheat straw is pretreated using steam explosion and fermented with the yeast *Saccharomyces cerevisiae* [37].

4. Hydrolysis of polysaccharides fraction of lignocelluloses for ethanol production

After pretreatment, the released cellulose and hemicelluloses are hydrolyzed into fermentable monomeric sugars (hexoses and pentoses). The hydrolysis of (hemi)cellulose remains a major bottleneck for the efficient production of ethanol [38, 39].

Two major methods can be employed to carry out the hydrolysis process. The first method involves acids as catalysts, while the second uses enzymes known as cellulases [40, 41]. The acid hydrolysis degrades the cellulose and hemicelluloses polymers into monomeric sugars with-

out preliminary pretreatment of biomass. It can be carried out using concentrated or diluted acids. The concentrated acid hydrolysis, with sulphuric and hydrochloric acids, is the mainly used method for the hydrolysis of lignocellulosic biomass [27] producing high hydrolysis yields of cellulose [40]. However, this process requires large quantities of acids, expensive acid recycling and causes degradation of monosaccharides.

It follows that enzymatic hydrolysis is the generally preferred method due to the mild operation conditions, higher conversion yields, low energy cost, and less corrosive and toxic conditions compared to the acid hydrolysis [20, 42, and 43]. However, the enzymes involved in the enzymatic hydrolysis have higher costs and this represent the main bottleneck in lignocellulosic ethanol production [44].

4.1. *Enzymatic hydrolysis*

As reviewed in Kumar and Murthy [42], the enzymatic hydrolysis process can be divided into two phases. The first step is represented by enzymatic depolymerization in which long polysaccharide chains are hydrolyzed to soluble oligomers. In the second step, oligomers are hydrolyzed to sugar monomers. The rate–limiting step in the process is considered the first hydrolysis phase.

Due to the complexity of the lignocellulosic carbohydrates, the activities of multiple hydrolytic enzymes are required for complete deconstruction of the various components of the lignocellulosic biomass; these enzymes are produced as either *free cellulases* or *complexed cellulases*, called cellulosomes [43, 45]. The efficient enzyme mixture comprises ten different enzymes. Among these, at least 6 can be considered crucial, depending on the composition of the raw materials [46]. Enzymes involved in lignocellulosic biomass are following described.

Cellulase enzymes

Cellulases are glycosyl hydrolases (GH) involved in cellulose hydrolysis, whose classification is available on the CArbohydrate–Active EnZymes web site (CAZY: www.cazy.org). These enzymes are con-

ventionally divided in three major classes: endoglucanases, exoglucanases and β–glucosidases [44, 47, and 48].

Endoglucanases (EG) or 1,4–beta–glucanases (EC 3.2.1.4) randomly hydrolyze intramolecular β–1,4–glucosidic linkages, creating new chain–ends; exoglucanases (CBH) or cellobiohydrolases (EC 3.2.91) hydrolyze the 1,4–glycocidyl linkages from the reducing or non–reducing ends to form cellobiose; β–glucosidases (BG) (EC 3.2.1.21) hydrolyze cello–oligosaccharides and cellobiose into glucose eliminating cellobiose inhibition.

Fungi and bacteria have been reported to be able to produce cellulases. In particular, the fungus *Trichoderma reesei* is the preferred industrial source of cellulases and hemicellulases due to the high level of secreted enzymes. This fungus produces at least two CBHs, five EGs, and two BGs whose expression is finely regulated [49]. However, this fungus produces low levels of BGs if compared to *Aspergillus sp.* *Trichoderma* strains engineered with extra β–glucosidase have been studied. In particular Nakazawa *et al.*[50] reported a recombinant *T. reesei* strain expressing *A. Aculeatus* BGL1. The resulting strain appears more suitable for cellulose hydrolysis. Among bacteria, several *Bacillus* strains isolated from natural habitats were shown to be able to produce cellulase activities [51].

Cellulosome systems are multi–enzymatic complexes produced mainly by anaerobic bacteria but also by other bacteria and few anaerobic fungi [40, 48, and 52]. In these systems there are two subunit types, the scaffolding and the enzymatic subunits. The structural scaffolding subunits contain a carbohydrate–binding module that binds the cellulose surface and multiple copies of cohesins (named dockerins) that interact with the enzymatic subunits, CBD (cellulose binding domains) and CBM (carbohydrates binding modules).

Hemicellulase enzymes

In addition to the three major groups of cellulose enzymes, there is also a number of hemicellulases which hydrolyze hemicellulose. Due to the more varied composition of this polysaccharide, enzymes degrading hemicellulose are divided into two major groups: depolymerising enzymes, which cleave the backbone, and enzymes that remove substituents. These enzymes act synergistically to hydrolyze lignocellulosic polysaccharides [48].

Xylanase

Most studies on hemicellulases were focused on enzymes that hydrolyze xylan. According to the CAZY database, these enzymes include GH 3, 30, 39, 43, 52, 54, 116 and 120.

The complete degradation of xylan requires the cooperative action of endoxylanase and xylosidase.

Endo–1,4–β–xylanase (1,4–b–d–xylan xylanohydrolases, EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone releasing shorter xylo–oligosaccharides; β–xylosidase (1,4–b–d–xylan xylohydrolase, EC 3.2.1.37) cleaves the small xylo–oligosaccharides and cellobiose into xylose. These enzymes are produced by several microorganisms such as fungi, bacteria, yeast, and marine algae. Filamentous fungi are interesting producers because the enzymes are secreted at levels higher than those of yeasts and bacteria.

As reviewed by Van Dyk and Pletschke, [48] endoxylanases have different specificities. For example, family 11 xylanases prefer cleaving the xylan backbone in unsubstituted regions, while family 10 xylanases are able to cleave the xylan backbone closer to the substituents.

The synergic activity of several accessory enzymes which remove the various substituents linked to the backbone is also essential.

α–arabinofuranosidase

α–L–arabinofuranosidase (EC 3.2.1.55) removes the L–arabinose substituents from the xylan backbone. According to the CAZY database, arabinofuranosidases are present in GH 3, 43, 51, 54 and 62. All these families, except for GH 43 and 62, perform hydrolysis with retention of the anomeric configuration.

As reviewed by Van Dyk and Pletschke [48], α–L– arabinofuranosidases have different specificities cleaving 1,2 linkages or 1,3 linkages. Moreover, these enzymes are able to cleave doubly substituted arabinose residues from arabinoxylan. As reported by Lagaert et al [53], complete degradation of arabinoxylan needs the synergistic action of arabinofuranosidases and also of β–xylosidases. Rasmussen *et al.* [54] demonstrated that optimal hydrolysis of soluble wheat arabinoxylan requires the combined action of an endoxylanase, a β–xylosidase and the two types of arabinofuranosidases.

α–glucuronidase

α–glucuronidase (EC 3.2.1.139) removes 4–O–methyl glucuronic acid substituents from the glucuronoxylan. This enzyme hydrolyses the α–1,2 bonds between the glucuronic acid residues and β–D–xylopyranosyl backbone units [44].

Esterases

Esterases hydrolyse the ester linkages between xylose units of the xylan and acetic acid (Acetylxylan esterase, EC 3.1.1.72) or between arabinose side chain residues and phenolic acids such as p–coumaric acid (p–coumaric acid esterase EC 3.2.1.73) or ferulic acid (ferulic acid esterase, EC 3.2.1.73).

Acetylxylan esterase removes the O–acetyl groups from positions 2 and / or 3 on the β–D–xylopyranosyl residues of acetyl xylan.

Ferulic acid and p–coumaric acid esterases (EC 3.1.1.73) hydrolyze ester linkages on xylan, liberating the respective phenolic acids linked to the arabinofuranoside residues [48].

Mannanases

Mannose residues have α–1,6–galactose as side groups and these are acetylated at the O–2 and O–3 positions. The core enzymes for complete degradation of mannan into simple sugars are the endomannanase and β–mannosidase.

Endo–β–1,4–mannanases (EC 3.2.1.78) catalyze hydrolysis of the β–1,4–linked backbone within different mannans. In the CAZy database enzymes are classified into three different glycoside hydrolase families: GH5, GH26 and GH113 [55].

β–mannosidase (EC 3.2.1.25) is the key enzyme responsible for catalyzing random hydrolysis of manno–glycosidic bonds in the main chain. All plant mannanases belong to family GH5 subfamily 7 (GH5_7) [56].

Additional enzymes, such as α–galactosidases (EC 3.2.1.22), β– glucosidase (EC 3.2.1.21) and acetyl mannan esterases are required to remove side chain sugars on mannans.

5. Sugars fermentation for bioethanol production

Once lignin has been removed and the saccharification of the free accessible (hemi)cellulose portions of the biomass has been carried out, the final step to be performed is the fermentation of sugars into bioethanol.

Saccharomyces cerevisiae is the most widely used organism for ethanol production from hexoses, whilst *Pichia stipitis* and *Candida shehatae* are the main yeasts able to ferment both hexose and pentose sugars to ethanol. Bacteria belonging to the species *Clostridia* and *Zymomonas*, and fungi such as *Fusarium* spp. have been also demonstrated to be useful for ethanol production.

The current process is optimized for 6–carbon atoms sugars fermentation since most yeasts cannot ferment 5–carbon atoms sugars. Given that the economically competitive ethanol production from lignocellulosic materials requires the efficient use of both hexose and pentose monosaccharides, research is now strongly exploring new engineered yeasts able to ferment 5–carbon sugars with high yields. The main routes for ethanol fermentation are described below.

5.1. *Ethanol production by separate hydrolysis and fermentation (SHF)*

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, resulting in the quick and easy possibility to optimize each production step, with conduction of each process at the optimal conditions of pH and temperature.

An example of SHF process application for ethanol production is described by Erdei *et al.* [57], who reported an experiment of separate hydrolysis and co–fermentation (combining xylose and glucose fermentation) of steam–pretreated wheat straw (SPWS) combined with wheat starch hydrolysate feed, achieving an average yield of ethanol up to 86%.

5.2. *Ethanol production by simultaneous saccharification and fermentation, SSF*

In SSF, enzymatic hydrolysis and fermentation are carried out together, using a unique reactor. By 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, besides the possibility to make the fermentable sugars immediately available to yeasts.

However, the ideal pH or temperature conditions for the saccharification step may differ from those of the fermentation step. Thus, it is very difficult to find favorable conditions for both processes.

As generally stated, SSF is a much more competitive process in comparison to SHF from the economical point of view, since the use of a unique bioreactor results in a total reduction of investment and operational costs.

Recent works regarding SSF are those by Nahar and Pryor [58] and Rezic´ *et al.* [59], who used as feedstock for bioethanol production crushed whole sugar beets (ethanol yield up to 92%) and sugar beet pulp (ethanol yield up to 49%), respectively.

Hydrolysis and fermentation conditions for production of ethanol from very high–gravity cassava mash during a simultaneous saccharification and fermentation (SSF) process were optimized using a statistical methodology [60], reaching a final ethanol yield of 15.03% (wt.%) in 72 h.

5.3. *Ethanol production by consolidated bio processing, CBP*

Consolidated bio processing (CBP) has been so far recognized as the best system to reduce the cost of biomass processing, by combining the hydrolysis of the polysaccharides and the subsequent fermentation of the hexoses/pentose sugars.

Differently from SSF, besides the use of a unique reactor, in CBP a single engineered microorganism is used, which is able to directly convert (hemi)cellulose into ethanol using its own enzymatic machinery.

So far, there are no microorganisms able to perform both the enzymatic hydrolysis and the fermentative steps with high yield of both processes, thus genetic engineering must be applied to create suitable microorganisms for the CBP process.

It is worth noting that there are two main routes to perform CBP: CBP I route, characterized by the engineering of a cellulase producing microorganism to make it able to ferment sugars; CBP II route aimed at the engineering of an ethanologenic microorganism to make it able to produce cellulases or hemicellulases.

As recently reviewed by Olson *et al.* [39], progress in the development of genetic tools for fungal systems has been obtained, focusing mostly on the increase of cellulase production, especially for fungi like *Fusarium oxysporum* and *Trichoderma reesei*. There has also been a substantial progress in the development of genetic tools for free–enzyme bacterial systems, including *Clostridium phytofermentans*, *Clostridium japonicas*, *Thermoanaerobacter* and *Thermanaero–bacterium* sp. The latter, a thermophilic anaerobe that utilizes a broad range of substrates including xylan, is a prominent example of engineered organisms with recently developed genetic tools to produce a biofuel at high yield [61, 62].

CBP category I

Trichoderma, *Aspergillus*, *Rhizopus* and *Fusarium* are the potential filamentous fungi suitable for development of CBP type I. All of them produce high amounts of both cellulases and hemicellulases, leading to high yields of fermentable sugars. Particularly, *T. reesei* is the best producer of such activities, so far described.

These fungi have been reported as ethanol producers, even if with very low yield and high concentration of by–products, such as lactic acid.

As reviewed by Amore and Faraco [63], many efforts are focused on the elucidation of regulatory mechanisms, in order to advance the knowledge on the metabolic pathways involved in ethanol production by filamentous fungi recognized as potential candidates for CBP type I.

CBP category II

S. cerevisiae is the most used yeast for ethanol production from C6 sugars and it represents the best candidate for CBP type II. Thus, a big challenge is to confer to this microorganism the capability to produce cellulase and/or hemicellulase activities.

S. cerevisiae has been so far described as a suitable host for recombinant production of different enzymes, among which are also enzymes involved in the conversion of polysaccharides.

As previously reported, for the complete conversion of cellulose there is the need of three different enzymes namely endoglucanases, cellobiohydrolases and β–glucosidases, whose most common sources are fungi, especially *Trichoderma reesei*, and bacteria.

Cellobiohydrolases (CBH) and endoglucanases have been successfully expressed in *S. cerevisiae*, whilst very low yields of fungal β–glucosidase have been achieved.

Kluyveromyces marxianus is another interesting candidate for CBP II. It has been used with good results as host for heterologous proteins, including enzymes involved in cellulose hydrolysis.

Among bacteria, *E. coli* represents the main candidate for the CBP II category. It is able to metabolize a wide spectrum of sugars and a well–know microorganism to be genetically engineered.

Despite the extensive research performed to optimize *E. coli* recombinant cellulase production and reduce the addition of external cellulases, the potential of the obtained recombinant strains to directly grow on plant biomass cannot be proved, mainly due to the complex cocktail of enzyme needed for the complete lignocelluloses conversion into fermentable sugar, as reviewed by Amore *et al.* [64].

Zymomonas mobilis, *Klebsiella oxytoca* and *Bacillus subtilis* are other candidates for the CBP II process, being known for ethanol production and tolerance to the toxicity of the final products.

6. Conclusions

To overcome bottlenecks associated to first generation bioethanol, produced from traditional agricultural crops, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting strong interest, as it minimizes the conflict between land use for food (and feed) and energy production. Lignocelluloses are the most abundant renewable resources on Earth and include residual biomass such as agro–industrial wastes, agricultural and forest crop residues and the organic and paper fractions of municipal solid waste (MSW). Moreover, second generation ethanol

production and use show lower green–house gas emissions than the first generation fuels, reducing environmental impact. However, the process currently adopted for second generation bioethanol production is not competitive as yet. Lignocellulose consists of three types of polymers — cellulose, hemicellulose and lignin — bonded by both non–covalent and covalent cross linkages. Bioethanol production from lignocellulosic materials takes place in three phases, namely a pretreatment step to remove the lignin barrier, hydrolysis of cellulose and hemicellulose to generate fermentable sugars (saccharification) and fermentation of mixed hexose and pentose sugars to produce ethanol.

As regards the pretreatment step, all the techniques so far used (physical, physico–chemical and chemical processes), are not eco–friendly and generate inhibitors which negatively affect the next step of lignocellulose saccharification into fermentable sugars. Biological pretreatment could represent a valid alternative, but the long treatment time and the low final yield do not make it a feasible process, as yet.

Despite the high costs of enzyme production, enzymatic hydrolysis is preferred to the acid one. A wide spectrum of enzymes is required to completely convert pretreated lignocellulose into fermentable sugars, both cellulases, acting on the cellulose polymer for C6 sugars release, and hemicellulases, for C5 sugars production.

Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) are the main routes used to produce bioethanol by fermenting the hydrolysate from cellulose, even if consolidated bioprocessing (CBP) has been recognized as the best system to reduce the cost of biomass processing, by combining the hydrolysis of the polisaccharides and the subsequent fermentation of the hexose/pentose sugars.

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The following paper **“Consolidated Bioprocessing for Improving Cellulosic Ethanol Production” (Paper II)** reviews recent efforts aimed at developing category II CBP process, which involved genetic manipulation of yeasts naturally able to ferment sugars.

1.4.2 PAPER II: CONSOLIDATED BIOPROCESSING FOR IMPROVING CELLULOSIC ETHANOL PRODUCTION

Chapter 9
Consolidated Bioprocessing for Improving Cellulosic Ethanol Production

Antonella Amore, Simona Giacobbe and Vincenza Faraco

Abstract Consolidated bioprocessing (CBP) is a potential system for reducing costs of production of bioethanol from lignocelluloses, combining hydrolysis and fermentation into a unique reactor, and exploiting the ability of engineered microorganisms to perform both the reactions. CBP would represent a breakthrough for low-cost biomass processing, due to economic benefits of process integration and, mainly, avoiding the high costs of the cellulolytic enzymes. This chapter discusses the progresses achieved in the development of both the CBP category I and category II, where CBP category I deals with engineering of a cellulase producer to make it ethanologenic and category II consists in engineering an ethanologenic microorganism to render it cellulolytic.

Contents

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9.1 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. Being produced in huge amounts all over the world, lignocellulose represents the best alternative to fossil sources as feedstock for production of energy and fuels. Bioethanol production from lignocelluloses requires several steps such as the pre-treatment, for lignin removal and cellulose/hemicelluloses accessibility, the hydrolysis of the polysaccharides to achieve both hexoses and pentose fermentable sugars, the fermentation step for conversion of sugars into ethanol.

One of the major problems in producing ethanol from lignocellulosic biomass is the high production costs, particularly those associated with the hydrolysis step.

Consolidated bioprocessing (CBP) has been so far recognized as a potential system to reduce the cost of biomass processing, by combining the main events required for lignocellulose conversion into one reactor, particularly the hydrolysis of the polysaccharides and the subsequent fermentation of the hexoses/pentose sugars. However, no microorganism shows this combined ability, thus the development of "CBP microorganisms" is required by using the genetic engineering.

There are two main routes to perform CBP: category I CBP is based on the engineering of a cellulase producing microorganism to make it able to ferment sugars, while category II CBP aims at engineering ethanologenic microorganisms to make them cellulolytic.

Filamentous fungi like *Trichoderma, Aspergillus*, *Rhizopus,* and *Fusarium* have been identified as good candidates for CBP category I, *Trichoderma reesei* being the best candidate due to the high level of cellulase activity production, the deep knowledge of its physiology, and the availability of genetic manipulation tools.

As far as CBP category II is concerned, bacteria and yeasts are the best candidates. *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are the most investigated among the yeasts, while *Escherichia coli* and *Zymomonas mobilis* are the most interesting bacteria for CBP type II. Tools for genetic manipulation of these microorganisms have been so far developed and definition of growth conditions for concomitant cellulose hydrolysis and ethanologenesis results to be easier for them than for fungi.

This chapter reviews the recent developments in production of CBP microorganisms, showing the advantages and disadvantages of this process and the challenges to be faced.

9.2 Potential of Fungi as CBP Microorganisms

Amore and Faraco (2012) recently reported an overview of the state-of-the-art of CBP I Category.

Some filamentous fungi belonging to the genera *Trichoderma, Aspergillus*, *Rhizopus,* and *Fusarium* have been reported to possess the ability to directly ferment cellulose to ethanol, even if with very low yield or high concentration of by-products. This conversion ability is thought to depend on two metabolic routes, one route involving the production of cellulases to degrade cellulose to soluble sugars under aerobic conditions, the other producing ethanol and other by-products, such as acetic acid, under anaerobic conditions.

T. reesei stands out as a good CBP I candidate, because of the extensive knowledge of its physiology and the wide range of tools for its genetic manipulation. It has been so far reported as the main producer of cellulases and it has been shown to possess all the metabolic pathways necessary to fully utilize the lignocellulose sugars for production of ethanol, as reported by Xu et al. (2009). It can produce ethanol in the range 0.2–4.8 g L^{-1}, where the maximum yield is achieved by fermenting glucose. Nevertheless, some difficulties have to be faced to make this fungus an effective CBP microorganism, such as the low ethanol yield and productivity, the low ethanol tolerance and the high energy required for mixing during fermentation, due to its morphology. Moreover, the obligate aerobe nature of *T. reesei* hinders its growth without oxygen, this being an important issue to be faced in order to develop an ethanologenic *T. reesei* strain.

Several manuscripts have so far reported the potential of *Aspergillus spp.* for (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. Most of *Aspergilli* have been shown to be able to produce ethanol from glucose. For instance, *A. terreus* has been demonstrated able to ferment fructose and mannose, with a yield of 2.16 % (w/v) (85 % conversion) and 1.98 % (w/v) (78 % conversion), respectively (Pushalkar and Rao 1998).

However, the main limitations of a cellulose CBP based on *Aspergillus spp.* consist of the very low ethanol yields that these microorganisms exhibit in comparison with *S. cerevisiae*, and formation of the by-product lactate.

Fusarium spp. has been shown to be a great cellulase and hemicellulase producer. Moreover, the ability of this species to convert biomass directly to ethanol was shown more than 20 years ago for the first time. 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 have been investigated (Ruiz et al. 2007; Panagiotu et al. 2005). The highest ethanol yield—1.66 mol ethanol/mol of glucose, corresponding to 80 % of the theoretical maximum yield—was achieved under anaerobic conditions. *F. oxysporum* wild-type strain F3 was able to grow at a maximum specific growth rate of 0.023 h^{-1} on cellulose in aerobic conditions, and to produce ethanol with a yield

of 0.35 g/g cellulose under anaerobic conditions. However, for this specie, the low yield of lignocellulosic hydrolysis is the major bottleneck that does not make it a microorganism ready to be used in CBP process.

R. oryzae has been generally reported able to produce ethanol, but lactic acid has been reported to be an abundant by-product. For example, Fujio et al. (1985) reported growth of *R. koji* on uncooked cassava starch with a maximum productivity of ethanol of 2.3 g ethanol/Lh, which corresponds to the 50 % of the general ethanol yield of a common yeast. *R. javanicus* and *R. oryzae* have been shown to produce a maximum of 33 g L^{-1} and 32 g L^{-1} of ethanol from glucose, respectively (Skory et al. 1997; Stevenson et al. 2002). To the best of our knowledge, few studies have been so far reported on the production and characterization of cellulases by *Rhizopus* spp.. In fact, in comparison with other filamentous fungi, *Rhizopus* genome contains a very low number of Glycoside hydrolases (GHs) coding genes.

Thus, even if there are several fungi potentially involved in CBP process, many efforts are still needed for developing a suitable CBP type I process which allows high yield of ethanol from lignocellulose raw materials.

9.3 Potential of Yeasts as CBP Microorganisms

9.3.1 Saccharomyces cerevisiae

The well-known ethanol producing yeast *Saccharomyces cerevisiae* has been long employed for the industrial production of ethanol from hexose sugars (Nissen et al. 2000; Van Dijken et al. 2000; Kuyper et al. 2004) and it has been the most exploited host for developing a CBP. *S. cerevisiae* plays a major role in applied research due to its capacity to produce ethanol and carbon dioxide from sugars with high productivity, titer and yield. Different process operation modes have been used for ethanol production by *S. cerevisiae*: Simultaneous Saccharification and Fermentation processes (SSF) (Jung et al. 2013), Simultaneous Saccharification and Co-Fermentation (SSCF) (Jin et al. 2012; Ohgren et al. 2006; Olofsson et al. 2010), Separate Hydrolysis and Fermentation (SHF) (Tomas-Pejò et al. 2008), and Pre-saccharification and Simultaneous Saccharification and Fermentation (PSSF) (Moreno et al. 2012).

S. cerevisiae has many positive traits making it a suitable host for CBP, such as the high ethanol yields (~0.45–0.50 g EtOH/g glucose) and productivity from glucose (around ~2–5 g EtOHL-1 h-1), its GRAS (Generally Recognized As Safe) status, the ability to grow at low pHs, the tolerance toward osmotic stress and inhibitors, robustness in industrial fermentation and amenability to genetic manipulations (Li and Elledge 2007; Shao et al. 2009; Gibson 2009; la Grange et al. 2010). Moreover, it produces several glucanase activities (Farkas et al. 1973; Larriba et al. 1995), such as a cell wall-bound endo-β-1,3-glucanase, encoded by

BLG2 (Mrsa et al. 1993), extracellular exo-β-1,3-glucanases EGX1 (BGL1) and EGX2 (Nebreda et al. 1986; Kuranda and Robbins 1987; Van Rensburg et al. 1997) with β-1,3- and β-1,6-activities (Nombela et al. 1988), an intracellular sporulation specific exo-β-1,3-glucanase (encoded by SSG1 or SPR1; San Segundo et al. 1993; Muthukumar et al. 1993), but no glucanase with β-1,4-activity.

However, this yeast has several shortcomings in terms of CBP-processing organism such as the inability to grow on complex substrates like lignocellulose, to hydrolyze polysaccharides (cellulose and hemicellulose) and to ferment pentose sugars. Thus, one of the main challenges to exploit *S. cerevisiae* as a CBP organism is to confer it the ability to hydrolyze the insoluble polysaccharides present in lignocellulosic biomass into monomeric sugars.

Heterologous expression of cellulases in *S. cerevisiae* has been an object of investigation for long time (Van Rensburg et al. 1998; Fujita et al. 2002, 2003) and there are several works concerning the expression of cellulase encoding genes in this yeast (Van Zyl et al. 2007, 2011; Ilmen et al. 2011; Den Haan et al. 2007a).

As reported in Table 9.1 where cellulase components expressed in *S. cerevisiae* are listed, cellobiohydrolases (CBH) and endoglucanases (EG) have been successfully expressed in *S. cerevisiae*, EG production being much more successful than CBH production (Den Haan et al. 2007a, b; Hong et al. 2003; Takada et al. 1998). Penttilä et al. (1988) expressed CBH genes from *T. reesei* in *S. cerevisiae*, achieving a yield of 100 and 2 mg L^{-1} for CBHII and CBHI, respectively. Recently, Den Haan et al. (2007a) reported the expression of four fungal individual CBH genes including two from *T. reesei* (*cbh1* and *cbh2*), one from *Aspergillus Niger* (*cbhB*), and one from *Phanerochaete chrysosporium* (*cbh1–4*). The activity expression level of the *cbh2* from *T. reesei* was significantly higher than the other CBHs, correlating well with the results of Penttilä et al. (1988). However, all studies demonstrated that the expression of fungal CBH genes in *S. cerevisiae* is too low to allow an efficient CBP and that *S. cerevisiae* is still unable to convert crystalline cellulose into fermentable sugars.

β-glucosidases (BGL) production in *S. cerevisiae* has been also reported by Pentillä et al. (1984). However, only one out of the five BGL genes from *Aspergillus* studied in this work was found to be expressed in the yeast, and even at a low level. Van Rooyen et al. (2005) showed the ability to ferment cellobiose by a recombinant *S. cerevisiae* strain possessing BGL encoding genes. In particular, the recombinant *S. cerevisiae* strain secreting BGL1 from *Saccharomycopsis fibuligera* (Y294[SFI]) was identified as the best strain with an ethanol yield of 0.41 g per gram of cellobiose consumed, which correspond to 89 % of maximum yield of the wild- type strain when it was grown on glucose. Cho et al. (1999) showed that during SSF experiments with a *S. cerevisiae* strain producing a BGL and an enzyme with both exo- and endo- cellulase activities, the need of additional cellulases decreases. The co-expression and surface display of EG II and CBHII from *T. reesei* and BGL from *Aspergillus aculeatus* resulted in a strain able to convert phosphoric acid swollen cellulose (PASC) to ethanol with a yield of 3 g L^{-1} from 10 g L^{-1} PASC in 40 h (Fujita et al. 2004). A *S. cerevisiae* strain co-expressing endoglucanase (EG) from *T. reesei* and BGL from *Saccharomycopsis fibuligera*

Table 9.1 Cellulases expressed in *S. cerevisiae*

Enzyme	Organism	References
CBHI	*Trichoderma reesei*	Penttila et al. (1988), Den Haan et al. (2007a)
CBHB	*Aspergillus niger*	Den Haan et al. (2007a)
CBH1–4	*Phanerochaete chrysosporium*	Den Haan et al. (2007a), Van Rensburg et al. (1998)
CBHI	*Thermoascus aurantiacus*	Hong et al. (2003)
CBHI	*Aspergillus aculeatus*	Takada et al. (1998)
CBH II	*Trichoderma reesei*	Penttila et al. (1988), Den Haan et al. (2007a), Fujita et al. (2004)
EGII	*Trichoderma reesei*	Fujita et al. (2002)
eg1	*Thermoascus aurantiacus*	Hong et al. (2003)
END1	*Butyrivibrio fibrisolvens*	Van Rensburg et al. (1997)
Endo/exo bifunctional enzyme	*Bacillus circulans*	Cho et al. (1999)
BEG1	*Bacillus subtilis*	Van Rensburg et al. (1997)
BGLI	*Aspergillus aculeatus*	Takada et al. (1998)
BGLI	*Saccharomycopsis fibuligera*	Den Haan et al. (2007b)
BGL	*Bacillus circulans*	Cho et al. (1999)
BGLI	*Endomyces fibuliger*	Van Rensburg et al. (1998)
CEL1	*Ruminococcus flavefaciens*	Van Rensburg et al. (1997)
BGL	*Aspergillus niger*	Pentillä et al. 1984)

was also produced, showing the ability to grow on and convert PASC to ethanol with a yield of 1 g L^{-1} from 1 g L^{-1} PASC (van Zyl et al. 2011). In order to optimize cellulase expression levels, different cellulase expression cassettes were integrated into *S. cerevisiae* chromosome in one step; the obtained strain expressed BGL, EG, and CBH and was shown able to hydrolyze PASC with a yield of 7.5 g L^{-1} ethanol in 72 h of fermentation at 37 °C (Yamada et al. 2011).

Several studies have been performed to develop *S. cerevisiae* strains able to ferment hemicellulose (Kuyper et al. 2005; Hahn-Hägerdal et al. 2001; Katahira et al. 2004). Hemicellulolytic enzymes, such as β-xylanase, β-xylosidase, and auxiliary enzymes, such as β-glucuronidase and arabinofuranosidase, have also been successfully produced in *S. cerevisiae* (Table 9.2) (La Grange et al. 1996, 1997, 2000; Crous et al. 1996; Ho et al., 1999). Sakamoto et al. (2012) showed that co-displaying genes encoding for endoxylanase from *T. reesei*, β-xylosidase from *Aspergillus oryzae* and BGL from *A. aculeatus* on the surface of xylose-utilizing *S. cerevisiae* cells, an ethanol titer of 8.2 g L^{-1} after 72 h of rice straw fermentation can be achieved. Furthermore, laboratory and industrial yeast strains have also been engineered to co-ferment D-xylose and L-arabinose (Becker and Boles 2003; Karhumaa et al. 2006) and to co-ferment xylose and cellobiose (Cho et al. 1999). In particular, Karhumaa et al. (2006) described genetical engineering of *S. cerevisiae* strains to co-ferment the pentose sugars D-xylose and L-arabinose, showing

Table 9.2 Hemicellulases expressed in *S. cerevisiae*

Enzyme	Organism	References
XYN2	*Trichoderma reesei*	La Grange et al. (1996)
XYNB	*Bacillus pumilus*	La Grange et al. (1997)
XYLA	*Aspergillus oryzae*	Katahira et al. (2004)
ABFB	*Aspergillus niger*	Crous et al. (1996)

that the co-utilization of arabinose together with xylose significantly reduced arabitol yield and increased ethanol yield from both xylose and arabinose.

Recently, the cellodextrin transporter of *Neurospora crassa* was expressed in *S. cerevisiae*, resulting in a strain able to grow on cellobiose (Galazka et al. 2010). Ha et al. (2011) reported that xylose fermenting strain, that also produces cellodextrin transporter, shows inhibition of xylose utilization by glucose. The strain expressing both cellodextrin transporter and BGL co-fermented cellobiose and xylose simultaneously and exhibited improved ethanol yield. In particular this resulting strain consumed 40 g L^{-1} of cellobiose within 24 h, producing 16.8 g L^{-1} of ethanol.

The expression of lactose permease encoding gene from *Kluyveromyces lactis* (*lac1*) facilitates the transport of cellobiose in *S. cerevisiae*, as shown by Sadie et al. (2011). Moreover, they demonstrated that a *S. cerevisiae* strain co-expressing *lac1* gene and *Clostridium stercorarium* cellobiose phosphorylase coding gene was able to grow on cellobiose as only carbon source.

Most of the cellulases produced in *S. cerevisiae* are non-complex or free enzymes, differently from the intricate hydrolytic enzyme complex—named cellulosome—that cellulolytic anaerobes use for biomass degradation (Devaux 2004; Doi 2008; Doi et al. 1998, 2003; Fierobe et al. 1999, 2005, 2008). Therefore, another approach to increase the hydrolytic activity of yeasts is to mimic the cellulosome complex. For instance, Lilly et al. (2009) reported the expression of *Clostridium cellulolyticum* mini-cellulosome on *S. cerevisiae* cells surface. The functionality of the minicellulosome was demonstrated by several experiments. Particularly, they demonstrated a two-fold increase of endoglucanase enzyme activity in *S. cerevisiae* cells expressing the minicellulosome compared with the wild-type strain.

Tsai et al. (2009) studied the in vitro assembly of a tri-functional mini-cellulosome containing an EG, an exoglucanase, and a BGL, thus obtaining a yeast able to hydrolyze cellulose and ferment PASC to ethanol. The levels of ethanol production and PASC hydrolysis were directly correlated with the number of cellulases docked on the cell surface and the maximum yield of ethanol was of 3.5 g L^{-1} (0.49 g ethanol/g sugar consumed) after 48 h, corresponding to 95 % of the theoretical ethanol yield.

A similar approach was followed by Wen et al. (2010) who engineered *S. cerevisiae* strains with uni, bi, and trifunctional minicellulosomes. These minicellulosomes consisted of a miniscaffold containing a cellulose-binding domain, three cohesion modules, and up to three types of cellulases. It was shown that the

recombinant yeast cells displaying the trifunctional minicellulosome had the ability to breakdown and ferment PASC to ethanol with a titer of 1.8 g L^{-1}.

Although several studies have reported coexpression of multiple genes in *S. cerevisiae* strains, many issues remain. In particular, the main challenges to be met are the poor growth of the engineered strains, the low yields, and titers of ethanol and limited substrate range. Moreover, these strains are often sensitive to the inhibitors present in lignocellulose biomasses and also have low ethanol tolerance.

9.3.2 Kluyveromyces marxianus

Kluyveromyces spp. yeasts are currently mainly used as an alternative to *S. cerevisiae* (Pecota and Da Silva 2005; Wolf et al. 2003). *Kluyveromyces marxianus* is a facultative fermentative and Crabtree-negative yeast (van Dijken et al. 1993), firstly described in 1888 (Lodder and Kreger-van Rij 1952). However, *K. lactis* was chosen by the scientific community as the model organism for the *Kluyveromyces* genus, probably due to the fact that strains belonging to the yeast species *K. marxianus* were isolated from a great variety of habitats, which results in a high metabolic diversity and a substantial degree of intraspecific polymorphisms that also represents one of the advantages of *K. marxianus*.

When compared to *K. lactis, K. marxianus* shows interesting properties that make it a potential candidate for a wide range of biotechnological applications, such as CBP. These properties include its GRAS status, its ability to grow on a broad variety of substrates and at high temperatures, its high specific growth rates (Singh et al. 1998), and its propension to produce ethanol even in the presence of sugar excess (Rouwenhorst et al. 1988; Steensma et al. 1988; Bellaver et al. 2004). Moreover, *K. marxianus* possesses the natural ability to excrete enzymes. This is a desired property for cost-efficient downstream processing of low- and medium-value enzymes (Hensing et al. 1994). Examples of *K. marxianus* application in industrial field include the production of ethanol (Singh et al. 1998; Kourkoutas et al. 2002), cell protein (Kim et al. 1998; Grba et al. 2002; Schultz et al. 2006), enzymes, such as inulinase β-galactosidase, β-glucosidase, and polygalacturonases (Rouwenhorst et al. 1988; Hensing et al. 1994, 1995; Passador-Gurgel et al. 1996; Rajoka and Shahid 2003; Rajoka et al. 2004), pectinase (Cruz-Guerrero et al. 1999). It is worth noting its use as baker's yeast (Caballero et al. 1995), and as a host for heterologous protein production (Swinkels et al. 1993; van den Berg et al. 1990). Physiological studies of this yeast started in the 1990s, focusing the attention on the regulation of respiration, fermentation, and on the so-called Crabtree-effect (van Urk et al. 1990; Verduyn et al. 1992). It was then shown that *K. marxianus* presents a strong Crabtree-negative character, since no ethanol production was observed after a glucose pulse applied to respiring cells, in contrast to what is commonly observed in *S. cerevisiae* (Kiers et al. 1998; Bellaver et al. 2004). Different process operation modes have been used for ethanol production by *K. marxianus*: batch cultures with elevated substrate concentrations (Grubb and

Mawson 1993; Barron et al. 1996), fed-batch systems (Ferrari et al. 1994; Gough et al. 1998; Love et al. 1996), continuous system (Love et al. 1998), membrane recycle bioreactors (Tin and Mawson 1993), two-stage fermentation (Hack et al. 1994; Banat et al. 1996), extractive fed-batch cultures (Jones et al. 1993), immobilization with β-galactosidase (Hahn-Hägerdal 1985), cells calcium-alginate immobilization (Bajpai and Margaritis 1987a, b; Marwaha et al. 1988; Nolan et al. 1994; Riordan et al. 1996; Barron et al. 1996; Brady et al. 1996, 1997a, b, 1998; Ferguson et al. 1998; Gough and Mchale 1998), immobilization of cells in poly(vinylalcohol) cryogel beads (Gough et al. 1998), or in Kissiris (a mineral glass foam derived from lava) (Nigam et al. 1997; Love et al. 1996, 1998).

K. marxianus has been also used in SSF processes with added hydrolytic enzymes (Barron et al. 1995b, 1996, 1997; Boyle et al. 1997; Nilsson et al. 1995; Ballesteros et al. 2002a, b, 2004; Kádár et al. 2004), cloning heterologous cellulase genes (Hong et al. 2007) or developing mixed cultures (Ward et al. 1995).

Ethanol production at elevated temperatures by *K. marxianus* has received much attention.

K. marxianus was shown to ferment carbohydrates to ethanol at temperatures above 40 °C with an efficiency in the range of 85–90 %. Moreover, it was shown to possess a maximum growth temperature in the range of 47–52 °C (Anderson et al. 1986; Hughes et al. 1984; Banat et al. 1992). Lower ethanol tolerance was observed when *K. marxianus* was compared to *S. cerevisiae*, and this was correlated with the activity of the plasma membrane ATPase (Rosa and Sa-Correia 1992; Fernanda and Sa-Correia 1992).

Hacking et al. (1984) screened *K. marxianus* strains for their ability to ferment glucose to ethanol at high temperatures. *Kluyveromyces* strains were shown more thermotolerant than *Saccharomyces*, which in turn can produce higher ethanol yields.

Sakanaka et al. (1996) reported protoplasts fusion of a thermotolerant strain of *K. marxianus* with a high ethanol producing strain of *S. cerevisiae*. The ethanol fermentation ability of fusants was as same as that of *K. marxianus* parental cells at 30 °C and was best at 43 °C. However, the amount of ethanol produced by fusants at 43 °C was about 3.0 % in 60 h, which was inferior to that at 30 °C. Moreover, the thermostability of the fusants was found to be lower than for either of the parental cells.

Schwan and Rose (1994) studied the effect of medium composition on ethanol production. They reported that growth and ethanol production in media containing galactose was not as high as in the presence of glucose as the carbon source and also the endopolygalacturonase (PG) secretion was lowered, while Duvnjak et al. (1987) found that galactose was a better carbon source for ethanol production than glucose; however, the strains employed in both works were different.

Xylose has also been described as a suitable carbon source for ethanol production by *K. marxianus* strains (Margaritis and Bajpai 1982). In particular, Margaritis and Bajpai (1982) reported a *K. marxianus* strain able to ferment xylose with an ethanol yield of 0.28 g per gram of d-xylose, which represents 55 % of theoretical yield.

K. marxianus strains used for SSF experiments at 42–45 °C in the presence of cellulases gave good results (Abdel-Banat et al. 2010a; Ballesteros et al. 2004; Boyle et al. 1997; Gough et al. 1996; Krishna et al. 2001; Lark et al. 1997; Oliva et al. 2004; Tómas-Pejó et al. 2009). In particular, Boyle et al. (1997) described ethanol production by *K. marxianus* strain in a SSF system at 45 °C; the ethanol yields amounted to 20 g ethanol from 100 g of straw.

Several heterologous genes have been expressed in *K. marxianus* using antibiotic resistant genes or auxotrophic marker genes (Pecota and Da Silva 2005).

Hong et al. (2007) reported the expression of thermostable cellulase genes encoding EG, BGL, and cellobiohydrolase in *K. marxianus* strain, resulting in a strain able to grow in synthetic medium containing cellobiose or carboxymethylcellulose (CMC) as the single carbon source. However, this strain was not able to ferment cellulosic material (CMC), probably due to a feedback inhibition of endoglucanase activity (Bok et al. 1998; Mosolova et al. 1993) or to a cell growth inhibition caused by CM-glucose (Rasmussen et al. 1988).

Recently, Yanase et al. (2010) showed the ability of a recombinant *K. marxianus* strain to convert cellulosic materials to ethanol. This strain was genetically engineered with a *T. reesei* endoglucanase (EG) and a *A. aculeatus* β-glucosidase (BGL) displayed on the cell surface, thus being able to convert a cellulosic β-glucan to ethanol directly. The maximum production of ethanol was achieved at 48 °C with an ethanol yield of 0.47 g per gram of β-glucan consumed, corresponding to 92.2 % of the theoretical yield; while in glucose fermentation, the wild-type strain of *K. marxianus* showed the highest production of ethanol at 40 °C. The recombinant strain was also able to produce ethanol from cellobiose with a yield of 43.4 g L^{-1} ethanol from 10 % cellobiose at 45 °C. Recombinant strains of this study have higher EG activity than previous strains (Hong et al. 2007). In particular, the high-BGL activity strain completely consumed cellobiose after 12 h of fermentation between 45 °C and 48 °C. In the case of cellobiose fermentation at elevated temperatures, glucose fermentation ability seems to be very important because of its reduction at above 48 °C and high-BGL activity in yeast.

Recently, Serrat et al. (2004) studied the combination of environmental and nutritional variables on ethanol and endopolygalacturonase co-production by *K. marxianus*. The authors reported the optimal conditions of simultaneous ethanol and endopolygalacturonase production achieving an ethanol yield of 47.6 g L^{-1}, corresponding to an increase of 22 % compared to the concentration obtained in suboptimal conditions. Recently, Yuan et al. (2011) reported for *K. marxianus* a CBP strategy that integrates inulinase production, saccharification of inulin contained in Jerusalem artichoke tubers, and ethanol production from sugars achieving 71 g L^{-1} ethanol in 48 h. Moreover, they demonstrated that *K. marxianus* possesses a distinctive superiority in the thermotolerance and utilization of inulin-type oligosaccharides reserved in Jerusalem artichoke tubers in comparison to *S. cerevisiae* (Yuan et al. 2011). For instance, *K. marxianus* was able to ferment Jerusalem artichoke tubers flour without any nutrients addition, achieving 90 % of theoretical ethanol yield at 40 °C, while *S. cerevisiae* produced 79.7 % of theoretical ethanol yield at 40 °C.

Besides *S. cerevisiae* and *K. marxianus*, several other yeasts are able to produce ethanol, as reported below.

9.3.3 Other Yeasts

Some native yeasts such as *Pichia stipitis* could be used in place of *S. cerevisiae* due to its ability to utilize xylose and hexoses (Jeffries 1996). In particular, *P. stipitis* (du Preez 1986) can utilize glucose, galactose, mannose, cellulose, and xylan as carbon source to produce ethanol. *P. stipitis* is superior to all other yeast species for ethanol production from xylose and it can convert xylose to ethanol at a high yield without xylitol formation. However, this native yeast has low ethanol tolerance. *P. stipitis* genome sequence has been recently published (http://www.jgi.doe.gov/pichia) (Jeffries 2007), showing several genes coding for enzymes involved in lignocellulose conversion such as α-glucosidase, xylanase, β-mannosidase, endo-1,4-β-glucanase, and exo-1,3-β-glucosidase, but low amounts of these enzymes are produced (Jeffries 2007). *P. stipitis* mainly produces β-glucosidases which degrade cellobiose to glucose, in turn fermented to ethanol. However, the ethanol production rate from glucose is at least five times lower than that obtained with *S. cerevisiae* (Jeffries 2007; Chandrakant 1998). *P. stipitis* is able to growth in the presence of L-arabinose as carbon source, while it does not show the ability to use polymeric cellulose as a carbon source (Nigam 2002). Xylose utilization by *P. stipitis* is regulated both by end-product repression by ethanol as well as by catabolite repression by glucose. Several glycoside hydrolases have been successfully expressed in *P. stipitis* in order to increase its capability to degrade polysaccharides. For instance, endoglucanase from *C. thermocellum* was produced in *P. stipitis* (Piotek et al. 1998). Den Haan and Van Zyl (2003) enhanced the xylanolytic ability of *P. stipitis* by co-expressing both xylanase of *T. reesei* and *Aspergillus kawachii* and xylosidase of *Aspergillus niger* encoding genes. The resulting strains were shown able to grow on medium containing on medium birchwood glucuronoxylan as sole carbohydrate source.

P. stipitis has an ethanol yield in the range of 33–57 g L^{-1}, however, 30 g L^{-1} is known as a critical concentration above which cells can not grow at 30 °C (du Preez 1987; Slinger et al. 1982). Although mutant strains of *P. stipitis* with increased ethanol tolerance were recently isolated, *P. stipitis* is not a very potent ethanol producer (Watanabe et al. 2011) and its maximum ethanol productivity is around 0.9 g L^{-1}/h (Jeffries 1996).

Hansenula polymorpha (*Pichia angusta*) is a thermotolerant methylotrophic yeast able to ferment a wide range of soluble sugars such as glucose, cellobiose, and xylose to ethanol (Ryabova 2003). It is worth noting that it has an optimal growth temperature of 37 °C or even higher up to 48–50 °C (Cabeca-Silva and Madiera-Lopes 1984; van Uden 1984). Moreover, it is resistant to both metals and

oxidative stress (Blazhenko et al. 2006; Mannazzu et al. 1998 and 2000). *H. polymorpha* is more ethanol tolerant than *P. stipitis* but more susceptible than *S. cerevisiae* (Ryabova et al. 2003). Several genes have been successfully expressed in *H. polymorpha* such as a thermostable endoglucanase and endoglucanases from *A. aculeatus* and *Humicola insolens* (Müller et al. 1998; Papendieck et al. 2002).

Since *H. polymorpha* cannot metabolize starchy materials or xylans, amylolytic and xylanolytic enzymes have been expressed in this yeast. For instance, both an alpha-amylase and a glucoamylase encoding genes from the yeast *Schwanniomyces occidentalis* have been expressed in *H. polymorpha*. *xyn2* gene encoding endoxylanase from *T. reseei* has also been successfully expressed in *H. polymorpha* and the co-expression of a *β*-xylosidase encoding gene from *A. niger* and the *xyn2* gene led to the production of transformants able to grow and perform alcoholic fermentation on a minimal medium supplemented with birchwood xylan as a sole carbon source (Voronovsky 2009).

In order to increase *H. polymorpha* fermenting ability, a pyruvate decarboxylase gene (*pdc1*) from *K. lactis* was expressed, resulting in increased ethanol production from xylose. Besides that, the introduction of multiple copies of the *H. polymorpha pdc1* gene showed a 20-fold increase in pyruvate decarboxylase activity and up to a 3-fold increase of ethanol production (Ishchuk 2008).

To improve both high-temperature resistance and fermentation ability of *H. polymorpha*, strains carrying deletion of acid trehalase gene (*ath1*) and overexpressing genes coding for heat-shock proteins were constructed. The recombinant strains have up to 12-fold increased tolerance to heat-shock treatment and to 5.8-fold improvement of ethanol production from xylose at 50 °C, even if the maximum ethanol concentration achieved from xylose was only 0.9 g L^{-1} (Ishchuk 2009).

Moreover, both its ethanol yield from xylose and ethanol resistance were increased through expression of *S. cerevisiae mpr1* gene encoding N-acetyltransferase. *H. polymorpha* recombinant strains harboring 1-3 copies of the *mpr1* gene showed enhanced tolerance to L-azetidine-2-carboxylic acid and ethanol (Ishchuk 2010).

Another candidate for CBP II is the thermotolerant yeast *Issatchenkia orientalis*. The *I. orientalis* MF-121 strain is a multistress-tolerant yeast, acid tolerant, salt tolerant, ethanol tolerant, and also thermotolerant. However, this yeast does not possess any genes coding for cellulase activity. Thus, recently it has been engineered with a *β*-glucosidase gene from *A. aculeatus* (Kitagawa 2010). The transformant strain produced 29 g L^{-1} of ethanol in 72 h at 40 °C during SSF in medium containing 100 g l^{-1} of microcrystalline cellulose and a commercial cellulase. Kwon et al. (2011) have recently isolated a new *I. orientalis* strain showing high ability to tolerate lignocellulosic inhibitory compounds and able to produce ethanol with a theoretical yield of 85 % per g of glucose at 42 °C.

9.4 Potential of Bacteria as CBP Microorganisms

9.4.1 Escherichia coli

Among bacteria, *E. coli* represents the main candidate for CBP II category since it is able to metabolize a wide spectrum of sugars and it is a well-know microorganism to be genetically engineered.

As reported by Ingram et al. (1987, 1998), in wild-type *E. coli*, pyruvate is converted to formate and acetyl-CoA, the latter being subsequently reduced to acetaldehyde by an aldehyde dehydrogenase and then to ethanol by an alcohol dehydrogenase, via Embden–Meyerhof–Parnas (EMP) pathway. Both these steps require NADH and the conversion of an equal amount of acetyl-CoA to acetate to maintain redox balance. Therefore, in native *E. coli*, only half of the available pyruvate is converted to ethanol differently from other ethanologenic microorganisms, like *Z. mobilis*, which produce one mole of ethanol for every mole of pyruvate thanks to the presence of a pyruvate decarboxylase.

Many efforts have been done to improve *E. coli* ethanol production yields, firstly the expression of the *Z. mobilis* pyruvate decarboxylase, achieving the *E. coli* KO11 strain which produces an amount of ethanol comparable to that by *Z. mobilis* which produces in fact the highest ethanol yields among Gram-negative bacteria (Ohta et al. 1991a). *E. coli* KO11 is able to utilize both mannitol and glucose. For instance, when the strain was cultured in *L. japonica* hydrolysate, mainly composed of mannitol and glucose, supplemented with Luria–Bertani medium and hydrolytic enzymes, a yield of 0.4 g ethanol per g of carbohydrate was obtained (Kim et al. 1998) .

However, *E. coli* strain KO11 still requires the addition of costly enzymes, such as cellulases, hemicellulases, pectinases to degrade the biomass into fermentable monomeric sugars and it is not able to ferment cellobiose which is known to have inhibitory effects on cellulose degradation (Holtzapple et al. 1990). Edwards et al. (2011) sequentially engineered *E. coli* KO11 strain with the *Klebsiella oxytoca* cellobiose phosphotransferase genes (*casAB*), a pectate lyase (*pelE*) from *Erwinia chrysanthemi*, the Sec-dependent pathway *out* genes from *E. chrysanthemi* and the oligogalacturonide lyase (*ogl*) from *E. chrysanthemi* to produce the strains LY40A, JP07, JP07C, and JP08C, respectively, which possess significant cellobiase activity, for ethanol production from pectin-rich lignocellulosic biomass. Particularly, *E. coli* strain LY40A produced 45 % more ethanol from model sugars than KO11, due to its capability to ferment cellobiose. JP07, JP07C, and JP08C produced concentrations of ethanol that were higher than those produced by KO11 (with an increase of around 15 %) but lower than those produced by LY40A.

The ethanologenic recombinant *E. coli* strain (FBR5) was created with the goal to produce ethanol from both hexoses and pentoses sugars, generated from pretreatment and hydrolysis of lignocellulosic biomass, such as glucose, xylose,513 arabinose, and galactose. The bacterium is stable without antibiotics and can tolerate ethanol up to 50 g L^{-1}, with a yield of around 45 g ethanol per L (Saha

and Cotta 2012) For instance, it has been shown to produce 40–50 g L^{-1} ethanol from 100 g L^{-1} xylose in batch reactors (Qureshi et al. 2012).

Several hydrolytic enzymes have been cloned into *E. coli* ethanologenic strains, but the main obstacle to an effective conversion of cellulose and hemicellulose biomass into fermentable sugars is the limited ability of the microorganism to secrete proteins which results in the accumulation of recombinant enzyme in the periplasmatic area.

However, most of the available studies so far reported describe the use of *E. coli* only as a suitable host of cellulase coding genes from different sources, for cellulase overproduction and characterization.

Among hydrolytic enzymes cloned in *E. coli*, a family 45 glycoside hydrolase from *Fibrobacter succinogenes* S85 (Park et al. 2007), CelC from *Salmonella typhimurium* UR (Yoo et al. 2004), Cel48Y, a noncellulosomic family 48 cellulase from *Clostridium thermocellum* (Berger et al. 2007) have been successfully produced.

Seven different bacterial genes, all producing cell-associated endoglucanase activity were expressed in *E.coli* KO11 strain by Wood et al. (1997). They demonstrated that *E. coli* KO11 can be used to produce recombinant endoglucanase as a co-product with ethanol. A production of 3,200 IU of recombinant *E. chrysanthemi* EGZ/L endoglucanase, equivalent to over 13 % of the commercial endoglucanase activity added to ferment crystalline cellulose, was achieved, demonstrating a new approach to reduce the amount of fungal cellulase required for the conversion of cellulose into ethanol. Amore et al. (2012) cloned a GH family 12 cellulase from a *Streptomyces* sp. G12 strain into *E. coli*, while recently, the first report on the cloning and expression of a GH family 43 β-xylosidase gene from thermophilic fungi in *E. coli* has been published by Teng et al. (2011) who cloned and extracellularly expressed a novel β-xylosidase gene (designated as PtXyl43) from thermophilic fungus *Paecilomyces thermophila.*

The secretion of the soluble cellulases/hemicellulases is the major prerequisite for CBP, thus many efforts have been done to increase secretion level of recombinant glycosyl hydrolases in *E. coli*.

Cellulase from *Bacillus subtilis* was cloned into *E. coli* and detectable extracellular secretion was achieved due to the presence of the signal peptide of the *B. subtilis* cellulase that might have specificity toward native protein secretion system in *E. coli* (Hinchliffe et al. 1984), while Lam et al. (1997) demonstrated an efficient secretion of an exoglucanase from *Cellulomonas fimi,* when fused to *ompA* sequence and expressed under a weak promoter (PlacUV5). Zhou et al. (1999) enabled *E. coli* to secrete more than 50 % of the recombinant Cel5Z from *E. chrysanthemi* by reconstructing the type II secretion system, encoded by the genes from *E. chrysanthemi*. In fact, at least three different types of protein secretion systems have been identified in Gram-negative bacteria, type II being the most widely used for protein secretion (la Grange et al. 2010).

Despite the extensive research performed to optimize *E. coli* recombinant cellulase production and reduce the addition of external cellulases, the recombinant strains so far obtained are still unable to directly grow on lignocellulosic

biomass, mainly due to the complex cocktail of enzyme needed for the complete lignocelluloses conversion into fermentable sugars. As a consequence, co-culture strategies have also been developed, to avoid the expression of multiple heterologous genes within a single cell.

For instance, Bokinsky et al. (2011) obtained the conversion of pretreated switchgrass through a co-culture consisting of *E. coli* strains capable of expressing either the cellulolytic or hemicellulolytic enzyme complexes, while Singh et al. (1998) developed a binary culture for efficient xylan conversion into fermentable sugars with two strains of *E. coli,* expressing a different combination of the six major xylanases.

However, the lack of a complete knowledge of the mechanism of glycosyl hydrolases limits the potential of *E. coli* as CBP II category microorganism. Metagenomic libraries represent an important tool for isolation of new genes which can be properly expressed in *E. coli,* thus overcoming the difficulty in design of suitable cellulase/hemicellulase expression system.

9.4.2 Zymomonas mobilis

Zymomonas mobilis is well known for its remarkably high ethanol production rate and tolerance to the toxicity of the final product (Lee 1980). It is able to ferment sugars at low pH and shows high resistance to the inhibitors produced during lignocellulosic pretreatment. Differently from *E. coli*, it ferments only glucose and fructose through the Entner–Doudoroff (ED) pathway but it has been successfully engineered to ferment also xylose and arabinose, by introduction of seven different genes encoding xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transaldolase, and transketolase (Zhang 1995a, 1995b; Deanda 1996).

Several strains of *Z. mobilis* possess extracellular glycosyl hydrolases active toward carboxymethylcellulose, suggesting the potential of *Z. mobilis* as a suitable host for cellulase recombinant expression.

Many manuscripts about ethanol production by SSF of *Z. mobilis* have been reported (Soleimani et al. 2012; Eklund and Zacchi 1995; Park et al. 1993; Yamada et al. 2002). Soleimani et al. (2012) reported an ethanol production of around 14.49 g L^{-1} (w/v) in the basal medium, which increased up to 86.26 g L^{-1} (v/v) in optimized nutritional conditions.

Few examples of cellulase recombinant expression in *Z. mobilis* are available. For instance, *Erwinia chrysanthemi cel5Z* was successfully expressed with a yield of 1,000 IU/L (Brestic-Goachet et al. (1989); *Acetobacter xylinum* cellulase gene and *Bacillus subtilis* endo-β-1,4-glucanase gene transfer into *Z. mobilis* has been reported by Okamoto et al. (1994) and Yoon et al. (2007), respectively. In most of the cases, the recombinant endoglucanases could not be secreted, mainly because of the protective outer membrane.

Expression of multiple cellulolytic enzymes is required for biomass conversion. Thus, Linger et al. (2010) expressed two cellulolytic enzymes, E1 and GH12 from *Acidothermus cellulolyticus*, in *Z. mobilis*. However, the lack of a secretion signal in their genes resulted in the localization of 96 % of GH12 activity within the cytoplasm, which decreased to 26 % when the *phoC* secretion signal was added.

9.4.3 Other Bacteria

Together with *E. coli*, *Klebsiella oxytoca* is one of the main promising Gram-negative bacteria for CBP II category. It is able to metabolize a wide spread of monomeric sugars present in the lignocellulose and it is known to possess four fermentative pathways: the pyruvate formate-lyase pathway, the lactic acid pathway, the succinate pathway, and the butanediol pathway (Ohta et al. 1991b).

K. oxytoca strain M5A1 has been engineered by introducing *Z. mobilis* genes for ethanol production, thus achieving the strain P2 able to utilize cellobiose and cellothriose for ethanol production. Maximum theoretical yields from glycolysis and fermentation were 0.51 g of ethanol per g of glucose, 0.536 g of ethanol per g of cellobiose, and 0.56 g of ethanol per g of cellulose (Wood and Ingram 1992).

It has been shown that the time taken by *K. oxytoca* P2 to produce up to about 33 g L^{-1} ethanol was much less than for any other organism investigated, including ethanol-tolerant strains of *Saccharomyces pastorianus*, *K. marxianus,* and *Z. mobilis*. However, *K. oxytoca* produces slightly less ethanol (maximum 36 g L^{-1}) than these organisms, reflecting its lower ethanol tolerance. Thus, co-cultures of *K. oxytoca* P2 with *S. pastorianus*, *K. marxianus,* or *Z. mobilis* have been developed demonstrating that the combination of *K. Oxytoca* P2 with thermotolerant yeasts or *Z. mobilis* is a good system for SSF (Golias et al. 2002).

Several cellulases have been expressed in *K. oxyotica* strains, in order to reduce the amount of commercial cellulase to be used in SSF. As a first attempt, a thermostable cellulase gene from *Clostridium thermocellum* has been expressed in *K. oxyotica* P2 strain by Wood and Ingram (1992), resulting in the hydrolysis of amorphous cellulose into cellobiose.

To improve *K. oxyotica* P2 strain SSF ability, Zouh et al. (2001) expressed *Erwinia chrysanthemi* genes *celY* and *celZ* encoding endoglucanases. Both were secreted with a total production level of 20,000 Units of CMCase activity per liter. Moreover, an increase in ethanol production up to 22 % more than the unmodified strain was achieved.

The *Z. mobilis* ethanol fermenting genes *pdc* and *adh* II were also cloned into three facultative anaerobic, Gram-negative cellulolytic bacteria, namely *Enterobacter cloacae* JV, *Proteus mirabilis* JV, and *Erwinia chrysanthemi* (Piriya et al. 2012). The latter was shown to be the best system for ethanol production in SSF experiments, due to both its higher tolerance to ethanol and its higher cellulase production.

Bacillus subtilis represents another candidate for CBP, being well known from the metabolic, biochemical, and physiological points of view. Moreover, it possesses polysaccharides degrading enzymes and it is able to produce secretory proteins. Romero et al. (2007) have produced an ethanologenic *B. subtilis* (strain BS36) through homologous recombination, disrupting the native *ldh* gene by chromosomal insertion of *pdc* and *adhB* genes from *Z. mobilis*, put under the control of the *ldh* native promoter, and inactivating the *alsS* gene involved in butanediol production.

9.5 Conclusions

This chapter discusses the advantages of consolidated bioprocessing as an alternative strategy to simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) techniques for reducing the cost of cellulosic ethanol production.

Both yeasts and bacteria are good candidate for CBP type II. However, the limited ability of these microorganisms to produce cellulolytic enzymes in sufficient quantity and quality for lignocellulose degradation is the main drawback in CBP type II process, thus pushing the interest toward filamentous fungi as CBP type I organisms, due to the ability of fungi to produce high amounts of cellulolytic enzymes. Development of new genetic tools for engineering filamentous fungi is needed to increase the ethanol yield and titers which are still low, mainly due to by-product formation and the slow rates of fermentation.

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1.5 MAIN CHALLENGES OF SECOND GENERATION ETHANOL PRODUCTION

The success in the commercial development of second generation biofuel requires to overcome the cost barriers of current technologies (Sims *et al*., 2010).

As described by Binod *et al*. (2011), the main factors that influence the enzymatic hydrolysis of lignocellulosic biomasses are substrate-related and enzyme-related factors.

The enzymatic hydrolysis of lignocellulosic biomass is affected by the nature of the substrate (Gao *et al*., 2013) such cellulose structure, accessible surface area, particle size and presence of lignin. In particular, the amount of lignin in the biomass plays an important role due to the fact that cellulases can irreversibly bind the lignin and consequently the release of monomeric sugars during enzymatic hydrolysis is low. Hence, the modifications occurring in the lignocellulosic biomass during pretreatment have a significant effect on sugar release and the enzymatic hydrolysis. Since lignocellulosic biomasses have different compositions depending on their source, an efficient and *ad hoc* pretreatment method is required to achieve their conversion into fermentable sugars.

Others important factors are related to the nature of the enzymes involved in enzymatic hydrolysis such as enzyme concentration, thermal and pH resistance and stability, synergistic action of enzymes and their irreversible binding to lignin. In the process of enzymatic hydrolysis, enzymatic have to work simultaneously towards the different components of lignocellulose; the synergism between the enzymes and their ratio/combination can increase the hydrolysis rate.

At present, the costs of (hemi)cellulolytic enzymes required to hydrolyze the polysaccharides account for more than 20% of the ethanol production costs. Due to high costs of enzymes involved in biomass hydrolysis, the use of tailor made enzyme cocktail, which contain all necessary activities with an optimal dosage of enzymes, is a possible strategy to decrease the cost of hydrolysis process (Gao *et al.*, 2011; Gao *et al*., 2010).

Improving both the effectiveness of (hemi)cellulolytic enzymes and the yield of the hydrolysis process, while reducing the needed amounts and their costs, are the main challenges that must be faced to make second-generation ethanol production commercially viable (Sun *et al*., 2002).

1.6 AIMS OF THE THESIS

This PhD is funded by the Industrial Research Project “Integrated agro-industrial chains with high energy efficiency for the development of eco-compatible processes of energy and biochemicals production from renewable sources and for the land valorization (EnerbioChem)” PON01_01966 (having Prof. Vincenza Faraco as scientific responsible). The research project EnerbioChem is aimed at the exploitation of no-food biomasses as raw-materials for industrial production of renewable energy and bio-chemicals, developing a new model of less dissipative system economy. A variety of investigations will be carried out on agronomic aspects for production of lignocellulosic and oleaginous biomasses and on optimization of processes for their transformation, minimizing waste production and optimizing energy efficiency and environmental/socio-economic impacts.

The activities of this project are mainly related to the work-package (WP) 3 of EnerbioChem “Definition of processes with high energetic efficiency for transformation of lignocellulosic biomasses at industrial level (including valorization of byproducts)”. The lignocellulosic biomasses for EnerbioChem are cultured on lands that are not suitable for food production such as hilly areas liable to erosion, high

salinity soils and polluted areas and they include *Arundo donax, Sorghum* (herbaceous), *Eucalyptus, Populus, Robinia pseudoacia* (woody) and *Cardoon*, *Tobacco, Sunflower* (agricultural residues). The main task of WP3 is the development of new biocatalysts for the optimization of bioprocesses for conversion of (hemi)cellulose into fermentable sugars to produce second-generation ethanol. This WP also includes the optimization of pretreatment of the lignocellulosic biomasses to remove lignin and improve accessibility of (hemi)cellulose. These activities will contribute to overcome the current constraints presently forbidding the industrial competitiveness of ethanol production, toward the concept of sustainable development.

The overall aim of this project is to improve the conversion of lignocellulosic biomass into sugars through i) **identification of novel accessory enzymes** (e.g. pectinases), ii) **development of tailor made enzymes** for transformation of (hemi)cellulosic fractions by directed evolution of selected hemicellulose, iii) **definition of tailor made enzymatic mixture** for biomass transformation.

As far as accessory enzymes are concerned, the strategy of this work was the selection of new microorganisms isolated from mature compost obtained from agro-industrial wastes.

The main goals concerning this task are:

1) Selection of new pectinolytic bacteria;

2) Evaluation of time courses of production of pectinolytic enzymatic activities involved in conversion of cellulosic biomass;

3) Identification and characterization of the pectinolytic enzymes produced by the selected bacteria in native form.

As regards the development of tailor made hemicellulases, the strategy of this work was the directed evolution of the enzyme α-L-arabinofuranosidase PoAbf produced by fungus *Pleurotus ostreatus* (Amore *et al.,* 2012a).

The main goals concerning this task are:

1) Construction of libraries of randomly mutated forms of PoAbf in the yeast S*accharomyces cerevisiae* by Error Prone PCR (EP-PCR);

2) Set up of a rapid high-throughput screening method to identify the mutants with beneficial mutations. The high-throughput screening was performed in multiwell plates by using an automated apparatus (Biomek® 3000 Laboratory Automation Workstation, Beckman Coulter);

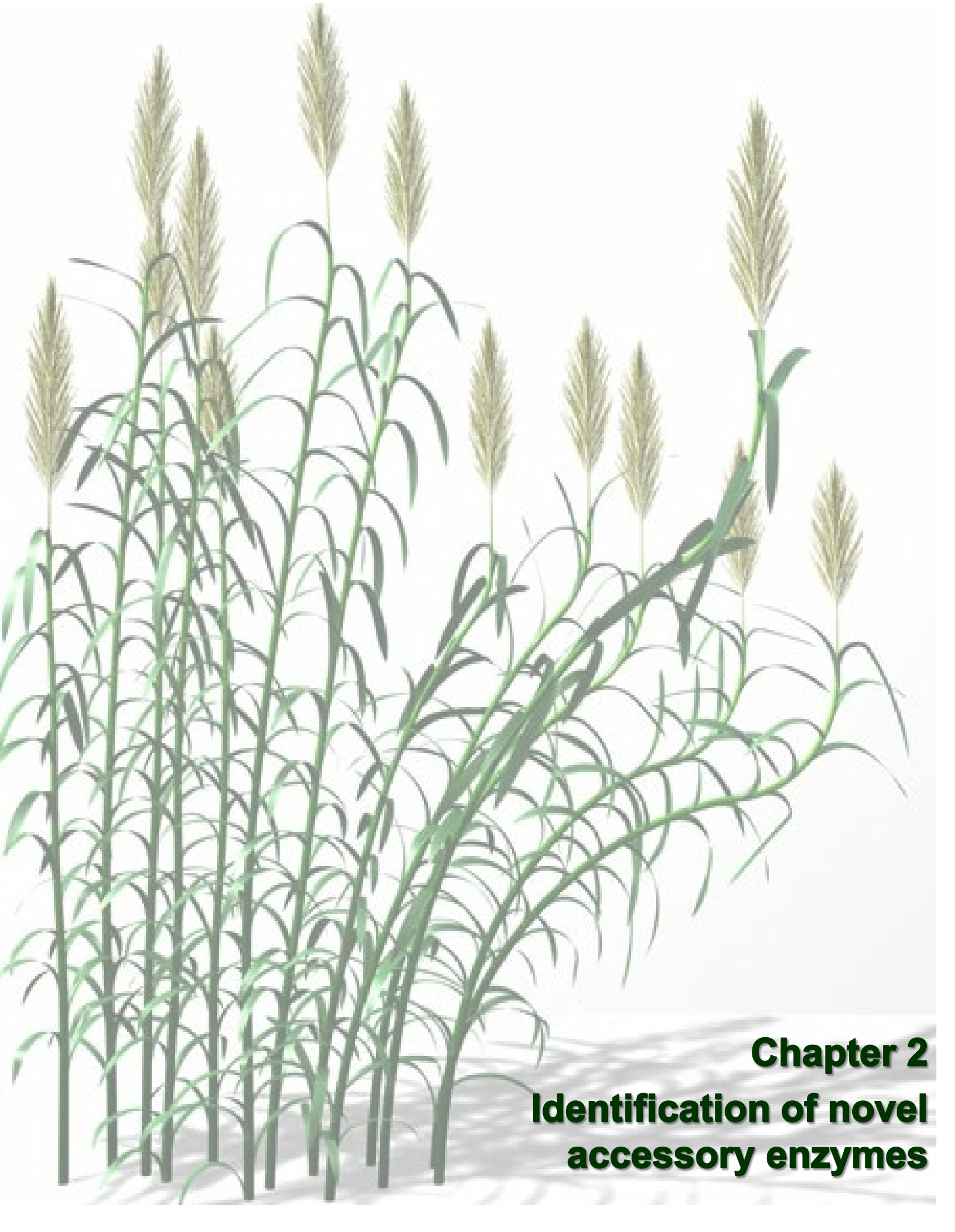
3) Purification and characterization of the best PoAbf directed evolved mutants.

The last task of this study was aimed at definition of synergistic enzymes necessary for lignocellulosic biomass transformation.The main purpose concerning this task are following reported:

1) Pretreatment and following saccharification of different lignocellulosic biomasses;

2) Evaluation of Ammonia Fiber Expansion (AFEX) as pretreatment method for *A. donax* biomass;

3) Evaluation of the ability of the cellulase CelStrep from *Streptomyces sp*. G12 recombinantly expressed in *Escherichia coli* (Amore *et al*., 2012b) and α-L-arabinofuranosidase PoAbf from the fungus *Pleurotus ostreatus* recombinantly expressed in *Pichia pastoris* (Amore *et al*., 2012a) and its evolved variant rPoAbf F435Y/Y446F (Giacobbe *et al.*, 2014) to hydrolyze lignocellulosic biomasses.



Chapter 2
Identification of novel accessory enzymes

2.1 INTRODUCTION

This chapter concerns the selection of new microorganisms producing accessory enzymes important for a complete saccharification of all the polysaccharides components of lignocellulose such as pectinases and mannanase. Among the accessory enzymes, pectinases have an important role in the bioconversion of agro-industrial cellulosic wastes (Adeleke *et al*., 2012; Van Dyk &Pletschke, 2012).

Pectin is a complex heteropolysaccharide presents in plant cell wall. D-galacturonic acid is the backbone of long pectin chains and it can be esterified with methyl/acetyl groups. The side chains branch are often arabinose, xylose and galactose. Pectic substances are classified in three groups: i) homogalacturonan, consisting in linear chains of α-(1–4)-linked D-galacturonic acid esterified with methyl or acetyl groups; ii) rhamnogalacturonan I, consisting in α-(rhamno)galacturonic acids; iii) rhamnogalacturonan II consisting in homogalacturonan chain with side chains attached to the galacturonic residues (Pedrolli *et al.,* 2009). Pectic polysaccharides can be hydrolysed by several pectinase enzymes such as hydrolases, lyases and esterases (Pedrolli *et al.,* 2009). Studies demonstrated that a mixture of pectinases with cellulases increased cellulose conversion (Bhatia *et al*., 2012).

The following paper “Identification and characterization of a pectinolytic enzyme from *Paenibacillus xylanolyticus*” describe results achieved starting from bacteria isolated from mature compost obtained from agro-industrial wastes by screening performed by team of Professor Olimpia Pepe of Department of Agriculture (DIA) of University of Naples “Federico II”.

2.1.1 PAPER III: IDENTIFICATION AND CHARACTERIZATION OF A PECTINOLYTIC ENZYME FROM *PAENIBACILLUS XYLANOLYTICUS*

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Identification and Characterisation of a Pectinolytic Enzyme from *Paenibacillusxylanolyticus*

Simona Giacobbe,[a] Olimpia Pepe,[b] Valeria Ventorino,[b] Leila Birolo,[a] Roberto Vinciguerra,[a] and Vincenza Faraco [a,*]

Pectinolytic enzymes play an important role in the processing of lignocellulosic materials because of their ability to improve the access of cellulases to their substrate by removing pectins. The strain *Paenibacillus xylanilyticus* 2-6L3 was isolated from mature compost obtained from agro-industrialwastes, and the enzyme pectate lyase from *P. xylanilyticus* 2-6L3, named PaenxylPel, was partially purified and subjected to structural and functional characterisation. The enzyme exhibited an optimum temperature between 60 and 70 °Cand optimal pH value of 9.0 for its pectinase activity on pectin from citrus fruit. PaenxylPel showed a thermoresistance and pH resistance higher than those of other pectate lyases so far described, with half-lives of 48 and 24 h at 60 and 70°C, respectively, a retention of around 80% of activity after 96 h at 40 and 50°C, and a half-life of about 15 days at pH 8.0. PaenxylPel followed Michaelis-Menten kinetics toward pectin from citrus fruit, pectin from sugar beet pulp, high-ester pectin extracted from citrus peel (>50% esterified), and polygalacturonic acid (PLA). The ability to act on both PLA and highly methylated pectins, together with a double peak in the graph of optimum pH at pH 5 and 9, suggest that pectate lyase from *P. xylanoliticus* shows an unusual activity, combining traits of pectatelyase and pectin lyase. This is the first manuscript on the pectinolytic activity of *P. xylanilyticus*.

Keywords:Paenibacillus; Pectatelyase; Pectin

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INTRODUCTION

Pectinolytic enzymes, or pectinases, are a group of enzymes involved in the breakdown of pectin, a polysaccharide present in the cell walls of plants, fruits, and vegetables.In plants, they are involved in cell wall extension and growth (Ward and Moo-Young 1989), the softening of some plant tissues during maturation, and the decomposition of dead plant materials (Sakai 1992). They are also responsible for the spoilage of fruits and vegetables (Collmer and Keen 1986; Lang and Dörnenburg 2000).

Pectinolytic enzymes are broadly distributed in plants and are also produced by both prokaryotic and eukaryotic microorganisms, mostly fungi that synthesize acid pectinases and yeasts (Jayani *et al.* 2005; Arunachalam and Asha 2010). The majority of studies are regarding pectinases from *Erwinia* and *Bacillus* within the bacteria, pectinases from *Aspergillus* within fungi, and pectinases from *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* within yeasts. Many pectic enzymes are recombinantly expressed in *Escherichia coli* (Soriano *et al.* 2000); Wang *et al.* 2011; Damak *et al.*2013).

Pectinolytic enzymes have an important role in the processing of lignocellulosic materials. In fact, bioconversion of agro-industrial cellulosic wastes, such as orange peels, citrus peels, rotten bananas, and grapefruit peels,requires synergistic actions of both pectinolytic and cellulolytic enzymes (Adeleke *et al.* 2012). Regarding ethanol production from cellulosic materials containing pectin, pectinolytic enzymes have been applied to pretreatment of various lignocellulosic biomasses to improve ethanol production (Spagnuolo *et al.* 1997; Hossain *et al.* 2011). In particular, Berlin *et al.* (2007) reported that pectins limit the access of cellulases to their substrate and demonstrated that improvement in hydrolysis is achieved using pectinases. Edwards *et al.* (2011) reported the engineering of an *Escherichia coli* strain with pectate lyase from *Erwinia chrysanthemi* and demonstrated that the recombinant strain was able to produce ethanol with a yield two-fold higher than that of the wild-type strain.

Among pectinolytic enzymes, lyases, classified into polysaccharide lyase family 1 (CAZY 2014), are depolymerising enzymes that cleave the α 1-4 glycosidic linkages in polygalacturonate or pectin by a trans-elimination reaction generating 4,5-unsaturated oligogalacturonates (Jayani *et al.* 2005). Pectin lyases or endo-polymethylgalacturonatelyase (EC 4.2.2.10) preferentially cleave glycosidic bonds in highly methyl esterified substrates, are stimulated by cations, and generally havean optimum pH close to 5.5 (Pedrolli and Carmona 2009); pectate yases are specific for demethylated or low-esterified forms of pectin, have an optimum pH from 8.0 to 9.8 (Nagel and Wilson 1970), and have an absolute requirement for calcium ions (Jayani *et al.* 2005). Depending on the mode of action, pectate lyases are classified as exo-PL (EC 4.2.2.9) that catalyse the substrate cleavage from the non-reducing end and are specific for pectate, but not for citrus pectin; or endo-PL (EC 4.2.2.2), which are more abundant than exo-PLand cleave the chain randomly (Jayani*et al.* 2005). Pectate most generally is used for endo pectate lyase studies because the enzymatic activity on pectate is higher than that on commercial high-methoxyl pectin (about 70% esterified).

In this manuscript, a pectate lyase produced by a strain of *Paenibacillus xylanolyticus* isolated from raw compost materials was characterised determining optimal temperature and pH for its activity on pectin from citrus fruit, its Michaelis-Menten constants toward pectin from citrus fruit, pectin from sugar beet pulp, high-ester pectin extracted from citrus peel (> 50% esterified), and polygalacturonic acid (PLA), thermoresistance and pH resistance.

EXPERIMENTAL

Isolation and Selection of Pectinolytic Bacteria

Pectinolytic microorganisms were isolated in the Campania region from mature compost obtained from agro-industrial wastes consisting of pomace with kernel (65%), liquid sewage sludge from industrial processing of potatoes and carrots (22%), and borland from the distillation of molasses (13%) (Pepe *et al.* 2013). Pectinolytic isolates were detected on solid medium composed of 2 g L^{-1}citrus pectin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution (in 1 L: 5 g K_2HPO_4; 2.5 g $MgSO_4$; 2.5 g NaCl; 0.05 g $Fe_2(SO_4)_3$; and 0.05 g $MnSO_4$), with1 mL L^{-1} trace elements solution (in 1 L: 0.05 g $K_2MoO_4 \cdot 5H_2O$; 0.05 g $Na_2B_4O_7 \cdot 10H_2O$; 0.05 g $Co(NO_3)_2 \cdot 6H_2O$; 0.05 g $MnSO_4$; 0.05 g $CdSO_4$; 0.05 g $ZnSO_4 \cdot 7H_2O$; 0.05 g $CuSO_4 \cdot H_2O$; and 0.1 g $FeCl_3$), and 20 g L^{-1} bacteriological agar, pH 7.0. After incubation

at 28°C for 7 d, the plates were flooded with a 1% hexadecyltrimethylammonium bromide (Sigma-Aldrich) solution. After 20 to 30 min, pectinase activity was detected by the presence of a clear halo surrounding the colony (Egamberdiyeva 2007; Pepe *et al.* 2013).

In order to perform a preliminary comparison of the pectinase production by the selected pectinolytic strains, a semi-quantitative agar spot method was carried out.Briefly, bacterial cells were suspended in Ringer solution (Sigma-Aldrich) at a concentrationapproximately $1.5x10^8$ CFU mL^{-1} and spotted on solid agar medium in triplicate. The enzymatic activity was recorded as the "indices of relative enzyme activity = diameter of clearing or halo zone/colony diameter" (Saini and Tewari 2012), and pectinase activity was reported as I_{PEC} (index of pectinase enzyme activity).

Screening on Liquid Media

The liquid medium adopted for analysis of pectinase production levels contained 0.5 g L^{-1}pectin from citrus fruit (Sigma-Aldrich), 1 g L^{-1} NH_4NO_3, 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution(as described above), and 1 mL L^{-1} trace elements solution (also as described above).

Inoculum Preparation and Submerged Fermentation

The bacterial strains were pre-inoculated by dissolving a single colony in 3 mL of liquid medium having the composition described in the previous paragraph and incubated overnight at 37 °C. Cultures were further grown in 100-mL plugged Erlenmeyer flasks, each containing 20 mL of medium and inoculated with volumes of pre-inoculum corresponding to 0.1 optical density (O.D.).

The inocula were incubated at 37°C on a rotary shaker at 225 rpm for 48 h. From time to time, samples of the liquid culture were withdrawn and used to measure $O.D._{600nm}$ and extracellular pectinase activity. The results of these determinations reported in the figures and tables correspond to mean values of three independent experiments, each of which was performed in duplicate.

Molecular Identification of Selected Pectinolytic Strains

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, fD1 and rD1, were used to amplify the 16S rRNA gene (Pepe *et al.* 2011). The PCR amplification fragment was purified in agarose 1.5% (wt/vol) by gel electrophoresis using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequencing was conducted at Primmsrl (Milan, Italy). The DNA sequences were determined and analysed as previously reported (Amore *et al.* 2013) and compared to the GenBank nucleotide data library using the Blast software (NCBI 2014) to determine their closest phylogenetic relatives.

The partial 16S rRNA gene sequences of the two selected strains 2-6L3 and 1-6H5 were deposited in the GenBank database under accession numbers KF923402 and KF923403, respectively.

Determination of Protein Concentration

Protein concentrations of crude enzyme preparationswere determined by the Bradford method using Bio-Rad reactive (München, Germany) following the procedure suggested by the supplier. Bovin serum albumin (BSA) was used for the standard curve.

Pectinase Assay

Pectinase activity was determined using pectin from citrus fruit as a substrate as described in Okafor *et al.* (2010). The reaction mixture, containing equal amounts (0.5 mL) of 1% pectin prepared in sodium acetate buffer 0.05 M (pH5.5) and suitably diluted crude enzyme, was incubated at 50°C in a water bath for 30 min. The reaction was stopped with 1.0 mL of dinitrosalicylic acid solution (Miller 1959), and the mixture was boiled for 5 min and cooled. The absorbance at 540 nm was measured using a spectrophotometer. The amount of reducing sugar released was quantified using galacturonic acid as a standard. The enzyme activity (IU) was calculated as the amount of enzyme required to release one micromole (1 µmol) equivalent of galacturonic acid per minute under the assay conditions.

Enzyme Identification

Protein fractionation

Culture media were harvested based on the optimal pectinase production time bysedimentation of cells through centrifugation at 5,000×g for 10 min at 4°C. Secreted proteins in the supernatant were filtered through Whatman filter paper. Filtrateswere concentrated with ultrafiltration devices with a molecular weight cut-off of 10 kDa (Millipore S.p.A., Vimodrone, Italy).

Zymogram analyses

To identify the proteins putatively responsible for pectinase activity of the 2-6L3 strain, zymogram analysis was performed following a method similar to that reported by Schneider *et al.* (2010) with some modifications. Semi-denaturing gel electrophoresis was carried out by loading non-denatured and non-reduced samples on a SDS polyacrylamide gel (12.5%) with 0.1% pectin from citrus fruit. After electrophoresis, the proteins in the gel were renaturated *in situ* by removing SDS through washing in Tris-HCl and isopropanol buffers. After renaturation, the zymogram was incubated at 40°C for 3 h to allow the enzymatic degradation of pectin. Subsequently, the gel was stained with 0.05% Ruthenium red for 10 min and destained with water until pectinase activity could be detected as colourless bands against a purple background.

Protein identification by mass spectrometry

Slices of interest from the semi-denaturing PAGE were cut and digested *in situ* after extensive destaining with 0.1 M NH_4HCO_3, pH 7.5 and acetonitrile, reduction of disulphide bonds for 45 min in 100 µL of 10 mM dithiothreitol, 0.1 M NH_4HCO_3, pH 7.5, and carboxyamidomethylation of thiols for 30 min 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 h at 4 °C. The buffer solution was then removed, and 50 µL of 10 mM NH_4HCO_3, pH 7.5 was added and incubated for 18 h at 37 °C. Peptides were extracted with 20 µL of 10 mM NH_4HCO_3, 1% formic acid, and 50% acetonitrile at room temperature.

Peptide mixtures were filtered on 0.22-µm PVDF membranes (Millipore) and analysed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a 6520 Accurate-Mass Q-TOF LC/MS (liquid chromatography mass spectrometry) System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 high-performance liquid chromatography (HPLC) system and a chip cube (Agilent Technologies). After loading, the peptide mixture was concentrated and washed in in a 40-nL enrichment column (Agilent Technologies), 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) at a flow rate of 400nL/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% over 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 m/z) followed by MS/MS scans of the five most abundant ions in each MS scan. The MS/MS spectra were measured automatically when the MS signal was over the threshold of 50,000 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 were used to query non redundant protein databases with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA). Additional search parameters consisted of a peptide mass tolerance set at 10 ppm, and a fragment mass tolerance of 0.6 Da, which will allow for up to 3 missed cleavages, carbamidomethylation of cysteines as fixed modification, oxidation of methionine, and cyclisation of N-term Q to pyro-Glu as variable modifications. Only doubly and triply charged ions were considered. The ions' score was -10 log(P), where P is the probability that the observed match is a random event. The threshold above which the individual ion's score indicated 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 ion scores as a nonprobabilistic basis for ranking protein hits (Matrix Science 2013). Trypsin, dithiothreitol, iodoacetamide, and NH_4HCO_3 were purchased from Sigma-Aldrich. Trifluoroacetic acid (TFA)-HPLC grade was from Carlo Erba (Milan, Italy). All other reagents and solvents were of the highest purity available from Baker.

Protein Purification

Proteins secreted by *P. xylanolyticus* after 24 h of growth were precipitated from the filtered medium (after cell removal by centrifugation at 5,000×g for 10 min) with 100% ammonium sulphate 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 a HiTrap Phenyl FF high sub column (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A (0.02 M Tris–HCl, 1.5 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 (10KDaMW cutoff).

Effects of pH and Temperature

The optimum temperature was determined by incubating the enzyme (30 min) at temperatures ranging from 30 to 70°C and using the pectinase assay procedure described in "Pectinase Assay" paragraph.

The thermal stability of the enzyme was studied by incubation at 40, 50, 60, and 70 °C. Enzyme samples were withdrawn periodically (every 30 min) and assayed to determine the residual enzyme activity, performing incubation (30 min) at the optimum temperature (60 °C) using the pectinase assay procedure described above.

To determine the optimum pH, the substrate, pectin from citrus fruit, was dissolved in 20 mM sodium acetate/HCl (pH 2 to 3); 20 mM sodium acetate (pH 4 to 5); 20 mM sodium phosphate (pH 6 to 8) and 20 mM sodium carbonate/bicarbonate (pH 9 to 11) as reported by Sukhumsiirchart *et al.* (2009). The incubation (30 min) was performed at the optimum temperature (60°C).

The stability of the enzyme at various pH values was determined by incubating the enzyme at the desired pH (2.0 to 11.0) at room temperature. Enzyme samples were withdrawn periodically and assayed to determine the residual enzyme activity, performing incubation (30 min) at 60°C using the pectinase assay procedure described above.

Substrate Specificity

The activity of the pectate lyase towards polygalacturonic acid was tested under standard assay conditions (Miller 1959). First, 50 µL of suitably diluted enzyme solution was incubated in the presence of 450 µL of 0.25% (w/v) polygalacturonic acid (PLA; Sigma-Aldrich) in 50 mM Tris-HCl buffer (pH 8.0) for 5 min. The reaction was stopped with 0.75 mL of dinitrosalicylic acid solution and subsequently boiled for 5 min, cooled on ice, centrifuged, and the absorbance read at 540 nm. The specific enzyme activity was calculated as the amount of enzyme required to release one micromole (1 µmol) equivalent of galactouronic acid per minute under the assay conditions.

Pectate lyase activity was also determined using pectins with different degrees of esterification: low ester pectin extracted from citrus peel, high ester pectin extracted from citrus peel, partly amidated low ester pectin from citrus peel, and pectin from sugar beet pulp (Megazyme International Ireland, Co. Wicklow, Ireland) following manufacturer instructions.

The activity was monitored spectrophotometrically by measuring the increase in absorption at 235 nm of the reaction mixture. First, 1 mL sample of substrate solution, 0.1mM $CaCl_2$, and 0.1% substrate in 20 mM Tris-HCl (pH 8) was pre-incubated for 5 min at 40°C; then, 0.5 mL of suitably diluted enzyme was added and incubated at 40°C for 15 and 30 min. One Unit of enzyme activity is the amount of enzyme required to release one micromole of product in 1 min under the described assay conditions.

Determination of V_{max} and K_M

For determination of the Michaelis-Menten constants, K_M, and V_{max}, the activity assay was performed at substrate concentrations from 0.025 gL^{-1} to 0.4 gL^{-1} of pectin from citrus fruit, pectin from sugar beet pulp, and PLA; and from 0.025 gL^{-1} to 10 gL^{-1} of high-ester pectin extracted from citrus peel. The enzyme activities were measured using the described assay procedure. The experiments were performed in triplicate and the reported values are the average of the values of three experiments.

RESULTS AND DISCUSSION

Selection and Identification of Pectinolytic Microorganisms

Seventy-two bacterial strains isolated from mature compost obtained from agro-industrialwasteswere screened for their pectinolytic activity on solid medium. From this preliminary selection, two pectinolytic strains, that showed the higher activity with an I_{PEC} values $\geq$ 10, were chosen for further characterisation.

The two selected strains 2-6L3 (I_{PEC} = 12) and 1-6H5 (I_{PEC} = 10) were identified by sequencing of the 16S rRNA gene. Results showed that both the strains belonged to *Paenibacillus xylanolyticus* (accession number KF923402 and KF923403).

Screening of Pectinolytic Microorganisms in Liquid Medium

A further screening of the two selected microorganisms, *P. xylanolyticus* 1-5H6 and *P. xylanolyticus*, 2-6L3 was performed by cultivating each in liquid medium containing 0.2% pectin from citrus fruit and assaying culture supernatants for pectinase activity production.Time courses of pectinase activity production by the strains *P. xylanolyticus* 1-5H6 (Fig. 1a) and *P. xylanolyticus* 2-6L3 (Fig. 1b) were monitored at 30°C and 37°C. Increasing the growth temperature from 30 to 37°C led to a 2-fold and 6-fold increase of pectinase activity production at 24h for *P. xylanolyticus* 1-5H6 (Fig. 1a) and *P. xylanolyticus* 2-6L3 (Fig. 1b), respectively. No difference in pectinase activity level was observed by changing pectin concentration in the culture medium (data not shown).

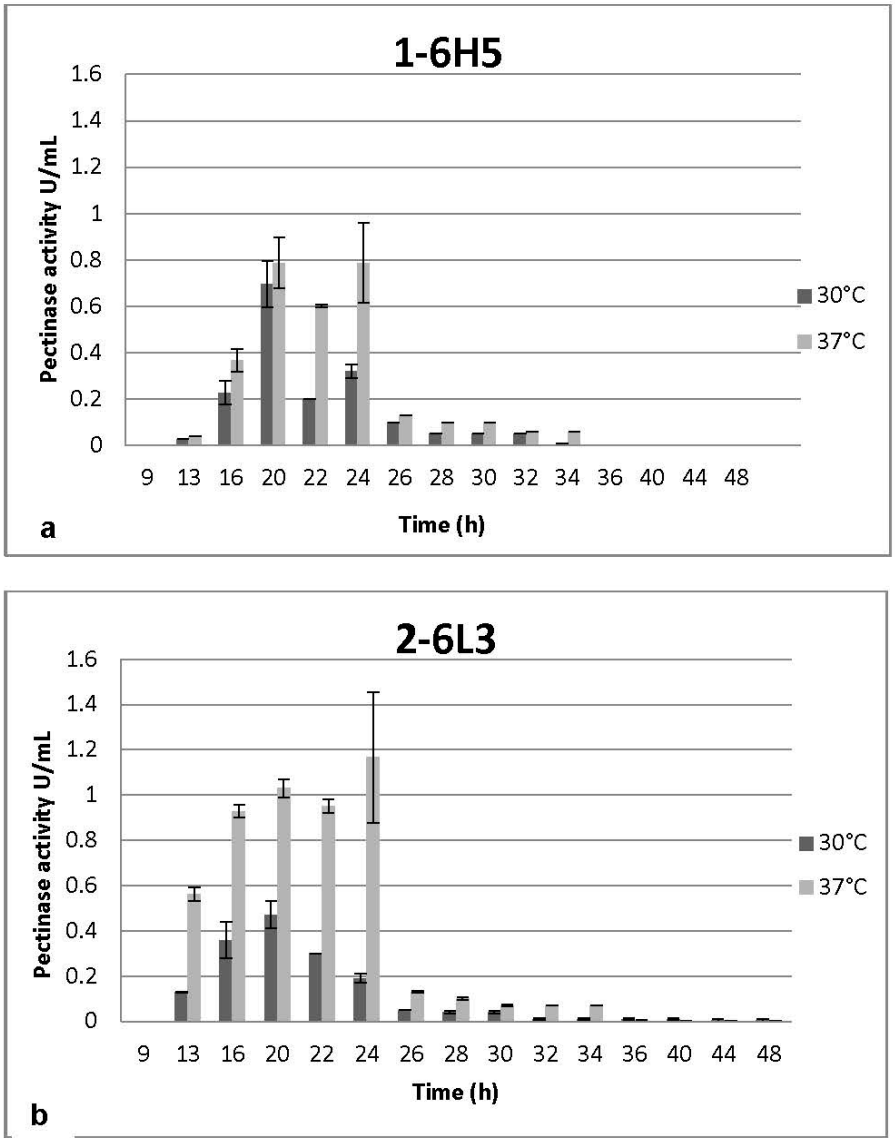

Fig. 1. Time course of production of pectinase activity assayed on pectin from citrus fruit for the strains 1-5H6 (**a**) and 2-6L3 (**b**) at 30 and 37°C

P. xylanilyticus 2-6L3 was found to be the more productive strain with a maximum of activity of 1.16 UmL^{-1} between 20 and 24 h of growth (Fig. 1b), while the *P. xylanolyticus* 1-5H6 strain showed a maximum activity of 0.78 UmL^{-1} at 24 h (Fig. 1a). Further studies were therefore focused on pectinolytic enzymes from *P. xylanilyticus*2-6L3. This is the first manuscript on pectinolytic activity of *P. xylanolyticus*. This microorganism was described for the first time by Rivas *et al.* (2005) as a new strain able to hydrolyse xylan. Recent studies were performed on polysaccharides-hydrolysing enzymes of *Paenibacillus* such as endo-glucanase, β-glucosidase, and xylanase (Park *et al.* 2012; 2013a,b), but pectinase has not yet been characterised from *P. xylanolyticus*.

Enzyme Identification

The proteins putatively responsible for pectinase activity of *P. xylanolyticus* 2-6L3 strain were tentatively identified after a fractionation on a semi-denaturing SDS-PAGE containing 0.1% pectin from citrus fruit where samples from the supernatant of the cell cultures were loaded without any denaturing treatment. An activity halo was visualised (Fig. 2a), and in correspondence to this halo, two different gel slices detected on the gel stained by Coomassie Blue (Fig. 2b) were excised and subjected to protein identification after *in situ* digestion and LC-MS/MS analysis of the peptide mixtures. Raw data were used to search the non-redundant NCBI database with no taxonomic restriction, with the MS/MS ion search program on a MASCOT server as described below.

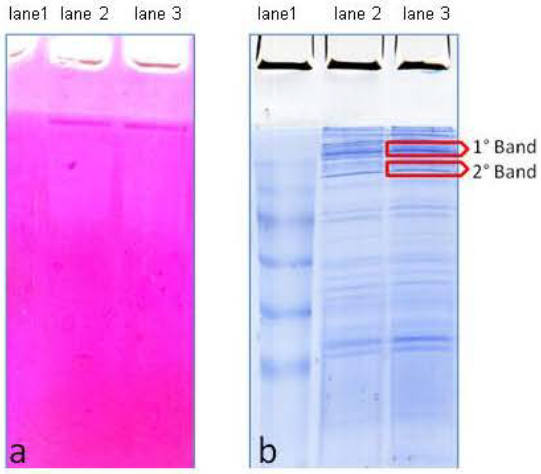

Fig. 2. Analyses of supernatant of *P. xylanolyticus* 2-6L3 strain by SDS-PAGE without denaturating treatments containing 0.1% pectin from citrus fruit stained with 0.05% Ruthenium red for pectinase activity detection (a) and with Coomassie Blue (b). Lane 1, protein molecular weight marker; lanes 2 and 3, culture supernatant

Several proteins were identified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analyses, and among these, three proteins putatively involved in pectin hydrolysis were identified in the first band (Fig. 2b): 9 peptides matched to peptides present in the sequence of a α-N-arabinofuranosidase from *Bacillus pumilus* SAFR-032 (identification number NCBI: gi 157693268); 7 peptides matched to peptides present in the sequence of a β-galactosidase from *Bacillus pumilus* ATCC 7061

(identification number NCBI: gi 194016271), and 4 peptides matched to peptides present in the sequence of a pectate lyase from *Bacillus sp.* (identification number NCBI: gi 4589753).

Enzyme Characterisation

The enzyme with pectate lyase activity from *P. xylanilyticus*2-6L3, named PaenxylPel, was partially purified (Fig. 3) and subjected to characterisation. The estimated molecular weight deduced from SDS-PAGE was around 37,000Da (Fig. 3), similar to the molecular masses, from 37 to 44 kDa, so far reported for other bacterial pectate lyases, *i.e.*, those from *Bacillus pumilus* BK2 (Klug-Santner *et al.*2006), *Erwinia chrysanthemi* (Preston *et al.* 1992), *Bacillus pumilus* BS22, *Bacillus subtilis* BS66, and *Bacillus fusiformis*BS90 (Ouattara *et al.* 2010).

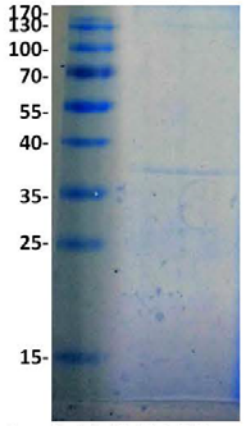

Fig. 3. SDS-PAGE profiling of the purified pectate lyase. Lane 1: protein molecular weight marker; lane 2: purified pectate lyase

An optimum temperature range of 60 to 70 °C was detected (Fig. 4a), in agreement with the optimum temperature found for other bacterial pectate lyases reported in literature (Ouattara *et al.* 2010; Soriano *et al.* 2006). When the effect of pH on the activity of PaenxylPel was investigated (Fig. 4b), an optimal pH of 9.0 was detected, which is in agreement with the optimum pH of other pectate lyases published in the literature (Klug-Santner *et al.* 2006). However, it is worth noting that PaenxylPel shows 60% of activity at pH 5.0. To the best of our knowledge, this is the first report of a pectate lyase with two optimum pHs. Yuan *et al.* (2012) reported recombinant pectate lyase from *Xanthomonas campestris* with optimum pH of 9.0 that retained more than 45% of its maximum activity from pH 3.0 to 12.0.

The thermoresistance and pH resistance of PaenxylPel were also determined (Table 1 and 2).

Table 1. Thermo-resistance of PaenxylPel

Temperature (°C)	$T_{1/2}$ (days)
40	7
50	6
60	2
70	1

Table 2. The pH resistance of PaenxylPel

pH	$T_{1/2}$ (days)
2	1
3	1
4	2
5	5
7	6
8	6
9	15
10	1
11	1

Table 1: Temperature resistance was studied by incubating the enzyme at 40°C, 50°C, 60°C and 70°C. The samples withdrawn were assayed for residual activity performing incubation (30 min) at 60°C. Table 2: Results were determined by incubating the enzyme at the desired pH (from 2.0 to 11.0) at room temperature. At different times, samples were withdrawn and immediately assayed for residual activity performing incubation (30 min) at 60°C.

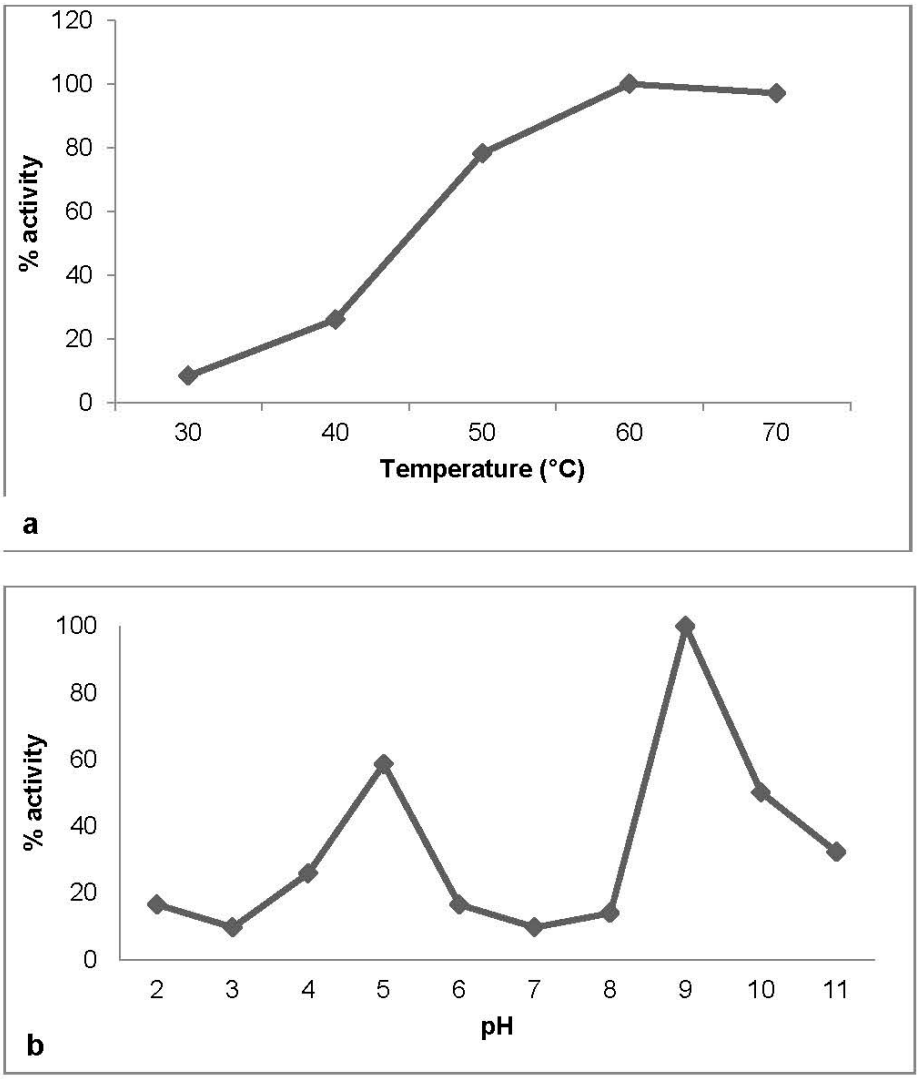

Fig. 4. Effect of temperature (a) and pH (**b**) on the pectatelyase activity of the strain 2-6L3. The pectatelyase activity was measured at the temperatures ranging from 30 to 70 °C, and pH ranging from 2 to 11

PaenxylPel showed a thermoresistance and pH resistance higher than those of the other pectate lyases so far described. In particular, at 60 °C, the half-life of PaenxylPel was 48 h, whilst the pectate lyases PL from *B. pumilus BK2* described by Klug-Santner *et al.* (2006), Apel from *B. subtilis* described by Liu *et al.* (2012), and thet hree extracellular pectate lyases (Pels) produced by *bacilli* described by Ouattara *et al.* (2010) showed a half-life of about 5, 20, and 30 min, respectively. Moreover, the pectate lyase of this study exhibited a half-life of around 24 h at 70°C, which was higher than that of pectate lyases described by Liu *et al.* (2012) and Ouattara *et al.* (2010). The thermoresistence of PaenxylPel at 50°C was higher than other pectate lyases reported in literature; indeed it showed a retention of around 80% of activity after 96 h at 50°C, whilst pectate lyase from non sporulating *Amycolata sp.* described by Bruhlmann (1995) lost its activity after 1 h of incubation at 50°C.

Table 3. Values of K_M and V_{max} of Pectate Lyase from *P. xylanolyticus* and other Bacterial Pectate Lyases towards Pectins

ENZYME	PLA		PECTIN from citrus fruit		PECTIN from sugar beet pulp		HIGH ESTER PECTIN (>50% esterification)		LOW ESTER PECTIN (< 50% esterification)		REFERENCE
	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	K_m (gL^{-1})	V_{max}	
PL from *Bacillus pumilus BK2*	0.24	0.72 $gl^{-1}min^{-1}$					1.34	0.21 $gl^{-1}min^{-1}$	0.35	0.35 $gl^{-1}min^{-1}$	Klug-Santner*et al.* 2005
PaenxylPel from *Paenibacillusxylanoliticus*	0.20	0.74 $gl^{-1}min^{-1}$	0.10	1,953 $gl^{-1}min^{-1}$	0.36	0.79 $gl^{-1}min^{-1}$	1.07	0.22 $gl^{-1}min^{-1}$			This study
Pel-22from *Bacillus pumilus* BS22	0.045	1.41 $\mu molmin^{-1}mg^{-1}$							0.043	1.45 $\mu molmin^{-1}mg^{-1}$	Ouattara*et al.* 2010
Pel-66 from *Bacillus subtilis* BS66	0.1	667 $\mu molmin^{-1}mg^{-1}$							0.1	714 $\mu molmin^{-1}mg^{-1}$	Ouattara*et al.* 2010
Pel-90 from *Bacillus fusiformis* BS90	0.125	909.9 $\mu molmin^{-1}mg^{-1}$							0.111	1136.36 $\mu molmin^{-1}mg^{-1}$	Ouattara*et al.* 2010
pelAfrom *Azospirillumirakense*	0.076	23 $\mu molmin^{-1}mg^{-1}$									Bekri*et al.* 1999
PL D from *Xanthomonascampestris*	4.9	30.1 $\mu molmin^{-1}mg^{-1}$									Yuan *et al.* 2012

As regards to pH resistance, the enzyme exhibited a half-life of about 15 days at pH 9.0, which is higher than the pectate lyase described by Klu-Santner *et al.* (2006) that showed a half-life of around 38 h.

The hydrolysing ability of PaenxylPel was tested *versus* pectins with different degrees of esterification, and all these compounds were hydrolyzed by the enzyme. The enzyme followed Michaelis-Menten kinetics towards pectin from citrus fruit, pectin from sugar beet pulp, high-ester pectin extracted from citrus peel (> 50% esterified), and PLA. The values of K_M and V_{max} of pectate lyase from *P. xylanolyticus*2-6L3 towards the abovementioned pectins are reported in Table 3.

The enzyme showed lyase activities on both polygalacturonic acid and highly methylated pectin with K_M and V_{max} similar to the PL from *Bacillus pumilus* BK2 reported by Klug-Santner *et al.* (2006).

These data suggest that pectate lyase in *P. xylanoliticus* shows an unusual activity by combining traits of pectate lyase and pectin lyase. These results are in agreement with those on other pectate lyases published by Soriano *et al.* (2000) and Boland *et al.* (2010). Soriano *et al.* (2000) studied pelA, from the strain *Paenibacillus sp.* BP-23, actives on pectins of any degree of esterification. Boland *et al.* (2010) described two pectinases from *P. amylolyticus,* PelA and PelB, that are pectate lyases that show a remarkable pectin lyase activity. The peculiar double peak observed in the graph of optimum pH at (pH 9 and 5) for Paenxyl-Pel can be explained by the ability of the enzyme to work as both pectate lyase, typically characterised by optimum alkaline pH, and pectin lyase, typically exhibiting an optimum acidic pH.

CONCLUSIONS

1. In this study, the strain *Paenibacillus xylanilyticus* 2-6L3 was isolated from mature compost obtained from agro-industrial wastes, and the enzyme pectate lyase from *P. xylanilyticus* 2-6L3, named PaenxylPel, was purified to apparent homogeneity and subjected to structural and functional characterisation.
2. PaenxylPel exhibited an optimum temperature of 60 to 70°C and optimal pH value of 9.0 for its pectinase activity on pectin from citrus fruit.
3. PaenxylPel showed a thermoresistance and pH resistance higher than those of the other pectate lyases so far described, with a half-life of 48 and 24 h at 60 and 70 °C, respectively, a retention of around 80% of activity after 96 h at 40 and 50°C, and a half-life of about 15 days at pH 8.0.
4. PaenxylPel follows Michaelis-Menten kinetics towards pectin from citrus fruit, pectin from sugar beet pulp, high ester pectin extracted from citrus peel (>50% esterified), and polygalacturonic acid (PLA).

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Chapter 3
Development of tailor made enzymes by directed evolution

3.1 INTRODUCTION

This chapter concerns the directed evolution of the enzyme α-L-arabinofuranosidase from *Pleurotus ostreatus* (PoAbf) previously identified and characterized in recombinant form, in the laboratory where this PhD project was performed (Amore *et al.,* 2012a).

Directed evolution mimics the Darwinian processes of evolution, since this approach reproduces, in vitro, the natural process of molecular evolution and it is able to generate a potentially infinite plethora of protein variants with new functions and properties (Valetti & Gilardi, 2004). Molecular diversity can be created by various methods such as combinatorial cassette mutagenesis, combinatorial saturation mutagenesis by *in vivo* overlap extension (IVOE) (Alcalde *et al*., 2006, *in vivo* DNA shuffling combined with random mutagenesis, *in vivo* assembly mutagenesis (IVAM) and error prone polymerase chain reaction (EP-PCR). EP-PCR, by far the most used approach to produce random mutants, uses a low fidelity polymerase to introduce random point mutations at each round of amplification. The EP-PCR method has the advantage to be simple and easy to use, moreover it does not require previous information on structure-function relationships. Yeasts were largely used as hosts for the directed evolution experiments, among these the yeast *S. cerevisiae* offering significant advantages for recombinant enzymes expression and directed evolution experiments (Bulter *et al*., 2003; Festa *et al*., 2008).

The following paper **"Development of an improved variant of GH51 α-L-arabinofuranosidase from Pleurotus ostreatus by directed evolution"** describes the construction of the first generation library of directed evolved mutants of PoAbf in *S. cerevisiae*, the selection of the best directed evolved variant and its recombinant expression and characterization.

3.1.1 PAPER IV: "DEVELOPMENT OF AN IMPROVED VARIANT OF GH51 A-L-ARABINOFURANOSIDASE FROM *PLEUROTUS OSTREATUS* BY DIRECTED EVOLUTION"

RESEARCH PAPER

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Research Paper

Development of an improved variant of GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus* by directed evolution

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In this study, the α-L-arabinofuranosidase from *Pleurotus ostreatus* was subjected to directed evolution by expressing a library of around 7000 randomly mutated variants by error prone Polymerase Chain Reaction. High-throughput screening of the library for the most active variants was performed by assaying activity towards p-nitrophenyl α-L-arabinofuranoside, and a variant with higher activity than the wild type was selected, purified and characterised. It exhibited a k_{cat} of $7.3 \times 10^3 \pm 0.3$ min^{-1}, around 3-fold higher than that of the wild type ($2.2 \times 10^3 \pm 0.2$ min^{-1}), and a K_M (0.54 ± 0.06 mM) 30% lower than that of the wild type (0.70 ± 0.05 mM) towards this substrate. The mutant also showed improved catalytic properties towards pNP-β-D-glucopyranoside (k_{cat} of 50.85 ± 0.21 min^{-1} versus 11.0 ± 0.6 min^{-1}) and it was shown able to hydrolyse larch arabinogalactan which is not recognised by the wild type. The mutant was also more active than the wild type towards arabinoxylan and was able to hydrolyse arabinan, which was not transformed by the wild type. The ability of rPoAbf F435Y/Y446F to hydrolyse these insoluble substrates expands its potential for application also to hemicelluloses, which in some types of pretreatment are recovered in solid fractions.

Introduction

The high cost of enzymes is one of the major economic bottlenecks for implementation of economically feasible lignocellulosic biorefineries [1]. A finely planned cocktail of enzymes, consisting not only of cellulases but also of xylanases, is required to achieve the highest yields of fermentable sugars from the polysaccharide components of lignocellulosic biomass. In particular, α-L-arabinofuranosidase has been detected as one of the crucial components for a more complete hydrolysis of the complex hemicellulolse matrix in pretreated biomass [2–4]. α-L-Arabinofuranosidases work synergistically with other enzymes, such as hemicellulases and pectinases, for the complete degradation of hemicelluloses and pectins, respectively [5–8]. In fact, Gao *et al.* [2] demonstrated that in the absence of specific activities, such as β-xylosidase, α-arabinofuranosidase and α-glucuronidase, it was not possible to achieve high xylose hydrolysis yields.

In a previous study, we reported on the identification of a new α-L-arabinofuranosidase produced by the fungus *Pleurotus ostreatus* (PoAbf) and the cloning and sequencing of the corresponding gene and cDNA [9]. Heterologous recombinant expression of PoAbf was carried out in the yeast *Pichia pastoris* and the recombinant enzyme (rPoAbf) was purified and characterised. rPoAbf is highly specific for α-L-arabinofuranosyl linkages and shows a robust enzymatic activity at pH 5 ($t_{1/2}$ = 51 days). This led us to adopt this hemicellulase as a scaffold for directed evolution experiments to improve its catalytic properties.

In this study, a library of around 7000 random mutants of rPoAbf was generated by error prone PCR of *poabf* cDNA and recombinant expression of the mutants in *Saccharomyces cerevisiae*. Screening for extracellular enzymatic activity towards p-nitrophenyl α-L-arabinofuranoside led to the selection of an improved enzyme variant. This mutant was overproduced by recombinant expression in *Pichia pastoris*, purified and characterised showing an improvement of its catalytic properties in comparison to the wild type.

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Materials and methods

Strains and culture media

The *Escherichia coli* strain used in DNA manipulations and conditions for its growth are reported in [9]. The *S. cerevisiae* strain and pSAL4 plasmid used for heterologous expression of the random mutants' library were kindly donated by Dr. T. Tron (CNRS UMR 6517, Marseille, France) and are reported in [10]. *S. cerevisiae* was grown on selective medium (SD) as described [10]. To overproduce the selected random mutant, the 'Easy Select Pichia Expression kit' (Invitrogen, Carlsbad, CA, USA) was used for expression in *P. pastoris* strain X33 with the vector pPICZB following manufacturer's instructions and using the growth conditions reported [9].

Vectors

The EMBL Data Library accession number of the sequence of *P. ostreatus* arabinofuranosidase [9] reported in this paper is HE565356. The vector pSAL4-poabf used for recombinant expression of PoAbf in *S. cerevisiae* was prepared by cloning *poabf* cDNA in pSAL4 (in *Eco*RI/*Hind*III) under control of copper-inducible CUP1 promoter [10]. The vector pPICZ-abf used for recombinant expression of PoAbf in *P. pastoris* had been previously prepared cloning *poabf* cDNA in pPICZB (in *Eco*RI/*Xba*I) under control of methanol-inducible *AOX1* promoter [9]. It was used as template for site-directed mutagenesis experiments (below) to prepare the vector for recombinant expression of the selected random mutant PoAbf (F435Y/Y446F) in *P. pastoris*.

Random mutagenesis

Random mutagenesis of *poabf* cDNA was performed by Error Prone PCR (EP-PCR), using pSAL4-poabf as template and Gene Morph II Random Mutagenesis Kit (Agilent, La Jolla, CA). Primers, pSal4Fw (CCAACGCAATATGGATTGTCAG) and pSal4Rev (CAAGTGTAGCGGTCACGCTGCG) used in amplification experiments are complementary to the both ends of polylinker pSAL4 sequence. Cycling parameters were 30 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 2 min.

Construction of a mutant library

cDNAs resulting from EP-PCR were ligated in pSAL4 vector digested with *Eco*RI/*Hind*III restriction enzymes, exploiting homologous recombination expression system of *S. cerevisiae*. Yeast transformation and selections was performed following the protocols reported in [10].

Library screening

Agar plate assays on SD medium supplemented with the chromogenic substrate 4-methylumbelliferyl-a-L-arabinofuranoside (MUA) (Carbosynth, Compton, Berkshire, UK) were used to select transformants expressing α-L-arabinofuranosidase activity as described previously [9]. High-throughput screening was performed in 96-well plates, assaying α-L-arabinofuranosidase activity with p-nitrophenyl α-L-arabinofuranoside (pNPA) as substrate following the method reported in [9] with some modifications. The assay mixture contained 70 μL of the substrate solution (2 mM pNPA in 50 mM sodium phosphate buffer pH 6.5) and 70 μL of appropriately diluted enzyme solution. After incubation at 30 °C for 10 min, the reaction was stopped by the addition of 150 μL of 1.6 M Na_2CO_3. The liberated p-nitrophenol in the mixture was measured by spectrophotometer at 405 nm ($\varepsilon = 17{,}000\ M^{-1}\ cm^{-1}$) after vigorous agitation. Cultures in shaken flasks were also performed for selected mutants as described [10]. Optical density and arabinofuranosidase activity were daily assayed.

DNA sequencing

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

Arabinofuranosidase activity assays

α-L-Arabinofuranosidase activity was measured spectrophotometrically with p-nitrophenyl α-L-arabinofuranoside (pNPA) (Gold Biotechnology, St Louis, MO, USA) as substrate as described in [9]. 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.

Site-directed mutagenesis for recombinant expression of selected random mutant in P. pastoris

The pPICZ-abf containing the cDNA encoding for PoAbf was used for recombinant expression in *P. pastoris* as previously reported [9]. Site-directed mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to introduce the desired mutations F435Y/Y446F, identified in the selected random mutants, and obtain the vector pPICZB-$abf_{F435Y/Y446F}$. The mutagenic primers for the mutant gene are the following (mutated nucleotides underlined and bold): Fw Y435F: CAGAACTCGTTCTTCT**T**CGATGATATGCCACGC; Fw F446Y: AATGGAGTCACCTTCT**A**CGAGGGTGAATATGC.

The mutated gene was confirmed by sequencing and the wild type and mutated proteins were overexpressed, purified and assayed for their activity on p-nitrophenyl α-L-arabinofuranoside (pNPA) (Gold Biotechnology, St Louis, MO, USA) as previously described [9].

P. pastoris *transformation, cultivation and enzyme production*

The recombinant plasmid pPICZB–abf and pPICZB-$abf_{F435Y/Y446F}$ were linearised by *Pme*I hydrolysis and introduced into *P. pastoris* strain GS115 by electroporation. The electroporation mixture was plated onto YPD–zeocin agar medium. After 72 h at 28°C, transformant colonies were screened for activity production on plates containing the chromogenic substrate MUA.

PoAbf purification

Proteins secreted by *P. pastoris* expressing rPoAbf and by *P. pastoris* expressing rPoAbf-F435Y/Y446F were purified following the protocol in [9].

Protein concentration determination

Protein concentration was determined by the Lowry method [11], using the BioRad Protein Assay (BioRad Laboratories S.r.l., Segrate, MI – Italy), with bovine serum albumin as standard.

Temperature optimum and temperature stability

The optimum temperature (among 30, 40, 50 and 60°C) and the thermo-resistance (at 40, 50 and 60°C) of the purified enzymes was determined as in [9].

Experiments were performed in duplicate and reported values are the average of three experiments.

pH optimum and pH stability

The optimum pH (among the pH values between 3.0 and 7.0) and pH stability (at pH 3–8 at 25°C) of the purified enzymes was determined as in [9]. Experiments were performed in duplicate and reported values are the average of three experiments.

Enzyme specificity assays

Activity of wild type and mutated rPoAbf was assayed against the substrates *p*NP-α-L-arabinofuranoside (Carbosynth, Berkshire, UK), *p*NP-β-D-xylopyranoside (Carbosynth, Berkshire, UK), *p*NP-α-D-glucopyranoside (Carbosynth, Berkshire, UK), *p*NP-β-D-glucopyranoside (Carbosynth, Berkshire, UK) and *o*NP-β-D-galactopyranoside (Carbosynth, Berkshire, UK) at concentrations in the range 0.1–6 mM in citrate phosphate buffer 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 as described in [9]. Activity against AZO-wheat arabinoxylan (Megazyme International Ireland, Co., Wicklow, Ireland) was assayed following supplier's instructions.

Endo-1,4-beta-D-xylanase and endo-1,5-α-L-arabinanase activity were determined against the insoluble substrates azurine-crosslinked (AZCL) wheat arabinoxylan (Megazyme International Ireland, Co., Wicklow, Ireland) and azurine-crosslinked (AZCL) arabinan (Debranched) (Megazyme International Ireland, Co., Wicklow, Ireland) respectively. 60 mU (measured on pNPA) of PoAbf wild type and PoAbf F435Y/Y446F were incubated with 1% (w/v) wheat arabinoxylan in 100 mM sodium phosphate buffer pH 6 or 1% (w/v) arabinan (Debranched) in 100 mM sodium acetate buffer pH 4.7 at 40°C for 5, 10 and 15 min. Reducing sugar content was measured by a modified Somogyi reducing sugar assay following the manufacturer's instructions. Reactions were terminated following the manufacturer's instructions and the absorbance was measured at 520 nm. One Unit of enzyme activity is the amount required to release one μmole of reducing sugar equivalents.

Experiments were performed in duplicate and reported values are the average 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 substrate concentrations from 0.1 mM to 10 mM *p*NPA and from 0.1 mM to 16 mM *p*NP-β-D-glucopyranoside at pH 5, performing incubations of 10 min at 40°C. The experiments were performed in triplicate and reported values are the average of three experiments.

Results and discussion

Construction and screening of mutant PoAbf libraries

To develop improved variants of *P. ostreatus* α-L-arabinofuranosidase PoAbf, it was subjected to directed evolution experiments. Random mutations were introduced into *poabf* cDNAs by EP-PCR. *S. cerevisiae* was chosen as the host for recombinant expression of the variants, being a more appropriate platform for directed evolution than *Pichia pastoris* [9], due to integrative phenomena taking place in the latter host. Two different PCR conditions were used to yield medium (MMF) and high (HMF) mutation frequency (4.5–9 mut/kbases and 9–16 mut/kbases, respectively). Mutated cDNAs were introduced into the expression vector pSAL4 using the homologous recombination system of *S. cerevisiae*. A total of 6400 transformants, 3300 and 3100 for MMF and HMF respectively, were obtained by *S. cerevisiae* transformation with pSAL4 vector and mutated cDNAs. The libraries were subjected to a selection designed to identify mutants exhibiting higher arabinofuranosidase activity than the wild type PoAbf. The first screening, performed on MUA-containing plates, to select only transformants expressing extracellular α-L-arabinofuranosidase activity, led to selection of 2100 mutants (1100 with MMF and 1000 with HMF), identified under UV light by the presence of fluorescence around the colonies after incubation at 28°C for 3 days. High-throughput screening of these active 2100 mutants in multiwell plates allowed the selection of 24 mutants, exhibiting at least 7-fold higher activity values towards pNPA than the wild type. Further screening was performed in shaken flasks monitoring time course of growth and activity production of the 24 clones, for 4 days (data not shown). Among the 24 mutants, four clones (H4B4, H4C4, H5C4, H13G9) exhibiting 2-fold higher activity values than the wild type at 48 h, were chosen for further analyses.

Characterisation of mutated and wild type PoAbf

Sequence analysis showed that all four selected clones have the same amino acid substitutions: F435Y/Y446F. A total of 509, 595, 592, and 562 nucleotide substitutions were identified in H4B4, H4C4, H5C4 and H13G9 respectively. In order to characterise the F435Y/Y446F mutant, it was expressed in *P. pastoris*, which had been shown to give higher rPoAbf expression levels than *S. cerevisiae* [9], and time course of production of arabinofuranosidase specific activity was investigated at 20 and 28°C in comparison to that of wild type (data not shown). These temperatures were chosen as typical for recombinant expression in *P. pastoris* [9]. It was found that the specific enzymatic activity of rPoAbfF 435Y/Y446F towards pNPA was two-fold higher than that of wild type at both 20 (36.8 ± 1.8 versus 16.7 ± 0.0 mU/OD) and 28°C (8.0 ± 0.3 versus 4.8 ± 0.4 mU/OD).

The recombinant PoAbf F435Y/Y446F was purified to apparent homogeneity (Fig. 1) and structurally and functionally characterised in comparison to the recombinant PoAbf wild type protein. The electrophoretic mobility of the mutant in a SDS-PAGE is similar to that of the wild type (Fig. 1). The catalytic properties of the mutants were determined towards the substrate pNPA and compared to those of the wild type. The mutant F435Y/Y446F showed a k_{cat} of $7.3 \times 10^3 \pm 0.3$ min $^{-1}$, around 3-fold higher than that of the wild type $2.2 \times 10^3 \pm 0.2$ min $^{-1}$. The K_M of rPoAbf F435Y/Y446F towards pNPA was lower (0.54 ± 0.06 mM) than that of the wild type (0.70 ± 0.05 mM).

In order to determine the enzymatic properties of the selected mutant rPoAbf F435Y/Y446F, a panel of experiments was performed to define the conditions and the field of its activity. The effect of pH and temperature on the activity of rPoAbf F435Y/Y446F towards pNPA (Fig. 2) was investigated in order to establish the optimal reaction conditions. Thermoresistance (Fig. 3) and pH resistance (Fig. 4) of the mutant were also determined and compared to the wild type, enzyme stability being a crucial property for applications.

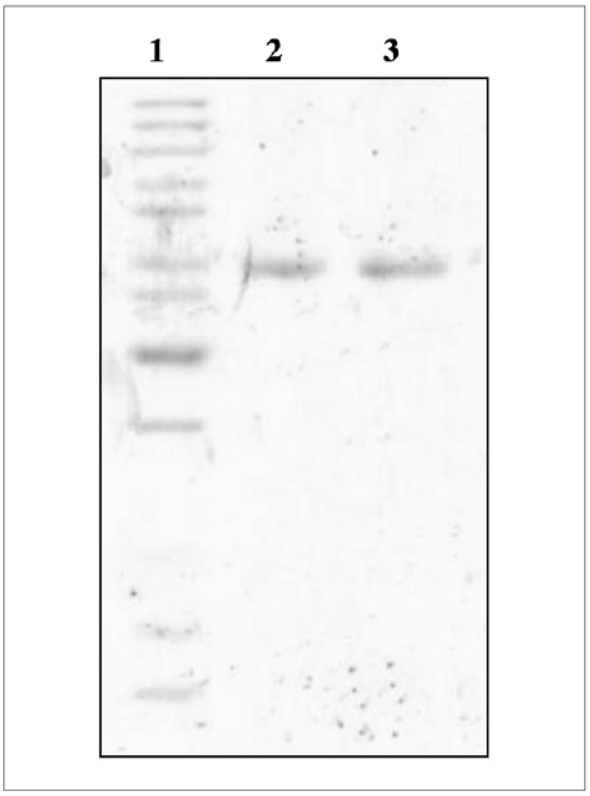

FIGURE 1

SDS-PAGE profiling of the purified rPoAbf wild type and rPoAbf F435Y/Y446F expressed in *P. pastoris*. Lane 1: protein molecular weight marker; lane 2: purified rPoAbf; lane 3: purified rPoAbf F435Y/Y446F.

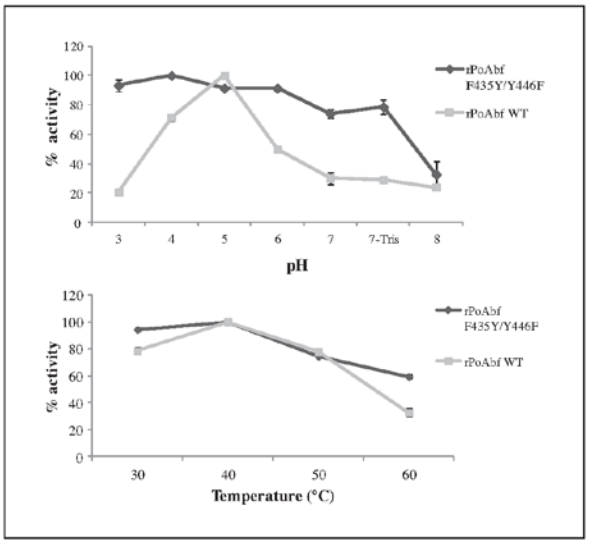

FIGURE 2

Effect of (a) pH and (b) temperature on activity of rPoAbf wild type (rPoAbf WT) and rPoAbfF435Y/Y446F towards pNPA. To determine the optimum pH of the purified enzyme, the substrate of the activity assay (2 mM pNPA) was dissolved in citrate phosphate buffers with pH values between 3.0 and 7.0 and the incubation (10 min) was performed at 40°C. 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 and 60°C. The percent activity reported in (a) and (b) was calculated as a ratio to the maximum activity at optimum pH (5) and temperature (40°C), respectively.

As regards the main significant improvements shown by the mutant, it exhibits a wide range of optimal pH values (3–6), differing from the wild type which shows an optimum at pH 5, and has 94% of maximum activity (exhibited like the wild type at 40°C) at 30°C. The selected mutant is therefore more versatile than the wild type, working over a larger range of operating conditions. Moreover, the mutant rPoAbf F435Y/Y446F was more thermoresistant than the wild type at all the tested temperatures (40, 50 and 60°C) and exhibited a pH resistance similar to, or higher than, that of the wild type. Thus, while the mutant F435Y/Y446F was selected for its higher activity towards pNPA, it also showed a higher resistance to temperature and pH than wild type. These observations further increase the applicative potential of the selected mutant.

To investigate the substrate specificity of the selected mutant, the hydrolytic ability of rPoAbf F435Y/Y446F was tested against a series of other nitrophenyl glycosides in comparison to the wild type, namely pNP-β-D-xylopyranoside, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside and oNP-β-D-galactopyranoside, in order to assess whether the enzyme is able to hydrolyse other types of glycosidic bonds that are present in hemicellulose matrix. Among these compounds, only pNP-β-D-glucopyranoside was recognised by the mutant enzyme similarly to the wild type and a K_M of 3.55 ± 0.08 mM (wt: 3.76 ± 0.15 mM) and a k_{cat} of $50.8 \pm 0.2\ min^{-1}$ (wt: $11.0 \pm 0.6\ min^{-1}$) were measured for this substrate.

Ability of the mutant to hydrolyse the arabinooligosaccharides 5-α-arabinotriose and 1,5-α-arabinohexaose were also analysed since these are intermediate products of hemicellulose hydrolysis, whose further hydrolysis is required during lignocellulose conversion to produce the fermentable pentose monosaccharides. The mutant was shown able to hydrolyse both the tested arabinooligosaccharides 1,5-α-arabinotriose and 1,5-α-arabinohexaose, with a slightly higher and similar activity than the wild type, respectively (Table 1).

rPoAbf F435Y/Y446F shows a similar activity to the wild type towards CM-linear arabinan. On the other hand, it is worth noting that rPoAbf F435Y/Y446F is able to hydrolyse larch arabinogalactan, which is not hydrolysed by the wild type as demonstrated by the assay (Table 1) in which less than the minimum detectable of arabinose ($0.346\ \mu g\ mL^{-1}$) was released when performed with the wild type. Moreover, when the enzyme was incubated with the AZO-wheat arabinoxylan, it was shown to possess an endo-1,4-β-xylanase activity of $0.81 \pm 0.03\ U\ mL^{-1}$, which was slightly higher to that of the wild type ($0.63 \pm 0.03\ U\ mL^{-1}$).

The ability of the mutant to hydrolyse the insoluble substrates arabinoxylan and arabinan was also analysed to verify its applicability to hemicelluloses which in some kinds of pretreatments are recovered in solid fractions. Towards AZCL-Arabinoxylan, the mutant exhibited an activity of $3.0 \pm 0.2\ mU\ mL^{-1}$, two-fold higher than that of the wild type, and it was also able to hydrolyse

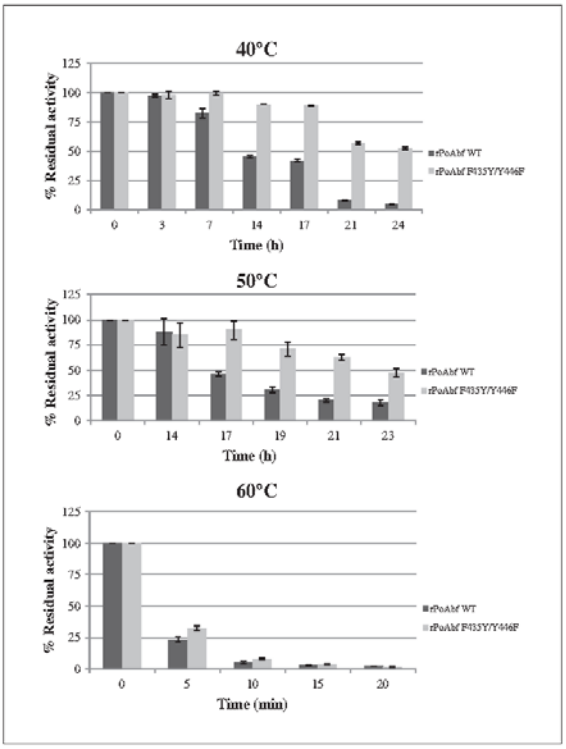

FIGURE 3

pH resistance of rPoAbf wild type (rPoAbf WT) and rPoAbf F435Y/Y446F. The pH resistance of the purified enzyme preparations was studied by dilution in citrate phosphate buffers, pH 3–8 and incubating at 25°C. At different times, samples were withdrawn and immediately assayed for residual α-arabinosidase activity performing incubation (10 min) at 40°C.

AZCL-Arabinan (debranched) showing an activity of 1.28 ± 0.05 mU mL ^{1}, whilst the wild type was unable to hydrolyse it, showing a negative (lower than the blank) absorbance. Hence, this work allowed isolation of an enzyme variant with higher arabinofuranosidase activity towards different substrates.

A 3D structure model of PoAbf was obtained by homology detection and structure prediction by HMM-HMM (Hidden Markov Models) comparison to localise the two mutations Y435F and F446Y (Fig. 5). Five 3D structures of GH51 family proteins are available: PDB: 1QW9 from *Geobacillus stearothermophilus* showing 8% sequence identity (id.) to PoAbf; PDB: 2C7F from *Clostridium thermocellum* (6% id.); PDB: 2VRQ from *Thermobacillus xylanilyticus* (14% id.); PDB: 2Y2W from *Bifidobacterium longum* (14% id.); and PDB: 3S2C from *Thermotoga petrophila* (14% id.). They have been used as templates to calculate a model for PoAbf, using HHpred and Modeller [12,13]. Although each template shares very low sequence identity with PoAbf, this enzyme is expected to adopt the same fold as the GH51 members, since all they belong to the same GH family, even though with distant homologies [14]. In the model, the key catalytic residues Glu acid/base and Glu nucleophile are conserved and superimpose well with the templates.

The structural model obtained for PoAbf suggests that the two mutations Y435F and F446Y should be located far from the active site and therefore no hypothesis regarding the role of these mutations on catalysis can be made. However, unlike the templates, the molecular surface of the model shows a channel starting from the bottom of the active site and crossing the protein. The wall of the channel creates a hydrophilic environment suggesting the presence of a water channel. Residue Y446 stands at the bottom of the central β-barrel and is close to the channel, such that the mutation of F446 into Y could impact on the water-relay inside this putative channel and would favour the release of water molecules in the active site.

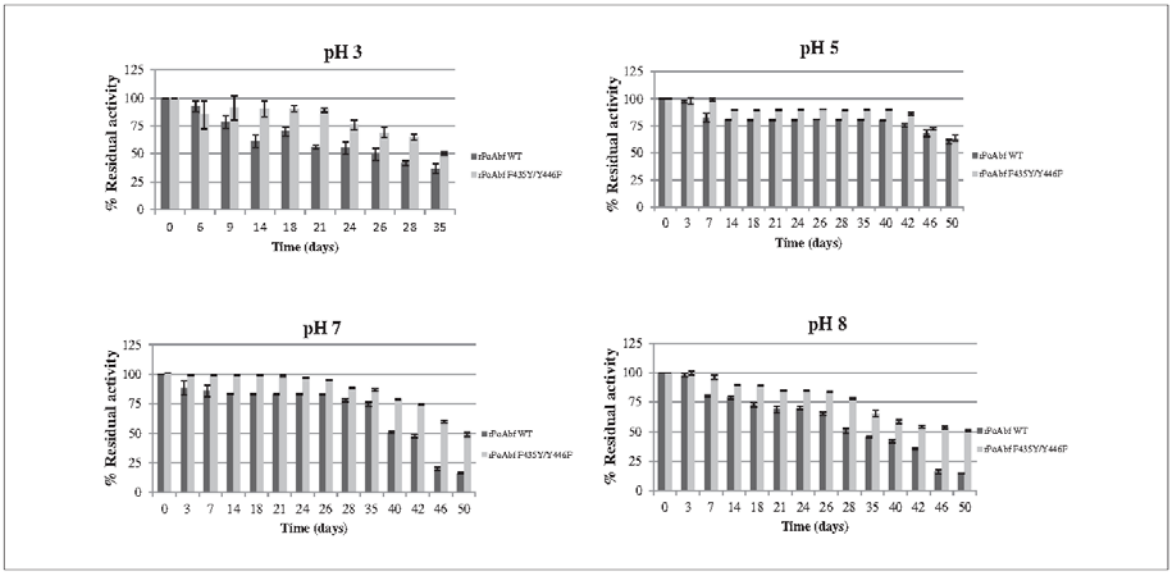

FIGURE 4

Thermo-resistance of rPoAbf wild type (rPoAbf WT) and rPoAbf F435Y/Y446F. The temperature resistance was studied by incubating the purified enzyme preparations in 50 mM citrate phosphate buffer pH 5.0, at 40°C, 50°C and 60°C. The samples withdrawn were assayed for residual α-arabinosidase activity performing incubation (10 min) at 40°C.

TABLE 1

Arabinose liberation from natural substrates and arabinooligosaccharides by rPoAbf wild-type and rPoAbf F435Y/Y446F. 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)

Substrate	Amount of released sugar (μg mL^{-1})	
	rPoAbf wild-type	rPoAbf F435Y/Y446F
Larch arabinogalactan	0	205.4 ± 13.8
CM-linear arabinan	240 ± 8	288.7 ± 4.6
Arabinotriose	112.7 ± 2.9	111 ± 4
Arabinohexaose	107.4 ± 8	132.06 ± 18.61

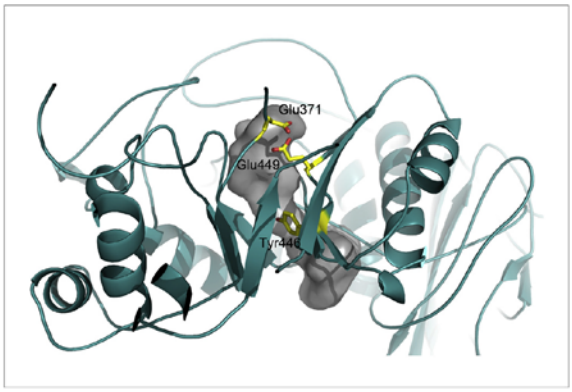

FIGURE 5

Cartoon and surface representation of the model of PoAbf. The conserved catalytic residues Glu371 and 449, as well as Tyr446, are shown in yellow. The channel crossing the modeled protein is represented with a blue arrow. The penta saccharide bound to the homolog from *Thermobacillus xylanilyticus* has been fitted into the active site of PoAbF and indicates the entrance of the channel.

Summary

A library of 6400 random mutants of the enzyme α-L-arabinofuranosidase from *Pleurotus ostreatus* was generated by error prone PCR of the *poabf* cDNA and expression of mutated cDNAs in *S. cerevisiae*. 24 variants exhibiting higher activity than the wild type enzyme towards p-nitrophenyl α-L-arabinofuranoside (pNPA) in multiwell microplates were selected and further analysed for production of extracellular α-L-arabinofuranosidase activity in shaken flasks. Four of the more active mutants were selected and their analysis showed the presence of the mutations F435Y/Y446F in all the variants. Characterisation of the mutant rPoAbf F435Y/Y446F over-expressed in *P. pastoris* showed a k_{cat} towards pNPA around 3-fold higher than that of the wild type. Moreover, analysis of the effect of pH and temperature on activity of rPoAbf F435Y/Y446F towards pNPA showed that it exhibits a wide range of optimal pH values (pH 3–6) differing from the wild type that shows an optimum pH of pH 5 and thus revealing more versatile than the parental enzyme. Furthermore, the hydrolytic abilities of rPoAbf F435Y/Y446F were shown to be improved towards other substrates. In fact, rPoAbf F435Y/Y446F shows a k_{cat} towards pNP-β-D-glucopyranoside around 5-fold higher than that of the wild type, and it is able to hydrolyse larch arabinogalactan which is not recognised by the wild type. The mutant was also more active than the wild type towards AZCL-arabinoxylan and was able to hydrolyse AZCL-arabinan (debranched) which was not transformed by the wild type.

Based on these results, the mutant rPoAbf F435Y/Y446F could be used as a template for new directed evolution rounds aimed at improving its ability to hydrolyse these substrates and increase its potential of application. Moreover, while the mutant rPoAbf F435Y/Y446F was selected for its higher activity, it showed also a higher resistance to temperature and pH than the wild type. Thus, the potential of *in vitro* evolution of the α-L-arabinofuranosidase PoAbf as a strategy to achieve improved variants of this hemicellulase was demonstrated. Conservative mutations were identified in the selected mutants indicating their importance for the evolutionary selection. To the best of our knowledge, this is the first manuscript of directed evolution of a α-L-arabinofuranosidase giving selection of enzyme variants with improved hydrolytic activity, the only other recently published work being aimed at improved transglycosidase activity [15].

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3.1.2 SECOND GENERATION LIBRARY OF ARABINOFURANOSIDASE FROM *PLEUROTUS OSTREATUS*

This section is related to a second generation library of directed evolved variant of PoAbf. The cDNA coding for PoAbf F435Y/Y446F was used as template to create a new library of variants. Mutated cDNAs library with medium mutation rate was created, and homologous recombination experiments were performed.

3.1.2.1 RESULTS

-Construction and screening II generation library

To develop novel improved variants of PoAbf, EP-PCR experiments with medium mutation frequence (MMF) were performed to introduce random mutations into *poabf* F435Y/Y446F cDNA. Mutated cDNAs were cloned into the expression vector pSAL4 by using the *in vivo* homologous recombination system of *S. cerevisiae* and a total of 5500 transformants were obtained. The library was subjected to a high-throughput screening to catch mutants exhibiting higher arabinofuranosidase activity towards p-nitrophenyl-α-L-arabinofuranoside (pNPA) as substrate than the PoAbf F435Y/Y446F as described in Giacobbe *et al*., 2014. The first screening, performed on MUA-containing solid growth medium led us to select around 3000 mutants. These were further subjected to a high-throughput screening in liquid medium within multiwell plates allowing the selection of 76 mutants, exhibiting at least 3-fold higher activity values towards pNPA than the mutant PoAbf F435Y/Y446F used as template. Among these 76 mutants, 19 clones, showing 4 or 5-fold higher activity values towards pNPA were selected and further analyzed in liquid medium in shaken flasks monitoring time course of growth and activity production for 4 days. Six out of these 19 mutants, exhibiting 2-fold higher activity values than the wild type at 48h, were chosen for sequence analyses (Figure 3). The sequence analysis showed that three out of selected clones have not new aminoacid substitutions, while the other three clones showed the aminoacid substitutions reported in the Table 6.

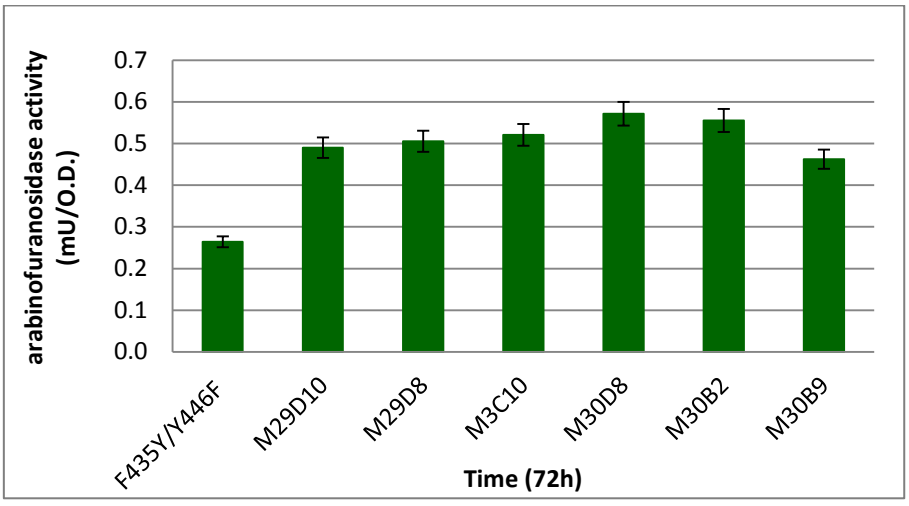

Figure 3: Arabinofuranosidase activity towards pNPA at 72 h of growth

Library	Screening in mutiwell plates	Screening in shaken flasks	Aminoacid sobstiitution
3100 mutants	**76** mutants	**6** mutants	**3** mutants **M30B2** (**T22S**/F435Y/Y446F) **M30D8** (F435Y/Y446F/**K533N)** **M3C10** (**F434L**/F435Y/Y446F)

Table 6: summary of II generation library screening

-Production of mutated PoAbfF435Y/Y446F/K533N and PoAbfF435Y/Y446F

In order to characterize the variant PoAbfF435Y/Y446F/K533N, this mutant was over-produced using Techfors-S 7L fermenter. Moreover, growth cultures in fermenter of the mutant PoAbfF435Y/Y446F were performed to compare the activity production levels.

A batch cultivation technique was adopted for the production of both enzymes expressed in *S. cerevisiae.* The cells were grown at 28°C using selective medium as described in Piscitelli *et al.* (2005). The highest arabinofuranosidase activity, 6mU/mL and 10 $mUmL^{-1}$ for PoAbfF435Y/Y446F and PoAbfF435Y/Y446F/K533N respectively, was reached after two days of growth. The arabinofuranosidase activity reached in fermentation was about 2 times higher than in shaken-flask culture (around 3 $mUmL^{-1}$ for PoAbf with two aminoacid substitutions and 6 $mUmL^{-1}$ for PoAbf with three substitutions) and was obtained 1 day before than in shaken-flask culture.

Utrafiltration was used to concentrate fermentation broths. After this step, different purification strategies were exploited for rPoAbfF435Y/Y446F/K533N and rPoAbf F435Y/Y446F. In the first strategy culture supernatant was dialyzed using 20mM Tris-HCl pH 7.5 containing 1.2M (NH4)2SO4. Hydrophobic interaction chromatography was sequentially tested to purify the enzyme rPoAbfF435Y/Y446F. The SDS-PAGE of active fraction, showed no homogeneity sample (Figure 4).

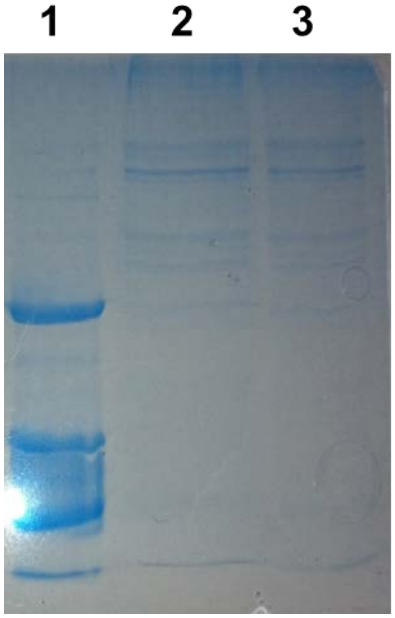

Figure 4: Electrophoretic analysis of recombinant PoAbf F435Y/Y446F (5 µg). Lane 1 molecular weight standards; lane 2 PoAbf F435Y/Y446F before HIC; lane 3 HIC active fraction.

In the second strategy culture supernatant was dialyzed using 20mM Tris-HCl pH 7.5 containing 200mM NaCl. Size exclusion chromatography was sequentially tested to purify the enzyme rPoAbf F435Y/Y446F. Also in this case no homogeneity sample was obtained (Figure 5).

Further purification strategies are needed to purify and sequentially characterize recombinant PoAbfF435Y/Y446F and its evolved variant.

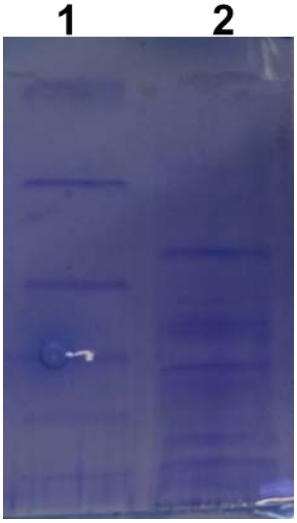

Figure 5: Electrophoretic analysis of recombinant PoAbf F435Y/Y446F (5 µg). Lane 1 molecular weight standards; lane 2 Size exclusion chromatography active fraction.

3.1.2.2 MATERIALS AND METHODS

-Strains and culture media

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 g L^{-1}: 10 bacto tryptone, 10 NaCl, 5 yeast extract), supplemented, when required, with 100 µg mL^{-1} of ampicillin.

The *S. cerevisiae* strain and the plasmid (pSAL4) used for heterologous expression of the random mutants are described in Giacobbe *et al*. (2014). *S. cerevisiae* was grown on a selective medium (SD) as described in Piscitelli *et al*. (2005).

-Random mutagenesis and construction of a mutant library

Random mutagenesis of *poabf* cDNA was performed by Error Prone PCR (EP-PCR) with Medium mutation frequencies, using pSAL4-*poabfF435Y/Y446F* as template and Gene Morph II Random Mutagenesis Kit (Agilent, La Jolla, CA). Primers and PCR condition used in amplification experiments are the same that used previously for the first generation library (Giacobbe *et al.,* 2014).

The cDNAs resulting from EP-PCR were ligated in pSAL4 vector digested with EcoRI/HindIII restriction enzymes, using homologous recombination expression system of *S. cerevisiae* as previously described for the first generation library

(Giacobbe *et al*., 2014). The cells were spread on SD medium and the plates incubated for 4 days at 28°C.

-Library screening

Agar plate assays on SD medium supplemented with the chromogenic substrate 4-methylumbelliferyl-α-L-arabinofuranoside (MUA) (Carbosynth, Compton, Berkshire, UK) were used to select transformants expressing α-L-arabinofuranosidase activity as described in Amore *et al*. (2012a). The high-throughput screening developed in Giacobbe *et al.,* (2014) was used to assay α-L-arabinofuranosidase activity with p-nitrophenyl-α-L-arabinofuranoside (pNPA) as substrate, following the method reported in Amore et al. (2012a) with some modifications. Cultures in shaken flasks and using Techfors-S 7L fermenter were also performed for selected mutants. Pre-cultures were grown in selective medium at 28°C on a rotary shaker (150 rpm). A volume of suspension sufficient to reach a final OD_{600} value of 0.5 was then used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of selective medium or fermenter containing at least 3L of selective medium. Optical density and arabinofuranosidase activity were daily assayed.

-DNA sequencing

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

-Arabinofuranosidase activity assays

α-L-Arabinofuranosidase activity was measured by spectrophotometric method with p-nitrophenyl-α-L-arabinofuranoside (pNPA) (Gold Biotechnology, St Louis, MO, USA) as substrate as described in Amore *et al*. (2012a).

-**PoAbf purification**

S.cerevisiae culture media expressing rPoAbf F435Y/Y446F and rPoAbf F435Y/Y446F/K533N were harvested on the optimal arabinofuranosidase production day, cells were sedimented by centrifugation at 7000 rpm at 4°C for 15 min. 1 mM PMSF was added to culture supernatant. After this step, different purification strategies were exploited for purification.

S.cerevisiae supernatant expressing rPoAbf F435Y/Y446F was dialyzed using 0.02 M Tris-HCl pH 7.5 containing 1.2M $(NH_4)_2SO_4$, and loaded on HiTrap Phenyl FF high sub (GE Healthcare, Uppsala, Sweden) equilibrated in buffer A (0.02M Tris-HCL, 1.2M $(NH_4)_2SO_4$, pH 7.5), and the proteins were eluted isocratically with buffer B (0.02M Tris-HCL pH 7.5). Fractions containing activity were combined and concentrated on an Amicon PM-10 membrane and analyzed by SDS-PAGE.

S.cerevisiae supernatant expressing rPoAbf F435Y/Y446F was dialyzed using 0.02 M Tris-HCl pH 7.5 containing 200mM NaCl, and loaded on HiLoad™ 16/60 Superdex™ 75pg (GE Healthcare, Uppsala, Sweden) equilibrated in buffer 0.02 M Tris-HCl, 200mM NaCl, 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.

Chapter 4

Definition of tailor made enzyme cocktails for biomass transformation

4.1 INTRODUCTION

This chapter concerns the enzymatic hydrolysis of lignocellulosic biomasses for bioethanol or bioproducts production. As described in many studies, enzymatic breakdown of lignocellulosic biomass is a complex process which requires synergistic/cooperative catalytic activities (e.g. cellulases, hemicellulases and accessory enzymes). Enzymes involved in biomass degradations have to work simultaneously towards different components of lignocellulosic matrix. Moreover, a pretreatment process is required to improve accessibility of substrates to enzymes involved into the enzymatic hydrolysis (Sweeney & Xu, 2012; De Vries & Visser., 2001; Yang et al., 2011). Synergistic action of enzymes are widely observed in cellulose hydrolysis and it depend on many factors such as enzymes sources, enzymes specificity and substrates structure and composition. It must be note that enzymes combination/ratio and kind of pretreatment depend on biomass composition (Yang *et al*., 2011).

The following paper **"The effect of Pleurotus ostreatus arabinofuranosidase and its evolved variant in lignocellulosic biomasses conversion" (Paper V)** describes the effect of PoAbf, its evolved vatriant and Celstrep on saccahirifiaction of different lignocellulosic biomasses.

4.1.1 THE EFFECT OF PLEUROTUS OSTREATUS ARABINOFURANOSIDASE AND ITS EVOLVED VARIANT IN LIGNOCELLULOSIC BIOMASSES CONVERSION

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The effect of *Pleurotus ostreatus* arabinofuranosidase and its evolved variant in lignocellulosic biomasses conversion

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ABSTRACT

The fungal arabinofuranosidase from *Pleurotus ostreatus* PoAbf recombinantly expressed in *Pichia pastoris* rPoAbf and its evolved variant rPoAbf F435Y/Y446F were tested for their effectiveness to enhance the enzymatic saccharification of three lignocellulosic biomasses, namely *Arundo donax*, corn cobs and brewer's spent grains (BSG), after chemical or chemical–physical pretreatment. All the raw materials were subjected to an alkaline pretreatment by soaking in aqueous ammonia solution whilst the biomass from *A. donax* was also pretreated by steam explosion. The capability of the wild-type and mutant rPoAbf to increase the fermentable sugars recovery was assessed by using these enzymes in combination with different (hemi)cellulolytic activities. These enzymatic mixtures were either entirely of commercial origin or contained the cellulase from *Streptomyces* sp. G12 CelStrep recombinantly expressed in *Escherichia coli* in substitution to the commercial counterparts. The addition of the arabinofuranosidases from *P. ostreatus* improved the hydrolytic efficiency of the commercial enzymatic cocktails on all the pretreated biomasses. The best results were obtained using the rPoAbf evolved variant and are represented by increases of the xylose recovery up to 56.4%. These data clearly highlight the important role of the accessory hemicellulolytic activities to optimize the xylan bioconversion yields.

1. Introduction

Lignocellulose represents a favoured feedstock for development of a 'no oil' bioeconomy since it is the most abundant biomass, representing near 70% of the total plant biomass, and its use does not provide competition with food industries. The efficient enzymatic hydrolysis of pretreated biomass to achieve both high glucose and xylose yields requires finely tuned mixtures of enzymes acting on the complex carbohydrate matrix. Cellulases, hemicellulases (xylanases) and accessory enzymes (e.g., arabinofuranosidases, pectinases, mannanases) are needed for conversion of pretreated lignocellulose into fermentable sugars. Hemicellulose represents about 20–35% of lignocellulosic biomass and it is a very complex polysaccharide composed of a wide variety of sugars including xylose, arabinose, mannose, and galactose (Saha, 2003). The importance of side-chain acting hemicellulases, such as α-L-arabinofuranosidase (EC 3.2.1.55), feruloyl esterase (EC 3.1.1.73), α-glucuronidase (EC 3.2.1.139), and acetyl xylan esterase (EC 3.1.1.72), in lignocellulosic biomass degradation has been clearly evidenced in literature demonstrating marked increases of the efficiency of lignocellulosic hydrolysis in presence of these auxiliary enzymes (Gao et al., 2011; Ravalason et al., 2012).

The fungi that obtain their nutrition from the breakdown of plant biomass are interesting producers of cell wall-degrading enzymes including side-chain acting hemicellulases (Van den Brink and de Vries, 2011). Several fungi such as *Aspergillus terreus* (Le Clinche et al., 1997), *Aspergillus awamori* (Kaneko et al., 1998), *Penicillium purpurogenum* (De Ioannes et al., 2000), *Aspergillus niger* (vd Veen et al., 1991) and *Aspergillus nidulans* (Ramón et al., 1993) have been reported to be able to produce α-L-arabinofuranosidases.

The white rot fungus *Pleurotus ostreatus* has been recently investigated for its hemicellulolytic capability (Iandolo et al., 2011) and an α-L-arabinofuranosidase, named PoAbf, was identified during its solid state fermentation on tomato pomace (Amore et al., 2012a). The enzyme PoAbf recombinantly expressed in *Pichia pastoris*, rPoAbf, was characterized and further

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experiments of its directed evolution allowed developing a variant, rPoAbf F435Y/Y446F, with improved catalytic properties (Giacobbe et al., 2014). As a matter of fact, this variant when assayed with p-nitrophenyl α-L-arabinofuranoside showed a k_{cat} about 3-fold higher and a K_M 30% lower than that of the wild type, and an improved ability to hydrolyze insoluble substrates such as larch arabinogalactan, arabinoxylan and arabinan, suggesting its improved potential for hemicellulose conversion.

Our work was aimed at assessing the ability of the α -L-arabinofuranosidase rPoAbf and its directed evolved variant rPoAbf F435Y/Y446F to improve the biodegradation of three different lignocellulosic biomasses, namely *Arundo donax*, corn cobs and brewer's spent grain. Since the commercially available hemicellulases are typically dominated by backbone-acting xylanases and β-xylosidases with low side-chain acting hemicellulases, the effect of the addition of PoAbf and its directed evolved variant to commercial cellulases, xylanases, β-xylosidase and β-glucosidase was analyzed. These enzymes were also tested in a cocktail containing the cellulase CelStrep from *Streptomyces* sp. G12 recombinantly expressed in *Escherichia coli* (Amore et al., 2012b) in substitution to the commercial cellulase.

2. Material and methods

2.1. Raw materials

Different agro-industrial raw materials including giant reed (*A. donax*), brewery's spent grains (BSG) and corn cobs were utilized for the saccharification experiments. Untreated *A. donax* biomass was kindly provided by Enea Trisaia Research Center – Italy and consisted of chips of approximately 3 × 2 × 0.5 cm of size obtained from whole plants, including leaves. The biomass was then dried at 50 °C, milled by an homogenizer to obtain a fine powder, and stored in sealed vials at room temperature until use. BSG were provided by Maltovivo micro-brewery, Avellino, Italy and corn cobs were obtained from Companhia das Lezírias (Samora Correia, Portugal). Both materials were dried up at 50 °C to a moisture content less than 10% (w/w), milled to particles smaller than 3 mm and stored in sealed vials at room temperature until use.

2.2. Pretreatments and determination of chemical composition

The different raw materials were subjected to a pretreatment with an aqueous ammonia solution on a lab-scale as described by Maurelli et al. (2013). The biomasses reduced to a fine powder, were soaked in 10% (v/v) aqueous ammonia solution at a solid loading of 5% at 70 °C for 22 h in screw-capped 25 mL bottles to reduce the evaporation. The alkaline mixtures were centrifuged at 800 × g, and the residues were extensively washed with 50 mM sodium acetate buffer until obtaining the required pH for the subsequent enzymatic saccharification process (pH 5.0).

The biomass from *A. donax* pretreated by steam explosion (Di Pasqua et al., 2014) was also analyzed.

Carbohydrate compositions of the biomass samples untreated and pretreated were determined according to the method of Davis (1998). This procedure involved an acid hydrolysis carried out in two step which fractionate the polysaccharides into their corresponding monomers. First, the samples were soaked in 72% v/v H_2SO_4, at a solid loading of 10%, at 30 °C, for 1 h; then the mixtures were diluted to 4% (v/v) H_2SO_4 with distilled water, fucose was added as an internal standard, and the secondary hydrolysis was performed for 1 h at 120 °C. After filtration through 0.45 μm Teflon syringe filters (National Scientific, Lawrenceville, GA), the hydrolysis products were analyzed via HPLC as described below. The acid insoluble lignin (Klason lignin) was determined by weighting the dried residue after total removal of the sugars.

2.3. Enzymatic activities

The following commercial enzymatic preparations: Accellerase 1500 (cellulase enzyme complex), Accellerase XY (xylanase enzyme complex), and Accellerase BG (β-glucosidase) were kindly provided by Danisco US Inc., Genencor Division (Palo Alto, CA, USA). Cellulase from *Trichoderma reesei* ATCC26921, Cellobiase from *A. niger*, Xylanase from *Trichoderma viride* and thermostable β-xylosidase were purchased from Sigma (St. Louis, MO). The cellulase CelStrep from *Streptomyces* sp. G12 recombinantly expressed in *E. coli* (Amore et al., 2012b), the α-L-arabinofuranosidase from *P. ostreatus* recombinantly expressed in *P. pastoris* (rPoAbf) (Amore et al., 2012a) and a mutant of rPoAbf indicated as rPoAbf F435Y/Y446F (Giacobbe et al., 2014) were utilized in combination with the commercial enzymatic preparations.

The enzymatic activities were measured at 50 °C in 50 mM sodium acetate buffer (pH 5.0). Cellulase activity was determined spectrophotometrically utilizing the soluble chromogenic substrate carboxymethyl cellulose-Remazol Brilliant Blue R (AZO-CM-Cellulose) (Megazyme Co., Bray Ireland). The standard assay mixture, containing 150 μL of 0.2% (w/v) substrate in 50 mM sodium acetate buffer, and 150 μL of properly diluted enzyme solution, was incubated for 30 min. The reaction was stopped by adding 750 μL of 96% ethanol to the mixture, followed by incubation at room temperature for 10 min and centrifugation at 9000 × *g* for 10 min. The absorbance of the supernatant was read at 590 nm, and the enzyme units were calculated from a standard curve constructed with known amounts of cellulase from *T. viride*. The xylanase activity was evaluated by the same assay method utilizing methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Azo-Oat Spelt Xylan) (Megazyme Co., Bray Ireland), as the substrate (Biely et al., 1985). One unit of xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 A min^{-1} under the experimental conditions. β-xylosidase activity was determined, according to Biely et al. (2000), utilizing the soluble chromogenic substrate *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXP) (Sigma, St. Louis, MO). The assay was started by adding 50 μl of appropriately diluted enzyme in 50 mM sodium acetate buffer pH 5.0 to 450 μL of 2 mM *p*NPXP, in the same buffer. After heating the assay mixture at 50 °C for 10 min, the reaction was stopped by adding 1 mL of 1.0 M sodium carbonate and the release of *p*-nitrophenol was detected by measuring the absorbance at 405 nm. One unit of β-xylosidase was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min under the assay conditions. α-Arabinofuranosidase and β-glucosidase activities were evaluated by the same assay method utilizing *p*-nitrophenyl (*p*NP)-α-L-arabinofuranoside and *p*NP-β-D-glucopyranoside (Sigma, St. Louis, MO) as the substrates, respectively. All the enzymatic measurements were performed in triplicates.

2.4. Enzymatic hydrolysis

The saccharification experiments were carried out at 50 °C for 72 h in a total volume of 5 mL (50 mM sodium acetate buffer pH 5.0 plus enzyme cocktail), at a solid loading of 5%. The hydrolysis of pretreated lignocellulosic materials was carried out with enzyme cocktails prepared with the following commercial products at the indicated amounts expressed as units per grams of pretreated biomass: 5.4 U g^{-1} of Accellerase 1500, 4000 U g^{-1} of Accellerase XY and 145 U g^{-1} of Accellerase BG (mix 1) or 5.4 U g^{-1} Cellulase from *T. reesei* ATCC26921, 145 U g^{-1} of Cellobiase from *A. niger*, 80 U g^{-1} of Xylanase from *T. viride*, 8 U g^{-1} of thermostable β-xylosidase (mix 2). Because of its high glucose content, Accellerase BG was dialyzed against 50 mM sodium acetate before addition to the pretreated biomass.

Subsequent experiments were performed utilizing cellulase CelStrep in each commercial cocktail, mix 1 or mix 2, in substitution of the cellulolytic component and the mixtures then named mix 1CS and mix 2CS, respectively.

The action of rPoAbf and rPoAbfF 435Y/Y446F mutant was evaluated in combination with mix1, mix 2, mix 1CS and mix 2CS.

The saccharification mixtures together with blanks (pretreated lignocellulosic materials without enzyme cocktail) were incubated in a shaking water bath at 50 °C and 140 rpm for 72 h. Samples were withdrawn at different time intervals, chilled on ice and centrifuged at 16,500 × g for 30 min at 4 °C. The supernatants were analyzed to quantify the amount of sugars released as described below. The saccharification yield was expressed as percentage of sugar production calculated respect to the amount of sugars present in the dry weight pretreated materials before the hydrolysis processes. Each saccharification experiment was run in triplicate.

2.5. Determination of sugar content

For estimation of the sugars released from untreated and pretreated biomasses prepared as described in Section 2.2 and enzymatic hydrolysates prepared as described in Section 2.4, the cleared supernatants were opportunely diluted, and analyzed by a high-performance liquid chromatographic (HPLC) system (Dionex, Sunnyvale, CA, USA), equipped with an anionic exchange column (Carbopac PA-100) and a pulsed electrochemical detector. Glucose and xylose were separated with 16 mM sodium hydroxide at a flow rate of 0.25 mL/min, and identified by the respective standards. Fucose was used as internal standard.

3. Results and discussion

3.1. Effect of rPoAbf and its variant in bioconversion of A. donax pretreated by steam explosion

The use of agricultural residues and non-food crops such as *A. donax* as alternative cheap substrates has considerable potentialities for the cost-effective biofuel production. *A. donax* crop is considered a valuable feedstock, being one of the highest yielding species for biomass production in southern Europe, with a high xylan content representing the 20% of its dry weight (Scordia et al., 2011). It has also been recognized as a very robust species with the characteristic to avoid competition with food crops for lands, due to its ability to grow well on marginal and non-agricultural soils (Krasuska et al., 2010).

A. donax pretreated by steam explosion (Di Pasqua et al., 2014) was subjected to saccharification using cocktails of commercial enzymes exclusively of fungal origin, with the addition of the *P. ostreatus* arabinofuranosidase recombinantly expressed in *P. pastoris* rPoAbf or its evolved variant rPoAbf F435Y/Y446F. These arabinofuranosidases were also tested in combination with the recently investigated cellulase from *Streptomyces* sp. G12 CelStrep recombinantly expressed in *E. coli* to verify a possible synergism with a bacterial cellulase. The conditions of pH and temperature adopted in saccharification experiments were those described in the Section 2.4 at which all the tested enzymes retained 80% of their initial activities. The following main effects were revealed in the different tested conditions.

3.1.1. Effect of addition of rPoAbf or its variant to the commercial enzymatic mix 1

It was shown that the use of wild-type rPoAbf in combination with the mix 1 from Genencor (Fig. 1) improved the xylose yield and speeded up xylan degradation. The use of the evolved mutant of rPoAbf allowed further increasing the xylose recovery and achieving the highest monosaccharide yield. This can be due to the increase of the xylan accessibility and hydrolysis caused by removal of the arabinosyl side chains from hemicellulose catalyzed by the arabinofuranosidases. The glucose yield, conversely, was not affected by the addition of the arabinofuranosidases (Supplementary Table 1).

3.1.2. Effect of addition of rPoAbf and its variant to the commercial enzymatic mix1 containing CelStrep instead of the commercial cellulase

When the recombinant enzyme CelStrep was used in the mix 1 instead of Accellerase 1500 (Fig. 1), the ability of rPoAbf and its evolved variant to improve the xylan degradation became even more evident. As reported by Gao et al. (2011), for evaluating the role of individual biocatalysts in biomass hydrolysis, the employment of a characterized enzyme, such as the recombinant CelStrep, is more advantageous than the utilization of enzymatic preparations lacking a defined composition. Using CelStrep, the xylose yield was highly reduced due to the absence of the hemicellulolytic activities reported for the Accellerase 1500 preparation. The addition of wild-type rPoAbf caused a marked increase in the xylose recovery that became closer to that obtained with Accellerase 1500. In particular, the xylose recovery after 48 h was 12.1% higher than that obtained with the mix 1. The evolved variant of rPoAbf was able to further improve the xylose yield and it is worth noting that the substitution of Accellerase 1500 with CelStrep plus the mutated rPoAbf enhanced the xylose yield up to 24% (after 48 h of conversion). The reported data clearly highlight that the limitation of the commercial preparation in xylan hydrolysis derives from the prevalence in their composition of backbone-acting xylanases at the expense of the auxiliary hemicellulolytic enzymes active on the side groups. Hence, the removal of side chains which impede endoxylanase activity are crucial to obtain high xylose yields (Banerjee et al., 2010). As concerning the glucose recovery, it was reduced in presence of CelStrep and was not affected by the addition of the arabinofuranosidases (Supplementary Table 1).

3.1.3. Effect of addition of rPoAbf or its variant to the commercial enzymatic mix 2

In order to identify enzyme combinations that could improve the hydrolysis yield, further trials were performed using a cocktail of commercial enzymes from Sigma (mix 2) including cellulolytic and xylanolytic activities (Table 1). This gave a 96.4% xylose recovery after 72 h. The addition of rPoAbf did not substantially influence the xylose recovery whilst the utilization of rPoAbf F435Y/Y446F speeded up the xylan degradation without modifying the maximum xylose yield.

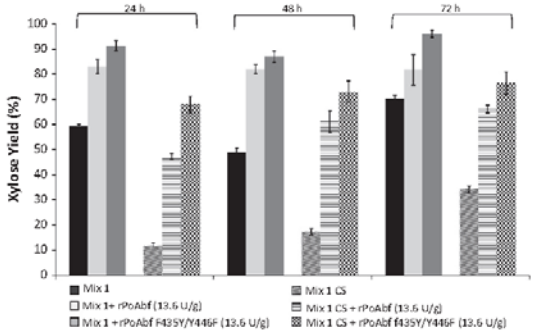

Fig. 1. Xylose yield of saccharification of *Arundo donax* pretreated by steam explosion obtained by adopting the mix 1 (5.4 U g^{-1} of Accellerase 1500, 4000 U g^{-1} of Accellerase XY and 145 U g^{-1} of Accellerase BG) or mix 1CS (mix 1 containing the recombinant enzyme CelStrep instead of Accellerase 1500) with or without addition of rPoAbf or rPoAbfF 435Y/Y446F mutant.

3.1.4. Effect of addition of rPoAbf to the commercial enzymatic mix 2 containing CelStrep instead of the commercial cellulase

The use of CelStrep in the enzyme mix 2 instead of the commercial cellulase reduced the glucose yield and the most evident effect was represented by halving of the maximum value (64%) at 72 h (Supplementary Table 2). Also the recovery of xylose decreased (Table 1), that can be explained by the lack, for CelStrep, of side xylanolytic activities present in the commercial cellulase. Differently from the results obtained with CelStrep in combination with Genencor enzymes (mix 1 CS), this situation was not modified by the addition of wild-type rPoAbf. These data indicate that, in presence of CelStrep, rPoAbf works better in combination with the Genencor commercial cocktail than with the Sigma preparation. Probably, the diverse specificities of the biocatalysts involved in the enzymatic cocktails towards the same substrate was at the basis of the different conversion yields reported.

3.1.5. Effect of addition of rPoAbf variant to the commercial enzymatic mix 2 containing CelStrep instead of the commercial cellulase

The use of the evolved mutant rPoAbf increased the xylose recovery and allowed to achieve, after 24 h, a yield of xylose by 14.1% higher than that obtained with the commercial enzymes mix 2 (Table 1). When the units of the evolved mutant of rPoAbf were doubled, the recovery of xylose achieved 90% after 72 h. This value was closer to that of 98.2% obtained with the mix 2, in presence of the evolved mutant. Doubling CelStrep gave an improvement in cellulose hydrolysis (Supplementary Table 2). Moreover, when double amount of CelStrep was used in combination with the evolved rPoAbf mutant, an acceleration of xylose recovery was also observed. In particular, a 21.6% increase in xylose yield was observed after 48 h compared to the results obtained when only a double amount of rPoAbf mutant was utilized. A possible synergism between the actions of CelStrep and the evolved rPoAbf mutant can be at the basis of the acceleration of the xylose recovery detected when the amounts of the cellulase and the rPoAbf mutant were increased at the same time. These findings highlighted that, besides the frequently cited effect of improvement in glucan conversion following the xylan removal (Polizeli et al., 2005), also the progress of the cellulose hydrolysis can facilitate the breakdown of the xylan fraction which is intimately adsorbed onto the cellulose microfibrils (Dammstrom et al., 2009).

3.2. Effect of PoAbf and its variant in bioconversion of A. donax and other biomasses pretreated by AAS

Different lignocellulosic biomasses, namely BSG and corn cobs, were also tested in the saccharification experiments in addition to *A. donax*. The former two materials are agro-industrial by-products characterized by a high xylan content of, that accounts for the 35% and 24.1% of their dry weights, respectively. In these trials, wild type rPoAbf or its evolved variant were used in combination with two kinds of enzymatic cocktails: the mix 2 and this cocktail containing CelStrep in substitution of the commercial cellulase. In all the experiments, the maximum amounts for both CelStrep and rPoAbf previously adopted were tested. In order to improve the xylan digestibility, the three biomasses BSG, corn cobs and *A. donax* were then subjected to pretreatment with aqueous ammonia soaking (AAS). As this kind of pretreatment modifies the structure and composition of lignocelluloses leaving behind a substantial amount of hemicellulose, it is conceivable that the effectiveness of side-chain acting hemicellulases is better highlighted on alkaline-treated materials. In Table 2, the macromolecular composition of all the materials before and after the pretreatments, in terms of percentage of glucans, xylans and Klason lignin, is reported. As it is possible to see, the pretreatment with AAS mainly caused the removal of lignin with a consequent enrichment of glucans and xylans. For *A. donax*, pretreated also by steam explosion, it was possible to compare the effects of the two pretreatment methods highlighting that steam explosion mainly provoked the removal of a part of the xylan.

3.2.1. Effect of PoAbf and its variant in bioconversion of A. donax

Analyzing the results of conversion of *A. donax* pretreated with AAS using the enzyme cocktail 2 in combination with wild-type rPoAbf or its evolved variant, it is possible to see that the yield of glucose remained unchanged (Supplementary Table 3) whilst a higher yield in xylose was obtained with the evolved variant of rPoAbf (Table 3).

The addition of the evolved variant of rPoAbf to the cocktail containing CelStrep allowed increasing the yield in xylose up to 14% respect to that obtained with the wild type. However, this effect was evident only after 48 h whilst the yield after 72 h remained unchanged (Table 3).

Comparing the results of conversion of *A. donax* pretreated with AAS and by steam explosion it is noticed that the saccharification of the biomass pretreated with AAS led to a lower yield of xylose expressed as percentage on the amount of sugars present before the hydrolysis (i.e., 90.5% instead of 62.9%, after 72 h of conversion with the mixture containing 27.2 U/g of mutated rPoAbf and Mix 2 CS2). This can be explained considering that the pretreatment with AAS causes the removal of a larger fraction of lignin, leading to an enrichment of glucans and xylans in the pretreated biomass, that leads to a lower fraction of xylose expressed in percentage on the total amount of these polysaccharides. On the other hand, if the absolute amounts of xylose are compared, the values after AAS pretreatment were higher than those achieved by steam explosion (i.e., 8.12 instead of 3.46 g $L^{-1}$of xylose, recovered after 72 h of conversion with the mixture containing CelStrep and

Table 1
Arundo donax pretreated by steam explosion (Di Pasqua et al., 2014).

	Composition	Xylose yield (%)		
		24 h	48 h	72 h
	Mix 2	50.1 ± 1.9	64.7 ± 2.6	96.4 ± 0.6
rPoAbf (13.6 U/g)	Mix 2	44.5 ± 5.4	59.9 ± 4.5	93.2 ± 3.7
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2	48.5 ± 6.1	77.7 ± 3.0	98.2 ± 1.3
	Mix 2 CS1	49.0 ± 4.0	51.0 ± 4.3	65.6 ± 3.2
rPoAbf (13.6 U/g)	Mix 2 CS1	48.6 ± 5.6	52.6 ± 4.2	60.8 ± 4.4
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2 CS1	64.2 ± 5.4	65.3 ± 3.2	68.4 ± 5.2
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS1	44.6 ± 2.8	45.5 ± 2.6	90.0 ± 4.0
rPoAbf F 435Y/Y446F (13.6 U/g)	Mix 2 CS2	53.0 ± 3.3	57.9 ± 5.8	63.8 ± 5.1
rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	50.6 ± 3.7	67.1 ± 5.5	90.5 ± 4.6

Biotransformations in 50 mM buffer acetate pH 5.0 T = 50 °C, for 24, 48 and 72 h.
Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g^{-1} of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g^{-1}); X3876 xylanase from *Trichoderma viride* (80 U g^{-1}); X3504 β-xylosidase, thermostable (8 U g^{-1}).
Mix 2 CS1 or Mix 2 CS2: Mix 2 containing CelStrep, cellulase from *Streptomyces* sp., (5.4 or 10.8 U g^{-1}) instead of C2730, cellulase from *Trichoderma reesei* ATCC 26921.

Table 2
Macromolecular composition of untreated and pretreated biomasses.

Chemical composition (% total dry weight)							
Polysaccharides	Corn cobs (untreated)	Corn cobs (after AAS)	BSG (untreated)	BSG (after AAS)	*Arundo donax* (untreated)	*Arundo donax* (after AAS)	*Arundo donax* (after steam explosion)
Glucan	38.0 ± 0.4	51.2 ± 1.2	26.3 ± 1.6	49.9 ± 0.3	37.8 ± 2.0	63.2 ± 1.5	38.2 ± 1.2
Xylan	35.0 ± 0.7	43.3 ± 0.8	24.1 ± 1.2	40.7 ± 0.6	19.3 ± 0.9	26.0 ± 0.7	5.7 ± 0.9
Klason lignin	19.1 ± 0.5	5.4 ± 0.7	9.8 ± 0.4	5.2 ± 0.3	24.9 ± 1.3	10.7 ± 1.1	36.1 ± 0.6

Table 3
A. donax 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0 $T = 50$ °C, for 24, 48 and 72 h incubation.

		Composition	Xylose yield (%)		
			24 h	48 h	72 h
Arundo donax	rPoAbf (27.2 U/g)	Mix 2	41.0 ± 2.2	49.9 ± 1.8	72.1 ± 2.0
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	42.8 ± 3.5	57.7 ± 2.2	82.5 ± 2.4
	rPoAbf (27.2 U/g)	Mix 2 CS2	29.8 ± 2.9	33.6 ± 3.7	64.9 ± 4.1
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	33.7 ± 3.5	47.5 ± 4.2	62.9 ± 4.3

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g $^{-1}$ of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g $^{-1}$); X3876 xylanase from *Trichoderma viride* (80 U g $^{-1}$); X3504 β-xylosidase, thermostable (8 U g $^{-1}$).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g $^{-1}$) from *Streptomyces* sp. instead of C2730 cellulase from *Trichoderma reesei* ATCC 26921.

Table 4
BSG 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0 $T = 50$ °C, for 24, 48 and 72 h incubation.

		Composition	Xylose yield (%)		
			24 h	48 h	72 h
BSG	rPoAbf (27.2 U/g)	Mix 2	57.6 ± 3.8	63.5 ± 3.0	73.1 ± 4.2
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	64.4 ± 1.5	72.7 ± 2.9	85.8 ± 3.6
	rPoAbf (27.2 U/g)	Mix 2 CS2	53.4 ± 2.3	58.4 ± 2.8	63.3 ± 4.2
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	58.5 ± 2.9	64.1 ± 2.4	66.4 ± 4.1

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g $^{-1}$ of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g $^{-1}$); X3876 xylanase from *Trichoderma viride* (80 U g $^{-1}$); X3504 β-xylosidase, thermostable (8 U g $^{-1}$).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g $^{-1}$) from *Streptomyces* sp. instead of C2730, cellulase from *Trichoderma reesei* ATCC 26921.

Table 5
Corn cobs 5% w/v, pretreated with NH_4OH. Biotransformation in 50 mM buffer acetate pH 5.0 $T = 50$ °C, for 24, 48 and 72 h incubation.

		Composition	Xylose yield (%)		
			24 h	48 h	72 h
Corn cobs	rPoAbf (27.2 U/g)	Mix 2	53.7 ± 1.0	64.5 ± 3.1	69.4 ± 5.0
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2	58.8 ± 2.0	64.0 ± 3.4	85.5 ± 4.2
	rPoAbf (27.2 U/g)	Mix 2 CS2	42.6 ± 3.0	47.9 ± 5.1	56.1 ± 4.5
	rPoAbf F 435Y/Y446F (27.2 U/g)	Mix 2 CS2	50.6 ± 2.8	57.5 ± 4.3	64.2 ± 6.8

Mix 2: C2730 cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g $^{-1}$ of pretreated biomass); C6105 Cellobiase from *Aspergillus niger* (145 U g $^{-1}$); X3876 xylanase from *Trichoderma viride* (80 U g $^{-1}$); X3504 β-xylosidase, thermostable (8 U g $^{-1}$).
Mix 2 CS2: Mix 2 containing cellulase CelStrep (10.8 U g $^{-1}$) from *Streptomyces* sp. instead of C2730 cellulase from *Trichoderma reesei* ATCC 26921.

mutated rPoAbf) (data not shown). This can be explained considering that the pretreatment with AAS reduce the content of lignin and the absorption of hydrolytic enzymes on it, thus facilitating the biodegradation of polysaccharides (Maurelli et al., 2013).

3.2.2. Effect of PoAbf and its variant in bioconversion of BSG and corn cobs

Analyzing the results of saccharification of BSG and corn cobs, it is possible to see that the addition of the evolved variant of PoAbf in comparison with the wild-type led to an improvement of the saccharification yield in terms of xylose and the best results were obtained when the enzyme was used in addition to the mix 2 (Tables 4 and 5). This outcome was more evident after 72 h of incubation when increases of 14.5% and 16.1% were observed for BSG and corn cobs, respectively. As concerning the glucose recovery, it was reduced in presence of CelStrep and unaltered after the addition of the arabinofuranosidases (Supplementary Tables 4 and 5). Moreover, even if the yields of xylose after 72 h were similar for the two tested materials with the mix 2 in combination with the evolved variant of rPoAbf (82–85%), the hydrolysis of BSG appeared to be the fastest. In fact, after 24 e 48 h of BSG saccharification, the highest xylose yields, corresponding to 64.4% and 72.7% respectively, were obtained. The explanation could be that the lower lignin content of this biomass can be at the basis of the higher biodegradability of its xylan fraction. This can also justify the similar absolute amounts of xylose obtained for BSG and corn cobs, despite the higher xylan content of corn cobs (at maximum 17.32 and 18.46 g L^{-1} of xylose obtained from BSG and corn cobs, respectively, after 72 h incubation with commercial enzymes and mutated rPoAbf) (data not shown). A similar situation was verified also analyzing the amounts (g L^{-1}) of arabinose obtained from the hydrolysis of both BSG and corn cobs (data not shown). The

highest arabinose recovery was obtained for BSG after 72 h with the evolved rPoAbf mutant that gave a two folds higher yield respect to those obtained with the wild type. The arabinofuranosidase gave better results in combination with the commercial cocktail (1.73 g L^{-1}) than in presence of CelStrep (1.65 g L^{-1}). Interestingly, it must be underlined that the evolved rPoAbf variant led to the best yields for arabinose and the xylose at the same time. These findings highlight the synergistic action of rPoAbf with the xylanolytic components of the enzymatic cocktails. The arabinose recovery from corn cobs was undoubtedly lower with all the enzymatic cocktails tested (around 1–1.2 g L^{-1}) and the worst results were obtained with wild type rPoAbf in combination with the mixture containing CelStrep (data not shown). The higher arabinose content for BSG (20–22%) in comparison with that of corn cobs (5.9%) and its lower lignin content (Musatto et al., 2006) can account for these data.

4. Conclusions

This study highlights the relevance of the white rot fungus *P. ostreatus* as source of side-chain acting hemicellulases and arabinofuranosidases, which play a crucial role in complete xylan hydrolysis via their cooperative action with the hydrolytic activities targeted to polysaccharides backbone.

The results obtained showed that the addition of wild-type or directed evolved variant of the arabinofuranosidase from *P. ostreatus* PoAbf improved the hydrolytic efficiency of the commercial enzymatic cocktails on *A. donax* pretreated by steam explosion and aqueous ammonia soaking and on BSG and corn cobs pretreated by aqueous ammonia soaking. It is conceivable that, due to the low content of auxiliary hemicellulolytic enzymes in the commercial preparation, the supplementation with enzymes, that removes side chain on xylan backbone, such as the arabinofuranosidases, are required to develop enzyme mixtures better adapted for lignocellulose hydrolysis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.07.003.

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4.1.2 ENZYMATIC SACCHARIFICATION OF ARUNDO DONAX AFTER OF AMMONIA FIBER EXPANSION (AFEX) PRETREATMENT

This section concerns the work carried out during the research experiences in foreign laboratories. The work was carried out in the laboratory of Dr. Venkatesh Balan at the Biomass Conversion Research Laboratory (BCRL) (Michigan State University). (Conversion of AFEX pretreated *Arundo donax* to Fermentable Sugars using a combination of Commercial and Research Enzymes. Manuscript in preparation).

4.1.2.1 INTRODUCTION

Biofuels produced from renewable feed stock are a good alternative to fossil fuels and have several environmental benefits. Several “eco-friendly clean” processes are being perused to produce produce biofuels and value-added bioproducts (Kajaste, 2014). However, the incentive given to produce energy crops could has devastating effects on agricultural markets, as several food grain producing lands will be taken away, reducing food production and consequently increasing food prices (Scheidel & Sorman 2012).

To avoid any competition for land between food and non-food crops, several efforts are under way to use cropland not suitable for the traditional food crops for growing dedicated energy crops. In Mediterranean environments, hilly areas are considered not sustainable for the traditional cereal production because of yield and gross income are very low and because of the traditional cropping system (deep soil tillage at the end of August and sowing at November) causes extreme vulnerability to soil erosion (Diodato *et al.,* 2009, 2011; Fagnano *et al.,* 2012). In these regions perennial biomass crops such as giant reed (*Arundo donax L*.) have proven to reduce soil erosion and to increase potential gross income of farmers (Fagnano *et al.,* 2015) with favourable environmental impacts as assessed by LCA (Forte *et al.,* 2015).

Giant reed is also suggested for other areas not suitable for growing food crops such as polluted soils and also act as a phyto-remediating agent (Ecoremed, 2011; Fiorentino *et al.,* 2010; 2013).

Many studies in the past have shown that sugar polymers (cellulose and hemicellulose) present in biomasses can be hydrolysed into fermentable sugars and then converted into fuels and chemicals (Kajaste, 2014; Wettstein *et al.,* 2012; Kobayashi & Fukuoka, 2013). The Pacific Northwest National Laboratory (PNNL) and National Renewable Energy Laboratory (NREL) identified 12 building blocks produced through sugars conversions which could be used to obtain variety of high value biobased products. They include, succinic acid, glucaric acid, aspartic acid, glycerol, sorbitol, and xylitol/arabinitol (Werpy & Petersen, 2004; Hermann & Patel, 2007).

The production of bioproducts from lignocellulosic biomass requires several steps (Kumar *et al*., 2009), including an initial pretreatment step to break the lignin barrier and make cellulose and hemicellulose accessible to enzymes during hydrolysis to produce fermentable sugars. Since lignocellulosic biomasses is naturally recalcitrant, it requires a thermo-chemical pretreatment step to open up the cell wall for enzymes to efficiently produce fermentable sugars. Enzymatic hydrolysis

of sugar polymers using biomass degrading enzymes such as cellulase and hemicellulase are preferred over dilute acid hydrolysis due to higher conversion, less corrosive process. Due to the complex composition of the polysaccharides present in the pretreated biomass a tailor made enzyme cocktail is needed to achieve a more efficient hydrolysis process with higher sugar yield in short period of time (Gao *et al.,* 2011; Gao *et al.,* 2010).

This study was aimed at evaluating the potential of *A. donax* as feedstock for bioethanol or bioproducts production. This was performed by testing Ammonia Fiber Expansion (AFEX) as pretreatment method and investigating the ability of the cellulase CelStrep from *Streptomyces sp.* G12 recombinantly expressed in *Escherichia coli* (Amore *et al.,* 2012b) and α-L-arabinofuranosidase PoAbf from the fungus *Pleurotus ostreatus* recombinantly expressed in *Pichia pastoris* (Amore *et al*., 2012a) and its evolved variant rPoAbfF435Y/Y446F (Giacobbe *et al.,* 2014) to improve the saccharification of AFEX pretreated *A. donax*.

4.1.2.2 RESULTS

-Characterization of biomasses

Analyses of macromolecular composition of the biomasses *A. donax* and corn stover were carried out as described in Materials and methods. The results, shown in Table 7, revealed that *A. donax* contains 61.41% of structural polysaccharides, 26.24% Klason lignin and 4.9% ash, while corn stover contains 63.23% of structural polysaccharides, 20.06% Klason lignin and 6.17% ash. Both the analysed biomasses contain significant percentages of C5 sugars mainly represented by xylan (~20%) while arabinan represents only around 2% of total dry weight and the C6 sugars consist mainly of glucan (>37%). These data are in agreement with data reported a previous studies (Caparrós *et al*., 2007; Li et al., 2011; Scordia *et al*., 2012).

COMPOSITION	*A. Donax* (weight %,on dry basis)	corn stover (weight %,on dry basis)
MOISTURE CONTENT	6.0±0.5	8.7±0.6
ASH	4.9±0.6	6.7±1.0
STRUCTURAL CARBOHYDRATE		
- Glucan	38.0±0.0	38.41±0.1
- Xylan	21.0±0.1	19.9±1.0
- Galactan	1±0.1	1.4±0.1
- Arabinan	1.5±0.0	2.5±0.2
- Mannan	N.D.	N.D.
LIGNIN		
- Acid Insoluble Lignin	26.2±0.1	20.1±0.7
- Acid Soluble Lignin	1.9±0.0	2.0±0.3

Table 7: *A. donax* and corn stover composition before AFEX pretreatment (Average values of duplicates)

-Selection of AFEX pretreatment conditions

AFEX pretreatment conditions were chosen based on the previous published worked on *Miscanthus* (Murnen *et al*., 2007). Table 8 summarized the pretreatment conditions reported for *A. donax* and their effect on biomass composition. Here, the composition of *A. donax* after different pretreatments is reported along with composition of untreated *A. donax*. *A. donax* from different sources showed similar structural polysaccharides composition, therefore only composition of one biomass is reported in the table. As shown in Table 8, most of the pretreatments solubilized large portions of xylan from *A. donax*. On the other hand, AFEX is a dry to dry process and able to preserve all the original carbohydrates (Gao *et al.,* 2011 and 2013; Li *et al.,* 2011; Harun *et al.,* 2013) without any loss. The composition of pretreated biomass is more or less similar to untreated biomass. Therefore, this pretreatment process was selected for this study.

Pretreatment	Pretreatment conditions	Composition of pretreated solid (%)				Reference
		Glucan	Xylan	Arabinan	Klason Lignin	
Untreated		35.15± 0.11	18.24±0.04	0.84±0.03	23.02±0.13	(Caparrós et al., 2007)
ACSEP (Water insoluble fraction)	196°C + acid	36 ± 2	8.6 ± 0.6	0.69 ± 0.06	27.1 ± 1.1	(De Bari et al., 2013)
	200 °C + acid	35.1 ± 1.7	6.0 ± 0.5	0.50 ± 0.04	25.7 ± 1.3	
	205 °C + acid	36 ± 2	4.8 ± 0.4	0.52 ± 0.04	23.5 ± 0.7	
	210 °C + acid	34 ± 2	3.5 ± 0.5	0.40 ± 0.04	23.0 ± 1.6	
ACSEP (after acid post hydrolysis)	196°C + acid	1.70 ± 0.05	6.0 ± 0.7	0.55 ± 0.05	N.D.	
	200 °C + acid	2.4 ± 0.2	9.5 ± 0.9	0.80 ± 0.07	N.D.	
	205 °C + acid	2.5 ± 0.3	5.2 ± 0.4	0.63 ± 0.06	N.D.	
	210 °C + acid	1.7 ± 0.2	4.3 ± 0.4	0.51 ± 0.05	N.D.	
Chemtex	Chemtex	46.5 ± 1.1	10.8 ± 0.8	0.25 ± 0.03	35.5 ± 0.9	
	Chemtex	45.4 ± 1.2	6.0 ± 0.4	0.14± 0.01	39.6 ± 0.7	
	Chemtex	45.8 ± 0.9	12.5 ± 0.9	0.14 ± 0.01	35.6 ± 1.1	
	Chemtex	45.1 ± 0.8	7.6 ± 0.6	0.25 ± 0.02	38.9 ± 0.8	
	1 day,20% HCl	54	9	N.D.	N.D.	(Van Den Brink et al., 2013)
	3 min 200°C	35	13	N.D.	N.D.	
AAS	10% (v/v) aqueous ammonia solution soak at a solid loading of 5%, 70°C, 22 h	63.2 ± 1.5	26.0 ± 0.7	N.D.	10.7 ± 1.1	(Marcolongo et al., 2014)
Steam explosion		38.2 ± 1.2	5.7 ± 0.9	N.D.	36.1 ± 0.6	

Table 8: Macromolecular composition of *A. donax* biomass before and after different pretreatment methods.

Corn stover was chosen as reference lignocellulosic biomass because AFEX pretreatment had been previously carried out on it in several works giving high yields of both glucose and xylose (Balan *et al*., 2009; Li *et al*., 2011). The yield of monosaccharides released during hydrolysis depends on various AFEX parameters such as moisture content, ammonia loading, and temperature. Therefore, in order to assess the best pretreatment conditions for *A. donax*, three different AFEX treatments were carried out (Table 9) and the samples of *A. donax* pretreated in the different conditions were then enzymatically hydrolysed for 168 hours using a commercial enzymatic preparation consisting of Novozymes Cellic® Ctec3 and Htec3. In Table 9, the glucan and xylan converison obtained after 24h, 72h and 168h of enzymatic hydrolysis of *A. donax* after the three different AFEX pretreatments are reported in comparison to corn stover. Both glucan and xylan hydrolysis reached their maximum level with the AFEX pretreatment condition 2: 130°C, 1 kg ammonia/1 kg dry biomass and 60% of moisture content. Based on these data, this condition was selected for further hydrolysis experiments. It is worthy of note that the selected AFEX condition is the same pretreatment condition used for corn stover (Gao *et al.*, 2011).

ARUNDO DONAX										
AFEX conditions	**Ammonia loading (Kg/Kg dry biomass)**	**Temperature (°C)**	**Moisture content (%)**	**Time (min)**	**Glucan conversion (%)**			**Xylan conversion (%)**		
					24h hydrolysis	**72h hydrolysis**	**168h hydrolysis**	**24h hydrolysis**	**72h hydrolysis**	**168h hydrolysis**
1	1:1	100	80	15	34.4	41.2	49.7	55.6	60.0	66.9
2	1:1	130	60	15	39.0	44.1	56.8	66.4	65.9	75.5
3	2:1	160	233 (water soak)	15	22.4	24.0	28.3	22.4	24.5	29.6
CORN STOVER										
	Ammonia loading (Kg/Kg dry biomass)	**Temperature (°C)**	**Moisture content (%)**	**Time (min)**	**Glucan conversion (%)**			**Xylan conversion (%)**		
					24h hydrolysis	**72h hydrolysis**	**168h hydrolysis**	**24h hydrolysis**	**72h hydrolysis**	**168h hydrolysis**
	1:1	130	60	15	71.9	79.1	90.4	70.0	90.0	100.0

Table 9: Experimental AFEX pretreatment conditions tested in this study and % of glucan and xylan conversion after 24 h hydrolysis of *A. donax and* corn stover by the commercial enzymatic preparation consisting of Novozymes Cellic® 60% Ctec3 and 40% Htec3 (15mg/g glucan).

-Saccharification of A. donax and corn stover using purified enzyme cocktail and commercial enzyme cocktail

Previous work have shown that an optimal cocktail of cellulases (CBH I, CBH II, EGI, βG) and a set of accessory hemicellulases and accessory enzymes (LX3, LX4, LArb, LbX) are necessary to enhance the hydrolysis of AFEX-treated corn stover (Gao *et al*., 2011; Gao *et al*., 2010). Based on these results, the enzymatic MIX-A (CBH I, CBH II, EGI, βG, LX3, LX4, LArb, LbX), previously optimized for corn stover (Gao *et al*., 2011) was used to hydrolyse *A. donax* biomass. The Figure 6 shows the glucan, xylan and arabinan conversion from corn stover and *A. donax* after AFEX-treatment in the selected condition 2, by 24 h and 72 h long hydrolyses with MIX-A.

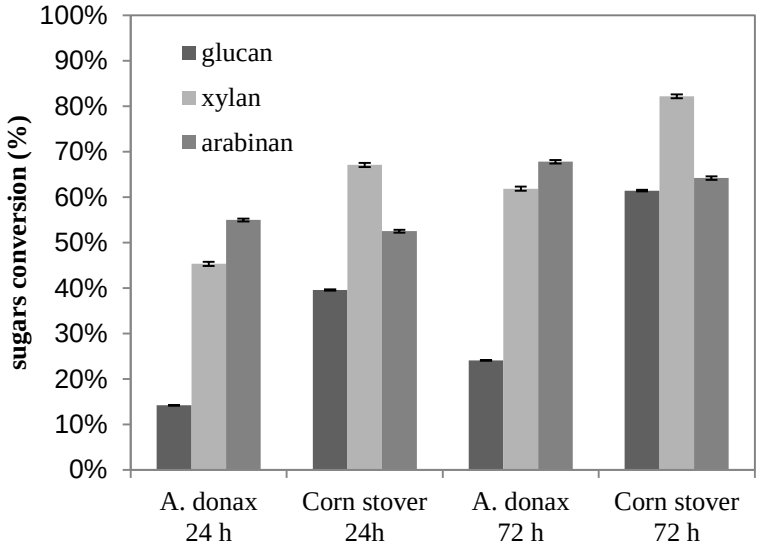

Figure 6: Glucan, xylan and arabinan converison after 24 h and 72 h of hydrolysis on AFEX treated corn stover and A. donax using the enzyme MIX-–A (15mg/g glucan). Cellulases (CBH I, CBH II, EG I) were loaded at 3.32 mg/g glucan each; βG was loaded at 2 mg/g glucan, endoxylanases (LX3 and LX4) and accessory hemicellulases (LArb and LbX) were loaded at 1.66 and 0.6 mg/g glucan each, respectively.

As far as *A. donax* is concerned, although the enzyme MIX-A gave similar xylan hydrolysis (~63 % after 72 h) compared commercial enzymatic preparation, Novozymes Cellic® (60% Ctec3 and 40% Htec3, reported in Table 9, lower glucan hydrolysis was achieved (less than 30% after 72h). As regards corn stover, the MIX-A gave sugar conversion (Figure 6) similar commercial enzyme cocktail (Table 9).

-Effect of the enzymes rCelStrep, rPoAbf and its variant on saccharification of A. donax and corn stover in comparison with the enzymes EGI or LArb

The effect of the arabinofuranosidase from the fungus *Pleurotus ostreatus* recombinantly expressed in *P. pastoris* (rPoAbf), its evolved variant rPoAbf F435Y/Y446F and the cellulase from *Streptomyces sp*. G12 CelStrep recombinantly expressed in *E. coli* in bioconversion of AFEX pretreated *A. donax* and corn stover was tested. In these experiments, the enzyme MIX-A, containing cellulases (CBHI, CBHII, EGI and βG), endoxylanases (LX3, LX4) and accessory hemicellulases (LArb

and LβX), was used as reference enzyme MIX, and the effect of substituting EGI with CelStrep and LArb with rPoAbf or rPoAbfF435Y/Y446F was assessed. More in detail, the cellulase CelStrep was used instead of the endoglucanase EGI in the enzyme MIX-B while the arabinofuranosidases rPoAbf or rPoAbfF435Y/Y446F were used instead of the arabinofuranosidase LArb in the enzyme MIX-C and D, respectively. Moreover, CelStrep was also tested in combination with rPoAbf (MIX-E) or its variant (MIX-F) to check a possible synergism between them.

Table 10 summarizes the conversion results of AFEX pretreated *A. donax* biomass using different purified enzymatic mixtures.

Enzyme Mixture	Purified Enzymes Cocktail	Glucan Conversion (%)	Xylan Conversion (%)	Arabinan Converseion (%)
24 hours	*hydrolysis*			
MIX-A	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & Larb	14.2 ±1.6	45.4 ±0.5	55.0 ±2.5
MIX-B	CBHI-CBHII-bG-LX3-LX4-LBX + rCelstrep & LArb	11.8 ±2.9	43.7 ±2.5	52.3 ±2.1
MIX-C	CBHI-CBHII-bG-LX3-LX4-LBX + EGI & rPoAbf	16.9 ±0.2	49.2 ±0.5	56.1±4.0
MIX-D	CBHI-CBHII-bG-LX3-LX4-LBX + EGI & rPoAbf F435Y/Y446F	17.5 ±1.8	52.5 ±4.2	64.9 ±6.1
MIX-E	CBHI-CBHII-bG-LX3-LX4-LBX + rCelstrep & rPoAbf	16.6 ±0.3	41.8±0.1	19.9 ±0.3
MIX-F	CBHI-CBHII-bG-LX3-LX4-LBX + rCelstrep & rPoAbf F435Y/Y446F	16.4 ±0.1	46.1 ±0.8	53.1 ±1.4
72 hours	*hydrolysis*			
MIX-A	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & LArb	24.1±1.1	61.8±0.9	67.8±0.6
MIX-B	CBHI-CBHII-bG-LX3-LX4-LBX+ rCelstrep & LArb	28.5±2.6	59.1±6.2	56.3±0.3
MIX-C	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & rPoAbf	24.6±1.5	61.5±0.9	57.8±2.6
MIX-D	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & rPoAbf F435Y/Y446F	61.6±0.4	62.7±4.1	78.2±8.2
MIX-E	CBHI-CBHII-bX-LX3-LX4-LBX+ rCelstrep & rPoAbf	20.6±1.2	48.5±0.7	27.9±0.7
MIX-F	CBHI-CBHII-bX-LX3-LX4-LBX+ rCelstrep & rPoAbf F435Y/Y446F	22.1±1.2	57.5±1.4	60.9±4.0

Table 10: *A.donax* sugars conversion in 50 mM citrate buffer pH 4.5, T = 50 °C, for 24 and 72 h.

The sugar conversion results reported in Table 10, showed that the substitution of EGI with Celstrep (MIX-B) gave higher glucan conversion (30%) after 72 h, instead of 24.1% obtained with the enzyme MIX-A (a 18% increase in sugar conversion). These data suggest a synergistic role of bacterial cellulase (rCelstrep) when mixed with fungal cellulases (CBHI, CBHII and βG) on glucan conversion. On the other hand, the use of rCelstrep (MIX-B) decreased both xylan and arabinanhydrolysis, giving a xylane conversion around 4% lower than that obtained

with the enzyme MIX-A after both 24 and 72h, and an arabinanconversion 5% and 17% lower than that obtained with the enzyme MIX-A after 24h and 72h, respectively.

When the rPoAbf wild type was used instead of LArb (MIX-C) no increase in glucan hydrolysis was observed. In the case of MIX-C after 24 h, a xylan conversion of around 49% (7.7% higher than that obtained with the enzyme MIX-A) was observed. This sugar conversion, after 72h hydrolysis was 62% (similar to MIX-A). The use of rPoAbf wild type decreased the arabinanhydrolysis (14% lower than that obtained with the Mix-A after 72h hydrolysis).

The use of the rPoAbfF435Y/Y446Finstead of LArb (enzyme MIX-D in comparison with the enzyme MIXA) and the wild-type rPoAbf (MIX-D in comparison with the MIX-C) led to improvement in sugar conversion. The use of the MIX- D gave 60% glucan conversion after 72 h (which 2.5 times higher than that obtained with the MIX-A and MIX-C). The use of the MIX-D gave 53% xylan conversion after 24 h, (which is 16% and 7.5% higher than that obtained with the MIX-A and MIX-C, respectively). Moreover, the xylan conversion after 72h hydrolysis with MIX-D was 63% (similar to MIX-A and MIX-C). The arabinan conversion was found to be 80% after 72 h (which is 15% and 26% higher than that obtained with the MIX-A and MIX-C, respectively) (Figure 7).

The effect of rCelstrep in combination with rPoAbf (MIX-E) and rPoAbf F435Y/Y446F (MIX-F) was also analysed (Table 10). Glucan conversion was found to be 16% after 24 h, for both MIX-E and MIX-F (14% higher than that obtained with the enzyme MIX-A). However, 21% glucan conversion was obtained after 72 h for both enzyme MIX-E and MIX-F (which is 12.5% lower than that obtained with enzyme MIX-A). For enzyme MIX-E , after 24 h hydrolysis, xylan conversion was 8% lower than that enzyme MIX-A; while after 72 h hydrolysis the xylan conversion was 21.5% lower than enzyme MIX-A. For enzyme MIX-F, 46% xylan conversion was found after 24 h (similar to enzyme MIX-A). However, after 72 h hydrolysis, 57.5% xylan conversion was achieved (7% lower than enzyme MIX-A).

Both enzyme MIX-E and MIX-F gave lower arabinan conversion. After 24 and 72 h enzyme MIX-E gave 20% and 28% arabinan conversion (64% and56% lower than enzyme MIX- A). While, MIX-F gave 53% and 61% arabinan conversion after 24 h and 72 h respectively (4% and 10% lower than enzyme MIX-A).

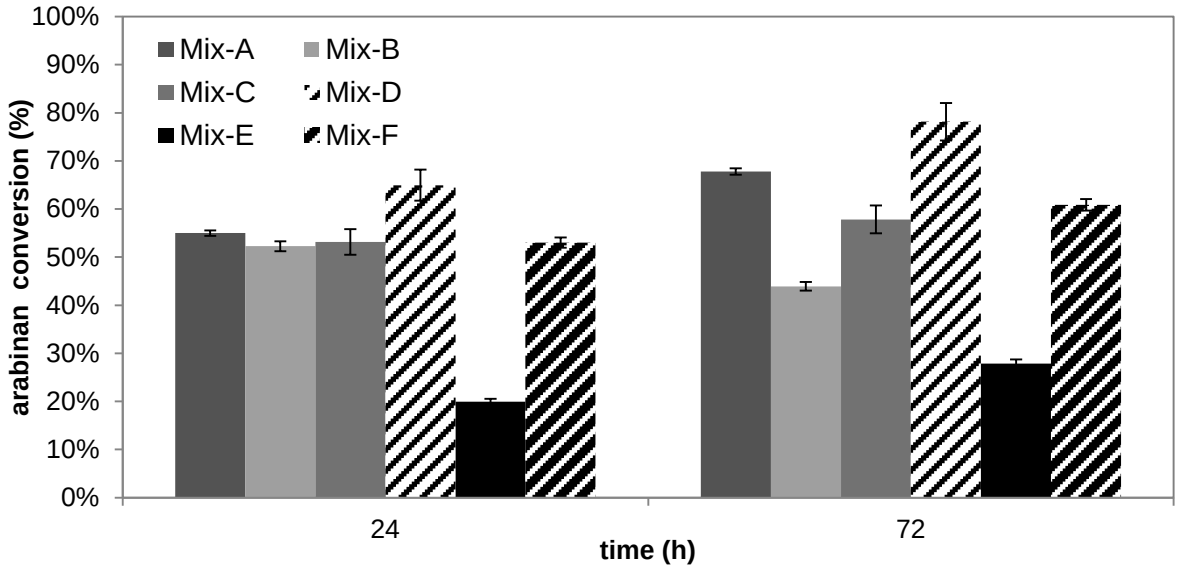

Figure 7: Arabinan conversion after 24 h and 72 h hydrolysis of ammonia fiber expansion (AFEX)-treated *A. donax*.

Hydrolysis of AFEX treated corn stover using purified enzyme cocktail mixtures showed that no increase in sugar conversion was achieved replacing enzyme MIX-A with the mix B, E and F (Table 11). On the other hand, enzyme MIX-C and D gave 8% higher glucan conversion after 24 h when compared to enzyme MIX-A. However, the sugar conversion decrease after 72 h hydrolysis. Enzyme MIX-C and D gave 57.4% and 63.2% arabinan conversion respectively after 24 h (which is 8.5% and 17% higher than MIX-A).

Enzyme Mixture	Purified Enzymes Cocktail	Glucan Conversion (%)	Xylan Conversion (%)	Arabinan Converseion (%)
24hour	*hydrolysis*			
MIX-A	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & LArb	39.6±0.8	67.1±1.2	52.5±1.3
MIX-B	CBHI-CBHII-bG-LX3-LX4-LBX+ rCelstrep & LArb	32.7±0.1	67.5±0.1	53.5±0.1
MIX-C	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & rPoAbf	42.8±0.7	72.8±1.1	57.4±0.8
MIX-D	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & rPoAbf F435Y/Y446F	42.7±0.2	73.6±0.4	63.2±0.2
MIX-E	CBHI-CBHII-bG-LX3-LX4-LBX+ rCelstrep & rPoAbf	38.3±1.1	59.6±1.2	54.1±2.0
MIX-F	CBHI-CBHII-bG-LX3-LX4-LBX+ rCelstrep & rPoAbf F435Y/Y446F	39.2±0.7	65.5±1.1	16.4±1.2
72hour	*hydrolysis*			
MIX-A	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & LArb	61.4± 1.2	82.2± 1.5	64.2±2.0
MIX-B	CBHI-CBHII-bG-LX3-LX4-LBX+ rCelstrep & LArb	56.3± 0.7	81.6±2.1	14.4±0.2
MIX-C	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & rPoAbf	41.4±0.1	77.0±1.2	56.0±0.1
MIX-D	CBHI-CBHII-bG-LX3-LX4-LBX+ EGI & rPoAbf F435Y/Y446F	42.2±0.6	73.4±0.4	63.5±0.2
MIX-E	CBHI-CBHII-bG-LX3-LX4-LBX+ rCelstrep & rPoAbf	47.2±0.8	57.9±0.2	59.2±1.4
MIX-F	CBHI-CBHII-bG-LX3-LX4-LBX+ rCelstrep & rPoAbf F435Y/Y446F	48.7±1.2	5.5±0.5	27.2±0.2

Table 11: corn stover sugar conversion in 50 mM citrate buffer pH 4.5, T =50 °C

-Synergy between commercial enzyme cocktail and purified enzymes (PoAbf and/ or Celstrep)

To understand synergy between Novozymes Cellic®, Ctec3 and Htec3 commercial enzyme cocktail and purified enzymes (rPoAbf, its evolved variant rPoAbfF435Y/Y446F and CelStrep), hydrolysis of AFEX pretreated *A. donax* and corn stover were carried out using different enzyme mixture combinations.

The glucan conversion commercial enzyme cocktail was found to be higher when compared to supplementing with wild-type rPoAbf or its evolved variant or rCelstrep or both enzymes (Figure 8A).

Supplementing rPoAbf and its evolved variant gave 70% xylan conversion (7% higher than that obtained by the commercial preparation) (Figure 8B). After 6 days of hydrolysis 80% xylan conversion was achieved when rPoAbf F435Y/Y446F was used along with commercial enzymes. The arabinan conversion was also improved by supplementing rPoAbf or variant to the commercial enzyme cocktail by 27% and 36% respectively after 24h of hydrolysis (Figure 8C). The maximum arabinan conversion achieved was 80%, after 6 days of hydrolysis when rPoAbf was supplemented with commercial enzymes.

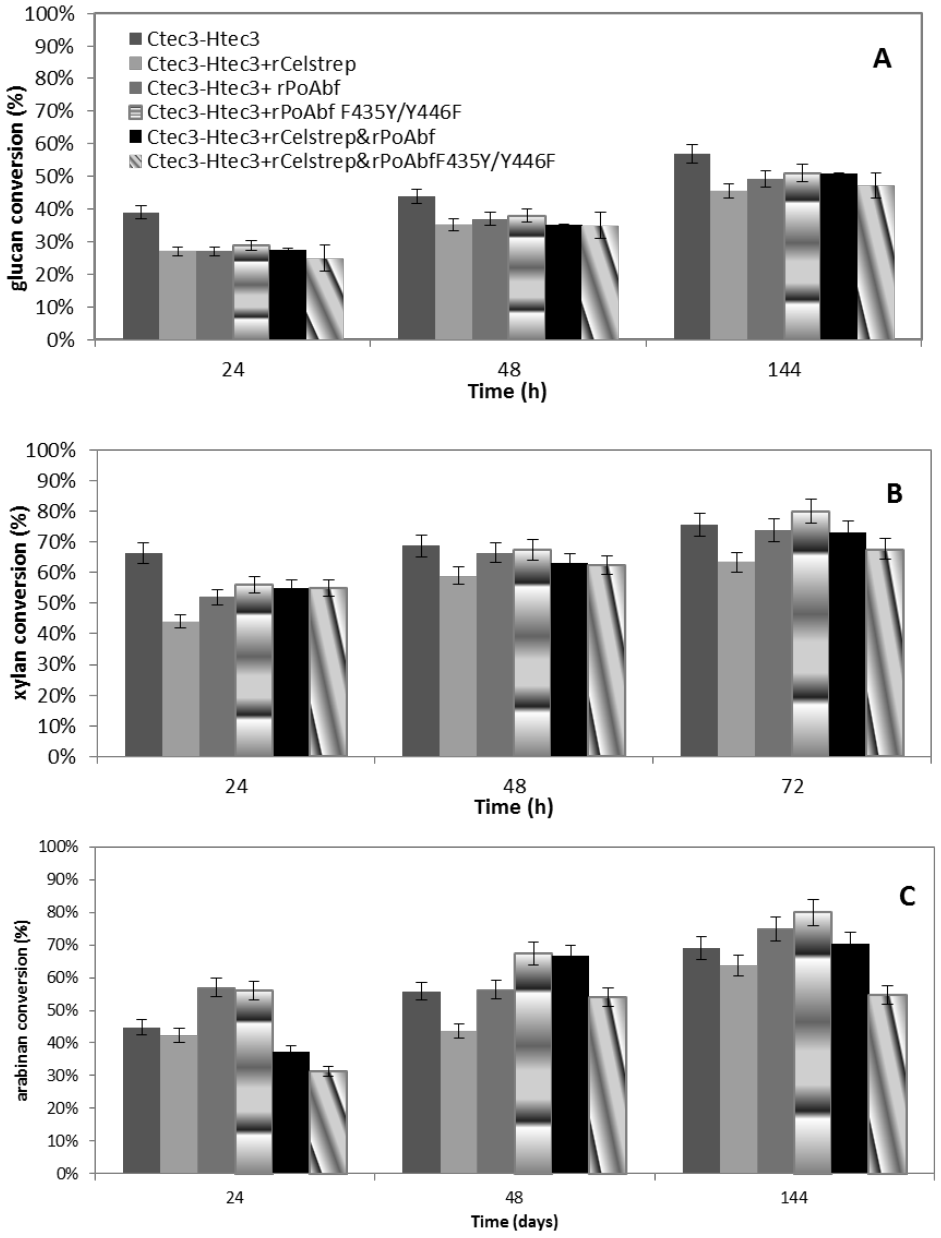

Figure 8: Glucan (A), xylan (B) and arabinan (C) conversion of AFEX-treated *A. donax* obtained by using commercial preparation with or without addition of rPoabf, its variant, Celstrep or a combination of these enzymes.

In the case of corn stover, no appreciable increase in glucan conversion was noticed when commercial preparation was supplemented with wild-type rPoAbf or its variant or rCelstrep or both enzymes (data not shown). However, 99% xylan conversion was achieved in 24 hours with rPoAbf wild-type was supplemented with commercial enzymes (Figure 9A).

The same xylan conversion was achieved using commercial preparation, and commercial preparation in combination with rPoAbf F435Y/Y446F or rCelstrep or

both enzymes after 6 days of hydrolysis. Supplementation of rPoAbf with commercial enzyme cocktail gave 70% of arabinan conversion after 24h of hydrolysis (40% higher than that obtained using commercial cocktail alone) (Figure 9B).

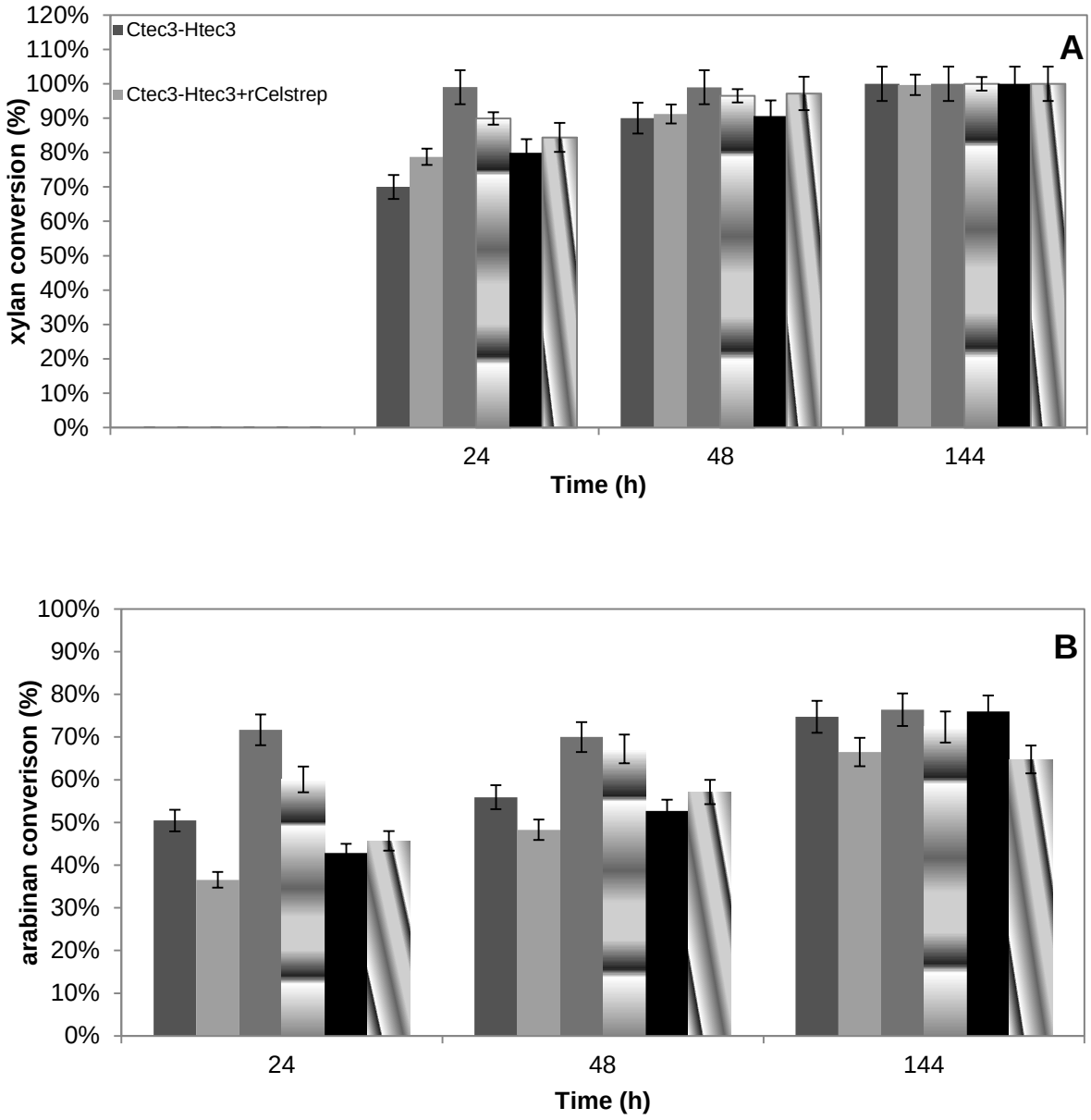

Figure 9: Xylan (A) and arabinan (B) conversion of AFEX-treated corn stover obtained by using commercial enzyme cocktail with or without addition of rPoabf, its variant, Celstrep or a combination of these.

4.1.2.3 MATERIALS AND METHODS

-Feedstock

Biomass used in this studies include, corn stover and giant reed (*Arundo donax* L.) for the doing saccharification experiments. *A. donax* was produced in marginal lands of Southern Italy with a low input cropping system (Forte *et al*., 2015) and corn stover was provided by Michigan State University (MSU). The biomasses were milled with a 2 mm diameter sieve and stored under dry conditions at room temperature until further use. The moisture content was measured using a moisture analyser (Sartorius MA35M, Elk Grove, IL).

-Compositional Analysis

Compositional analyses of corn stover and *A. donax* biomasses were performed following the National Renewable Energy Laboratory (NREL) standard protocols (Seling & Weiss, 2008; Sluiter *et al.,* 2005a; Sluiter *et al.,* 2005b), Laboratory Analytical Procedures (LAPs): ‘‘Preparation of samples for compositional analysis’’, ‘‘Determination of structural carbohydrates and lignin in Biomass”, “Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples”, “Determination of Ash in Biomass”. Monomeric sugars were quantified using a Biorad Aminex HPX-87H high-performance liquid chromatography (HPLC) column using 5mM sulphuric acid as mobile phase.

-AFEX pretreatment

Corn stover and *A. donax* was subjected to AFEX pretreatment by varying reaction temperature (100-160^{o}C), moisture (60-233% on dry weight basis), ammonia to biomass ratio (1:1 and 2:1) and fixed residence time (15 minutes). AFEX was done in a high pressure stainless steel vessel. Biomass is first loaded in to the vest with appropriate moisture after taking in to consideration the moisture content of original biomass. Then the reactor is closed and vacuum applied to remove residual air in the reactor. Require amount of liquid ammonia is loaded in to the reactor using a ammonia delivery pump. The vessel is heated by external mantle and the biomass is mixed during AFEX process for 15 minutes. As the temperature of reactor is increased, the pressure in the vessel raised (between 200-400 psi) depending on the ammonia to biomass loading. The pressure is released from the vessel and ammonia is vented in the hood. The pretreated biomass is transferred to a tray and dried in the hood overnight to remove residual ammonia present in the biomass. Then dry AFEX treated biomass is stored in a sealed polythene bag in a refrigerator until further use.

-Enzymatic hydrolysis

The hydrolysis experiments were carried out in 5 vials at 1% (w/w) glucan loading in 50 mM citrate buffer (pH 4.8) with the desired enzymes. 0.5 mM sodium azide was used to prevent microbial and fungal growth. The enzymes mixtures were prepared separately and added simultaneously at a loading of 15 mg/g of glucan. The hydrolysis was carried out at 50 °C, 250 rpm in a shaking incubator. Sampling was done out every 24h to evaluate the sugar composition using sugar assay kit or using High Performance Liquid Chromatography (HPLC) system. The commercial enzyme preparation Novozymes Cellic® (60% Ctec3 and 40% Htec3) was used for hydrolysis experiment to select the best pretreatment condition. The hydrolysis of pretreated lignocellulosic materials was carried out with the following enzymes. An

enzymatic mixture named MIX-A was prepared including: cellobiohydrolase I (CBH I; glycoside hydrolase (GH) family 7A), cellobiohydrolase II (CBH II; GH family 6A), endoglucanase I (EG I; GH family 7B) at a concentration of 3.32 mg/g glucan each, β-glucosidase (βG; GH family 3) 2 mg/g glucan, xylanases (LX3, GH family 10; LX4, GH family 11) 1.66 mg/g glucan each, β-xylosidase (LβX; GH family 52), and α-arabinofuranosidase (LArb, GH family 51) were loaded at 0.6 mg/g glucan each. In MIX-B, the EG I was replaced by cellulase CelStrep from Streptomyces sp. G12 recombinantly expressed in *E. coli* (Amore *et al*., 2012b); in MIX-C the LArb was replaced by the α-L-arabinofuranosidase from *P. ostreatus* recombinantly expressed in *P. pastoris* (rPoAbf) (Amore *et al*., 2012a); in the MIX-D the LArb was replaced by mutant of rPoAbf indicated as rPoAbf F435Y/Y446F (Giacobbe et al., 2014); in the MIX-E both EGI and LArb were replaced by rCelStrep and rPoAbf; in MIX- F both EGI and LArb were replaced by rCelStrep and rPoAbf F435Y/Y446F. Moreover rCelStrep, rPoAbf and rPoAbf mutant were utilized in combination with the commercial preparations Novozymes Cellic®.

-Glucose and xylose assays

Monomeric glucose and xylose concentration was determined by using D-glucose assay kit by r-biopharm (Roche) and D-xylose assay kit by Megazyme, respectively. All experiments were carried out in duplicates.

-HPLC Sugar Analysis

About 200 μl hydrolysate collected at different time points during enzyme hydrolysis were transferred to a centrifuge tube, heated to 100^{o}C to 10 minutes (to denature the enzymes), then spin down at 8,000 rpm for 10 minutes to remove the precipitates and then stored in a HPLC vial at -20^{o}C until further use. Monomeric sugars concentration in the hydrolysate were determined by HPLC using Biorad Aminex HPX-87P. All experiments were performed in duplicates. Shimadzu HPLC Prominence system (Columbia, MD, USA) with a refractive index detector (RID) were used for analysing the sugars. Water was used as the mobile phase at a fixed flow rate of 0.6 ml/min, with isocratic elution. The column temperature was maintained at 60°C and the HPLC sample injection volume was 20 µl. Standard curves were generated using different concentrations of mixed sugars. A guard column with similar packing was used throughout the chromatography experiments.

Concluding remarks

CONCLUDING REMARKS

This PhD project was aimed at the improvement of lignocellulosic biomasses conversion to release sugars which can be used to produce second generation ethanol and bioproducts.

A novel pectate lyase, named Paenxyl Pel was identified from *Paenibacillus xylanilyticus* 2-6L3 isolated from mature compost obtained from agro-industrial wastes. The conditions for native Paenxyl Pel production and purification were investigated. The new native enzymes was partially purified and characterized from the catalytic point of view. The results on the ability to act on both PLA and highly methylated pectins, together with data about optimum pH (pH 5 and 9), suggest that pectate lyase from *P. xylanoliticus* shows an unusual activity combining traits of pectate lyase and pectin lyase.

As far as the directed evolution of PoAbf is concerned, experiments of error prone Polymerase Chain Reaction were performed in order to obtain libraries of directed evolved variants. A first generation library of 7000 randomly mutated variants of PoAbf was obtained. The high-throughput screening of the library was developed by assaying the activity towards p-nitrophenyl-α-L-arabinofuranoside, and PoAbf F435Y/Y446 variant with higher activity than the wild-type was selected, recombinantly expressed in *Pichia pastoris,* purified and characterized. The mutant showed a K_{cat} towards pNPA around 3-fold higher than that of the wild-type. Moreover, analyzing the effect of pH and temperature on the activity of rPoAbf F435Y/Y446F towards pNPA it was shown that it exhibits a wide range of optimal pH values (3-6) differing from the wild-type. Furthermore, the hydrolyzing abilities of rPoAbfF435Y/Y446F were shown improved also toward all the other tested substrates. Interestingly, it is must be underlined that the mutant rPoAbf F435Y/Y446F was selected for its higher activity but it showed also a higher resistance to temperature and pH than the wild-type.

So, the potential of *in vitro* evolution of the α-L-arabinofuranosidase PoAbf as a strategy to achieve improved variants of this hemicellulase was demonstrated. Based on these data, this mutant was used as template for a new directed evolution library. A second generation library of 6000 randomly mutated variants of PoAbf F435Y/Y446F was obtained. Among these variants, the mutant PoAbf F435Y/Y446F/K533N, showing 2-fold higher activity towards pNPA than rPoAbf F435Y/Y446F, was selected, over-produced in *S.cerevisiae* using fermenter to be purified and characterized from a catalytic point of view.

The biocatalysts rPoAbf wild-type and its evolved variant and rCelStrep from *Streptomyces sp*. G12 recombinantly expressed in *Escherichia coli*, were tested for their potential role in lignocellulosic biomass conversion by using these enzymes in combination with enzymatic activities involved in the hydrolysis of lignocellulose. As far as bioconversions are concerned, the work was carried out in collaboration with group of Dr. La Cara, of CNR-IBP of Naples and with Dr. Balan of the Biomass Conversion Research Laboratory (BCRL) at Michigan State University. The results reveled the importance of arabinofuranosidases in the hydrolysis of xylan present in the lignocellulosic biomasses. The rPoAbf and its evolved variant improved xylose and arabinose conversion of tested lignocellulosic biomasses (*Arundo donax*, corn stover, BSG and corn cobs). As far as rCelstrep is concerned, data suggest

synergistic action of bacterial cellulase with fungal too in conversion of cellulose present in *A.donax* biomass.

The results obtained in this project support the use of second generation raw materials as feedstock to produce fermentable sugars for ethanol and other bioproducts production.

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Appendix I

List of communication

1. Faraco V, Amore A 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.
2. Faraco V., **Giacobbe S.** and Amore A. Improved variants of GH51 α-l-arabinofuranosidase from pleurotus ostreatus for lignocellulose conversio. Intervento orale al 2nd Iberoamerican Congress on Biorefineries 11/04/13
3. Faraco V, Amore A, Pepe O, **Giacobbe S** and Ventorino V. Development of new biocatalysts based on cellulases and hemicellulases for lignocellulose conversion. Intervento orale: Renewable Resources and Biorefineries (RRB-9) 5-7 Giugno, 2013, Antwerp, Belgium
4. **Giacobbe S**, Amore A and Faraco V. Improved variants of GH51 alpha-L-arabinofuranosidase from Pleurotus ostreatus for lignocellulose conversion. Intervento orale: 2-CIAB, 2nd Congreso Iberoamericano Sobre Biorrefinerias, Aprile 2013, Jaen, Spain.
5. Vincenza Faraco, A. Amore, **S. Giacobbe**, F. La Cara, L. Marcolongo, E. Ionata and O. Pepe. NEW BIOCATALYSTS FOR *Arundo donax* CONVERSION INTO FERMENTABLE SUGARS FOR SECOND GENERATION BIOETHANOL PRODUCTION. Intervento orale: Renewable Resources and Biorefineries (RRB-10) giugno 2014, Spain

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1. Amore A, **Giacobbe S,** Faraco V. **Regulation of cellulase and hemicellulase gene expression in fungi.** Current genomics, 2013. 14, 1- 20

2. **Giacobbe S**, Vincent F and Faraco V. **Development of an improved variant of GH51 a-l-arabinofuranosidase from *Pleurotus ostreatus* by directed evolution.** New Biotechnology, 2014 doi: 10.1016/j.nbt.2014.02.004

3. **Giacobbe S**., Pepe O., Ventorino V., Birolo L., Vinciguerra R., Faraco V.* **Identification and Characterisation of a Pectinolytic Enzyme from Paenibacillus xylanolyticus** BioResources. 2014. Vol 9, No 3 4873-4887

4. Marcolongo L., Ionata E., La Cara F., Amore A., **Giacobbe S**., Pepe O. and Faraco V.*. **The effect of Pleurotus ostreatus arabinofuranosidase and its evolved variant in lignocellulosic biomasses conversion**. Fungal Genetics and Biology. 2014. 72, 162-167

5. Amore A., **Giacobbe S**., Liguori R. and Faraco V.* **The second generation ethanol production** Rendiconti Accademia Nazionale delle Scienze detta dei XL Memorie di Scienze Fisiche e Naturali ISBN 987-88- 548-7171-7. 2014. DOI 10.4399/97888548717176 pag. 113–136

6. Ventorino V., Aliberti A., Faraco V., Robertiello A., **Giacobbe S**., Ercolini D., Amore A., Fagnano M., Pepe O. (2015). **Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application**. SCIENTIFIC REPORTS, vol. 5, ISSN: 2045-2322, doi: 10.1038/srep08161

Chapters in book

1. Amore A, **Giacobbe S,** Faraco V. Consolidated bioprocessing for improving cellulosic ethanol production. In: Lignocellulose conversion: enzymatic and microbial tools for bioethanol conversion (V. Faraco ed.) Springer, Springer-Verlag Berlin Heidelberg, 2013. 169-196.

Experiences in foreign laboratories

-stage at **Biomass Conversion Research Laboratory (BCRL**) of Michigan State University, Lansing (MI). The work was supervised by Dr Balan (30th May- 16th September, 2014).

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March 19, 2015

This is to certify that Ms. Simona Giacobbe Researcher at University of Naples "Federico II", Italy carried out research at Michigan State University in the Biomass Conversion Research Laboratory (BCRL) for the period of May 30, 2014 through September 16, 2014.

During her time at Michigan State University she worked in the lab of Dr. Venkatesh Balan of the BCRL. Her responsibilities were to test commercial enzyme cocktail along with arabinofuranosidase and its directed evolved mutants in conversion of the lignocellulose biomasses, Arundo donax and Newspaper waste in to fermentable sugars to produce biofuels, that have to be pretreated using Ammonia Fiber Expansion (AFEX) process.

Sincerely,

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Regulation of Cellulase and Hemicellulase Gene Expression in Fungi

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Abstract: Research on regulation of cellulases and hemicellulases gene expression may be very useful for increasing the production of these enzymes in their native producers. Mechanisms of gene regulation of cellulase and hemicellulase expression in filamentous fungi have been studied, mainly in *Aspergillus* and *Trichoderma*. The production of these extracellular enzymes is an energy-consuming process, so the enzymes are produced only under conditions in which the fungus needs to use plant polymers as an energy and carbon source. Moreover production of many of these enzymes is coordinately regulated, and induced in the presence of the substrate polymers. In addition to induction by mono- and oligo-saccharides, genes encoding hydrolytic enzymes involved in plant cell wall deconstruction in filamentous fungi can be repressed during growth in the presence of easily metabolizable carbon sources, such as glucose. Carbon catabolite repression is an important mechanism to repress the production of plant cell wall degrading enzymes during growth on preferred carbon sources. This manuscript reviews the recent advancements in elucidation of molecular mechanisms responsible for regulation of expression of cellulase and hemicellulase genes in fungi.

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1. INTRODUCTION

In order to enhance energy security and mitigate climate change, interest in finding renewable fuels to replace petroleum-based ones is enormously increasing. The biofuels ethanol and biodiesel represent potential options for meeting these needs in the transportation sector. The uniqueness of cellulosic ethanol as a sustainable liquid transportation fuel, which can be produced in high volumes and at low cost, and its many powerful benefits, have been recognized for decades [1-5]. A recent awareness of the urgent need to advance cellulosic ethanol production is evidenced by the number of reviews reported on the theme of ethanol fuel production from lignocellulosic biomass, with great attention to ethanol production from lignocellulosic residues, such as crop and wood residues and municipal solid waste [6-15]. The key step for conversion of lignocellulosic biomass into fermentable sugars for fuel production is represented by the hydrolysis of polysaccharides, resulting from biomass pretreatment, by cellulases and hemicellulases. Filamentous fungi are the major source of cellulases and hemicellulases. As far as cellulases are concerned, three main enzymatic activities are involved in cellulose hydrolysis: 1) endoglucanases (EC 3.2.1.4); 2) exoglucanases, including d-cellodextrinases (EC 3.2.1.74) and cellobiohydrolases (EC 3.2.1.91); and 3) β-glucosidases (EC 3.2.1.21). As far as hemicellulases are concerned, endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) are required for degradation of the xylan backbone, while auxiliary enzymes such as α-glucuronidases (EC 3.2.1), α-arabinofuranosidases (EC 3.2.1.55), acetylesterases or acetyl xylan esterases (EC 3.1.1.6) are required to achieve the complete degradation of complex substituted xylans. Research on regulation of cellulase and hemicellulase genes' expression may be very useful for increasing production of these enzymes in their native producers. Mechanisms of cellulase and hemicellulase genes regulation have been studied in filamentous fungi, mainly in *Aspergillus* [16, 17] and *Trichoderma* [18]. The production of these extracellular enzymes is an energy-consuming process, so the enzymes are produced only under conditions in which the fungus needs to use plant polymers as an energy and carbon source. Moreover, production of many of these enzymes is coordinately regulated, and induced in the presence of the substrate polymers. Induction mechanisms of cellulase and hemicellulase genes expression involve activation of gene expression by the respective hydrolysis and/or transglycosylation products of xylan and/or cellulose, such as gentiobiose for *Penicillium* [19], and sophorose for *A. terreus* and *T. reesei* [20, 21]. In addition to induction by mono- and oligosaccharides, genes encoding hydrolytic enzymes involved in plant cell wall deconstruction in filamentous fungi can be repressed during growth in the presence of easily metabolizable carbon sources, such as glucose. Carbon catabolite repression (CCR) is an important mechanism to repress the production of plant cell wall degrading enzymes during growth on preferred carbon sources [22-25].

This manuscript reviews the recent advancements in elucidation of molecular mechanisms responsible for regulation of expression of cellulase and hemicellulase genes in fungi.

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2. REGULATION OF PRODUCTION OF CELLULASES AND HEMICELLULASES IN *TRICHODERMA REESEI*

The cellulolytic machinery of *T. reesei* is one of the most widely studied [26]. *T. reesei* genome *(http://genome.jgi psf.org/Trire2/Trire2.home.html)* contains ten cellulase and sixteen hemicellulase genes [27]. The enzymes so far identified and characterized as responsible for the cellulolytic activity of *T. reesei* include five endoglucanases -EGI/Cel7B, EGII/Cel5A, EGIII/Cel12A [28, 29], EGIV/Cel61A [30], and EGV/Cel45A [31] and two exoglucanases -the cellobiohydrolases CBHI/Cel7A and CBHII/Cel6A [32]. These enzymes act synergistically to convert cellulose into cellobiose [28-33], whose hydrolysis into glucose involves then two β-glucosidases -BGLI/Cel3A [34] and BGLII/Cel1A [35]. An additional protein, swollenin (encoded by the gene *swo1*), has been described, that disrupts crystalline cellulose structures, presumably making polysaccharides more accessible to hydrolysis [36]. The cellulases CBHI/Cel7A, CBHII/Cel6A, EGI/Cel7B, and EGII/Cel5A are the most abundantly produced by *T. reesei* secreting them up to 40 g/liter [37]. Due to the enormous level of cellulase production, *T. reesei* revealed to be a potential candidate for advancing cellulosic ethanol by I Consolidated BioProcessing [38].

The *T. reesei* genome also contains sixteen hemicellulases including two GH43, one GH10, four GH11, one GH74, one GH62, two GH54, one GH67 and four GH95 [27]. Among these, two major endo-β-1,4-xylanases XYNI and XYNII (EC 3.2.1.8) [39]; and one β-xylosidase, BXLI (EC 3.2.1.37) [40] have been characterized.

The presence of cellulose, xylan or mixtures of plant polymers in the fungal culture medium causes abundant production of cellulolytic and xylanolytic activities by *T. reesei,* as already reported by the earlier studies [41-44]. Pure (oligo)saccharides, such as sophorose [20, 21], β-cellobiono-1,5-lactone, D-xylose, xylobiose, galactose, and lactose, have been also reported to induce cellulase and hemicellulase production in *T. reesei* (Table **1**) [24, 45-49].

Inability of the fungal cells to incorporate insoluble polymeric compounds, such as cellulose and xylan, aroused the question on how these polymers can initiate production of hydrolytic enzymes. Several studies investigating this aspect postulated the inducer function of a low molecular weight and soluble compound derived from cellulose. One of the proposed mechanisms is that the fungus produces basal levels of cellulase (mainly CEL7A and CEL6A) and that the activity of these extracellular enzymes on cellulose produces a soluble inducer, which can enter the cell and affect induction [50, 51] In support of this mechanism, it was shown that antibodies against CBHI, CBHII, EGI and EGII blocked the expression of *cbh1/cel7a* gene in the presence of cellulose but not the soluble inducer sophorose [50]. The constitutive levels of these cellulases and their role in cellulase induction were afterwards demonstrated by Carle-Urioste *et al.* [51]. These authors showed that the mRNAs *cbh1* and *egl1* are transcribed under uninduced conditions, and that induction with cellulose results in at least 1100-fold increase of both transcripts, as demonstrated by Northern blots. The basal activity of the *cbh1* promoter was also examined by using a chimeric vector in which the gene encoding hygromycin B phosphotransferase [52] was placed under the control of the 59-flanking DNA sequence of the *cbh1* gene. Under uninduced conditions, resistance to the antibiotic hygromycin B was observed with *T. reesei* cells transformed with this vector and grown on medium lacking cellulose. An antisense RNA strategy was also adopted by the same authors to gain *in vivo* evidence for the requirement of the basal expression of the cellulase in induction of the cellulase transcripts by cellulose [51]. The results demonstrated that the expression of this antisense RNA produced marked effects on the induction of the *cbh1* transcript using cellulose (reduction of the *cbh1* transcript expression between 80 and 90%) but not sophorose as an inducer. The authors also showed that the initial hydrolysis of cellulose is the rate-limiting step in the induction, as suggested by the observation that the addition of the cellulase system or its purified enzyme members to a culture of *T. reesei,* in the presence of cellulose, resulted in earlier detection of the *cbh1* and *egl1* transcripts. The time required for induction of *cbh1* and *egl1* transcripts using cellulose, cellulose – cellulase, or sophorose is 14, 10, and 4 h, respectively. This result supports the hypothesis that oligosaccharide(s) is(are) formed *in vivo* from cellulose by the activity of a low, constitutive, and extracellular cellulase activity. The relatively slow induction by sophorose could be explained by the fact that the inductive process is protein synthesis-dependent. In addition, it has recently been shown that a sophorose-inducible β-diglucoside permease is involved in the induction of the cellulase system in *T. reesei* [53]. Subsequently, Foreman *et al.* [54] identified further genes whose regulatory behavior is consistent with their role in primary inducer formation for cellulase expression. Among them, the mRNA of *cel5b* was moderately expressed during growth on glycerol, glucose, sophorose and lactose, and only slightly induced over this level by cellulose. It is worth noting that CEL5B contains the *consensus* sequence for membrane-anchoring via a glycosylphosphatidylinositol residue. All these properties make it an interesting candidate for generating the inducer of cellulase formation. Similarly, the acetyl xylan esterase Axe2, which is also predicted to contain a glycosylphosphatidylinositol anchor, may be involved in primary induction of some hemicellulases [54].

The surface-bound cellulolytic activity displayed by conidia of *T. reesei,* mainly due to CEL6A/CBHII [55, 56] is also considered important for cellulase induction since its elimination by detergents hinders germination of the conidia on cellulose. These conclusions were deduced by the observation that introduction of multiple copies of the *cel6a* gene into *T. reesei* caused an enhanced secretion of CEL7A and CEL6A on cellulose and an increased cellulase activity on cellulose corresponding to enhanced level of conidial-bound CEL6A [56, 57]. Consistently, a *cel6a* knocked out strain showed a delay in growth and cellulase formation on cellulose [58]. In more details, comparing strains in which the corresponding genes of the main cellulases (*cel6a, cel7a, cel7b, cel5a*) had been deleted, Seiboth *et al.* [58] showed that strains knocked out for *cel6* and *cel5a*, respectively, exhibited a significantly reduced expression of the remaining cellulase genes, while strains carrying the *cel7a* or *cel7b* deletion showed these transcripts. A strain showing both the cellobiohydrolases *cel6a* and *cel7a* deletion, was unable to initiate growth on cellulose. During growth on lactose,

Tabella 1. Inducibility of Cellulases, Hemicellulases and Related Enzymes in *T. reesei*, *N. crassa* **and** *Aspergillus spp. X: repressor; +: inductor; -: no action*

Substrate / Enzyme	Glycerol	Glucose	Sorbitol	Cellulose	Cellobiose	Xylose	Sophorose	Lactose	Xylobiose	Galactose	Laminaribiose	Gentiobiose	Aryl-β-glucosides	Maltose	Xylan	Galac Turonic acid	Fructose	Mannitol	Arabinose	Arabinitolo	Mannose	Sorbose	Tween 80	C18 Fatty acids	References
T. reesei																									
bgl1					+	+	+		−																[24]
bgl2						+	+		−																[48]
bxl1						+	+		−																[48]
cbh1	-		-	+	+	+	+	+							−										[51, 59]
cbh2	-		-	+	+	+	+		−																[59]
cel5b	-	+					+	+																	[54]
egl1	-		-	+			+																		[51, 59]
egl2	-		-	+			+																		[59]
egl3							+															−			[192]
egl5	-		-	+			+																		[59]
xynI		x			+	+	+		−						−							−			[192]
xynII		x			+	+	+		−						−							−			[192]
xyn3						-	+															−			[192, 193]
a-AF																						-			[192]
abf1		x																	+	+					[71]
abf2		x																	+	+					[71]
abf3		x																	+	+					[71]
agl1									−	+															[24]
agl2									−	+															[24]
glr1									−																[24]
axe1										+															[24]
N. crassa																									
Endocellulase					+																		−	−	[121]
β-glucosidases																							x	-	[121]
aryl-β-glucosidase		x			+	+				+	+	+	+												[127]
Cellobiase		x			+	+				+				+											[127]
Xylanase		-		+											−										[122]
xyr-1						+																			[143]
xdh-1						+																			[143]
xyk-1						+																			[143]
Cellulase				+											-								−	−	[121]

(Table 1) contd….

Substrate / Enzyme	Glycerol	Glucose	Sorbitol	Cellulose	Cellobiose	Xylose	Sophorose	Lactose	Xylobiose	Galactose	Laminaribiose	Gentiobiose	Aryl-β-glucosides	Maltose	Xylan	Galac. Turonic acid	Fructose	Mannitol	Arabinose	Arabinitolo	Mannose	Sorbose	Tween 80	C18 Fatty acids	References
N. crassa																									
gh 43-2				+											–										[143]
gh 51-1															–										[143]
gh 10-1															–										[143]
gh 43-5				+											–										[143]
arabinase															–										[143]
gh 11-2				+											+										[143]
gh 10-2				+											–										[143]
gh 53-1				+											–										[143]
gh 1-1						+																			[143]
β-galacto-sidases						+		+		+															[199]
gh5-1				+																					[143]
gh 61-7				+																					[143]
gh 61-4				+																					[143]
gh 61-1				+																					[143]
gh 61-3				+																					[143]
gh 61-6				+																					[143]
gh 7-1				+																					[143]
gh 6-3				+																					[143]
gh 61-13				+																					[143]
gh 61-5				+																					[143]
gh 11-1				+																					[143]
gh 74-1				+																					[143]
gh 10-1				+																					[143]
Aspergillus spp.																									
Endoglu-canase	x			+													+	+							[160]
β-glucosi-dase	x			+													+	+							[160]
Xylanolytic enzymes		+		+		+									–										[20, 161]
eglA/B					+	+																			[161, 200]
pe-1A/B/C/D/E/F																+									[168, 200]

(Table 1) contd....

Substrate / Enzyme	Glycerol	Glucose	Sorbitol	Cellulose	Cellobiose	Xylose	Sophorose	Lactose	Xylobiose	Galactose	Laminari-biose	Gentiobiose	Aryl-β-glucosides	Maltose	Xylan	Galac Turonic acid	Fructose	Mannitol	Arabinose	Arabinitolo	Mannose	Sorbose	Tween 80	C18 Fatty acids	References
Aspergillus spp.																									
plyA																+									[168, 200]
pga-A/B/C/D/E																±									[168, 200]
pgaX/I/II																+									[168, 200]
pmeA																+									[168, 200]
rglA																+									[168, 200]
afbA																±			±	±					[24, 169, 170]
afbB																+			+	+					[24, 169, 170]
abnA																+									[168, 169, 200]
lacA						+				+						+			+		−				[168, 169, 200]
aguA						+										+									[169, 200]
axeA						+																			[169, 200]
faeA						+																			[169, 200]
axhA																			+	+					[169, 200]
rhgA/B																±									[169, 200]
axhA						±										±		±							[24]

these strains showed no significant alterations in their ability to express the respective other cellulase genes. These data support the role of CEL6A and other conidial-bound cellulases (such as CEL5A, for which a conidial location is not yet known) in the induction of cellulases and germination on cellulose.

Ilmén *et al.* [59] investigated basic features of expression regulation of the *T. reesei* cellobiohydrolases *cbh1* and *cbh2* and endoglucanases *egl1*, *egl2* and *egl5* encoding genes, at the mRNA level, showing that these cellulase genes are coordinately expressed and the steady-state mRNA levels of *cbh1*/Cel7A is the highest. The highest induction level was achieved with cellulose and sophorose and moderate expression was observed when cellobiose or lactose were used as the carbon source. No expression could be observed on glucose-containing medium and that high glucose levels abolish the inducing effect of sophorose. However, derepression of cellulase expression occurs without apparent addition of an inducer once glucose has been depleted from the medium. This expression seems not to arise simply from starvation, since the lack of carbon or nitrogen as such is not sufficient to trigger significant expression. It was also found that glycerol and sorbitol do not promote expression but, unlike glucose, do not inhibit it either, because the addition of 1 to 2 mM sophorose to glycerol or sorbitol cultures provokes high cellulase expression levels.

The best inducer of cellulase expression so far known is sophorose (2-*O*-*β*-glucopyranosyl-D-glucos) [60, 21, 61], whose synthesis from cellobiose involves the transglycosylation activity of *β*-glucosidase [62]. Induction by sophorose is affected by various parameters such as its concentration and rate of uptake [61, 63]. Two pathways of sophorose utilisation were for the first time hypothesised by Loewenberg and Chapman [64]: a catabolic pathway characterized by a high capacity but low affinity for sophorose, and a cellulase inducing pathway endowed with a lower capacity but higher affinity for sophorose. As a matter of fact, Kubicek *et al.* [53] showed that sophorose is transported by a cellobiose permease, characterized by low Km and Vmax for sophorose, and thus competing with the extracellular β-glucosidase, which has a much higher Km but also Vmax for it.

Most authors implied a β-glucosidase in the process of sophorose production. *T. reesei* produces β-glucosidases having different cellular localizations [65-69]. The gene *cel3a* [65, 70] encodes the major extracellular β-glucosidase identified as one of the β-glucosidases involved in inducer formation. Knock-out of the *cel3a* gene causes a delay in induction of the other cellulase genes by cellulose, but not by sophorose, whilst a *cel3a*-multicopy strain is able to produce higher levels of cellulases than the wild-type strain under nonsaturating concentrations of sophorose, but both strains were comparably efficient at saturating concentrations [48].

The observation that the β-glucosidase inhibitor nojirimycin inhibits cellulase induction also in the *cel3a* disrupted strain suggests that the CEL3A is not the only β-glucosidase involved in inducer formation [48]. An additional β-glucosidase-encoding gene has been cloned [35] and properties and intracellular localisation of the corresponding enzyme have been characterised [69]. However, as no multicopy or gene deletion studies have yet been carried out, its involvement in cellulase induction warrants further investigation.

2.1. Transcriptional Factors Involved in Regulation of Cellulase and Hemicellulase Genes' Expression in *T. reesei*

Foreman *et al.* [54] performed investigations on regulation of cellulase and hemicellulase genes' expression in *T. reesei* by microarrays showing that most of the genes encoding known and putative biomass-degrading enzymes are transcriptionally co-regulated. This co-regulation indicates a tightly coordinated cooperation of the corresponding transcription factors, five of which have been so far identified (Fig. **1**): the positive regulators XYR1, ACE2, and the HAP2/3/5 complex, the repressor ACE1 and the carbon catabolite repressor CRE1 (Table **2**) [25].

The main positive regulator of cellulase and hemicellulase gene expression is represented by XYR1 (xylanase regulator 1) [48, 18], a zinc binuclear cluster protein binding to a GGCTAA-motif arranged as an inverted repeat [48]. *xyr1* deletion abolishes cellulase induction on cellulose and sophorose and impairs the induction of hemicellulase genes involved in xylan and arabinan degradation [71, 18], thus proving its essential role in the induction process. *xyr1* transcription seems not to be induced during growth on cellulose [72]. Most of eukaryotic transcriptional activators are present in cells only in small amounts required to start gene expression [73], and, in many cases, they are further induced by the conditions for which they are needed and are degraded once they are no longer required [74]. On the contrary, *xyr1* expression is regulated solely by CRE1-dependent CCR and by repression by the specific transcription factor ACE1, not by induction [72, 49]. Whether an increase in constitutive expression of *xyr1* would increase enzyme formation is not sufficiently understood. Aigner-Mach *et al.* [72] fused the *xyr1* gene under the regulatory signals of the *nag1* (N-acetyl-β-D-glucosaminidase) promoter, which resulted in a slightly earlier beginning of xylanase formation but did not significantly enhance the final enzyme titre. However, these studies used the uninduced, basal expression level of *nag1*, which is not much higher than that of *xyr1* itself, and studies using stronger expressed promoters (such as those for glycolytic or hydrophobin genes) must be used to clarify whether the constitutive expression of *xyr1* would enhance cellulase and/or xylanase formation.

The cellulase activator ACE2 also belongs to the class of zinc binuclear cluster proteins [75]. It has so far been shown to occur only in *Trichoderma* spp. Deletion of *ace2* lowers the transcript levels of the major cellulases and causes a decrease of cellulase activity during growth on cellulose [75, 76] whilst it does not affect cellulase induction by sophorose [75]. It is worth noting that the DNA-binding domain of ACE2 is able to bind to the promoter motif [GGC(T/A)A] present in the *cbh1* promoter also recognized by XYR1 [77]. Stricker *et al.* [76] suggested that phosphorylation and dimerization are needed for the binding of ACE2 to the corresponding promoter element.

The CCAAT motif is a common *cis*-acting element found in either orientation in the promoter and enhancer region of a large number of eukaryotic genes. Particularly, in yeasts, as well in filamentous fungi, the CCAAT box-binding proteins identified so far all belong to the group of HAP-like factors. Site-directed mutagenesis of the promoter of one of the most abundant cellulase produced by *T. reesei*, cbh2, revealed the existence of an undecameric nucleotide motif which is essential for gene expression *in vivo*. Moreover, experiments of promoter mutation and *in vivo* footprinting analysis allowed to show that expression from the *cel6a* promoter is dependent on a CCAAT box bound by the HAP2/3/5 protein complex [78]. The CCAAT motif is found in approximately 30% of the 5'- non-coding regions of eukaryotic genes [79]. In analogy to the mammalian NF-Y complex containing NF-YA, NFYB and NF-YC orthologues of HAP2, HAP3 and HAP5, respectively, they contain a histone fold motif, a structural feature of histones suggesting that NF-Y might be involved in the organisation of the chromatin structure [80]. Thereby the action of acetyltransferases may play a role in the local disruption of nucleosomes since an association of GATA-1 and NF-Y with acetyltransferases p300/CBP has been shown [81, 82].

The corresponding *hap2, hap3* and *hap5* genes from *T. reesei* were cloned by Zeilinger *et al.* [83] showing that they encode proteins similar to Hap homologues from other organisms and essential for binding to the CAE (*cbh2*-activating element) in the *T. reesei cel6a* promoter. The HAP2/3/5 complex is considered needed for generating an open chromatin structure required for full transcriptional activation [84]. The hypothesis that the CCAAT sequences in the cellulase promoters could play a conserved role in the generation of an open chromatin structure necessary for full transcriptional activation is supported by the detection of a nucleosome-free region around the XYR1/ACE2/HAP2/3/5-binding area in the *cel6a* promoter, which is flanked by strictly positioned nucleosomes [84]. Induction by sophorose results in a loss of positioning of nucleosomes -1 and -2 downstream of the binding area, thus making the TATA box accessible. A mutation in the CCAAT box shifted this positioning, thus proving the role of the HAP2/3/5 complex in this process [84].

These data provide an experiment based explanation of the advantage for clustering of cellulases in the genome of *T. reesei* and illustrate that chromatin regulation is a suitable target for strain improvement.

For instance, it is worth noting that Zou *et al.* [85] have recently demonstrated that replacement of the CRE1 binding sites within the *cbh1* promoter of *T. reesei* with the binding sites of transcription activator, namely the HAP2/3/5, besides the ACEII, led to improvement of promoter efficiency. The new developped promoter was shown able to induce expression of the green fluorescent protein reporter by 5.5-fold in inducing culture medium and 7.4-fold in repressing culture medium.

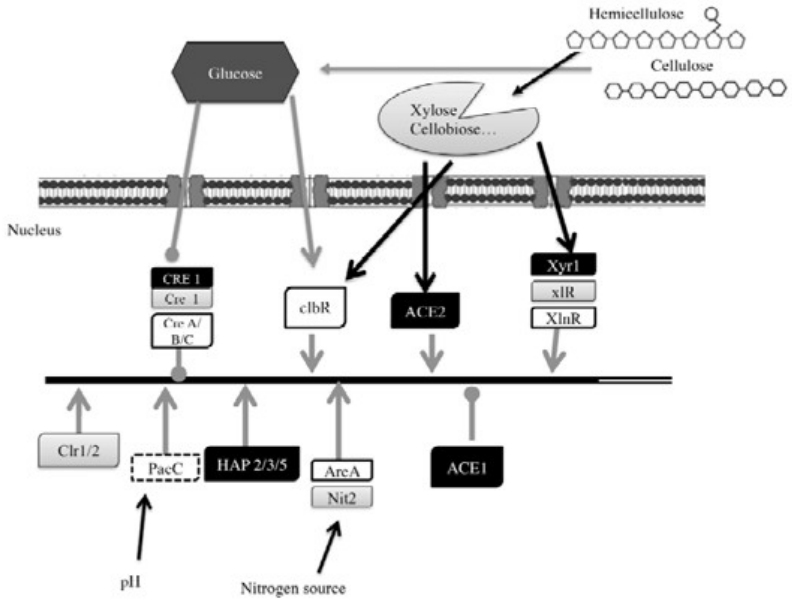

Fig. (1). Schematic representation of transcriptional factors affecting cellulases and xylanases expression in *T. reseei* (black box), *N. crassa* (grey box) and *Aspergillus* spp. (white box). The carbon catabolite repressor CRE, the activators clbR, Xyr/xlR/XlnR,Clr,ACE2, the repressor ACE1, the CCAAT binding Hap2/3/5 complex, the pH regulator PacC, and the nitrogen regulators AreA and Nit2 are shown. The repression activity (⊸), the induction activity (➔) and also promoter (▬) and coding region (═) are indicated.

Table 2. Positive and Negative Regulators of Expression of Genes Coding for (hemi)cellulolytic Enzymes and Their Binding *consensus* Sequences in the Target Promoters

Trichoderma Reesei			
Positive Regulators			
Name	**Structure**	**Consensus Region**	**References**
XYR1	Zinc binuclear cluster protein	5'-GGCTAA	[194] [25]
ACE2	Zinc binuclear cluster proteins	5'GGCTAATAA	
HAP2	Multimeric protein complex	5'- CCAAT	
HAP3			
HAP5			
Negative regulators			
Name	**Structure**	**Consensus region**	**References**
ACE1	Three Cys2His2-type zinc fingers	5'-AGGCA	[25] [195]
CRE1	Cys2His2 type transcription factor	5'-SYGGRG	
Neurospora crassa			
Positive regulators			
Name	**Structure**	**Consensus region**	**References**
CLR-1/-2	Two zinc binuclear cluster	-	[145]
PacC	Three Cys2His2 zinc fingers	5'-GCCARG	[147]

(Table 2) contd….

Neurospora crassa			
Positive regulators			
Name	**Structure**	**Consensus region**	**References**
XLR1	Zinc binuclear cluster protein		[148]
NIT2	Single zinc finger protein	5'- TATCTA	[149]
Negative regulators			
Name	**Structure**	**Consensus region**	**References**
CRE1	Cys2His2 type transcription factor	5'-SYGGRG-	[152]
Aspergillus spp.			
Positive regulators			
Name	**Structure**	**Consensus region**	**References**
AmyR	Zn(II)2Cys6-binuclear cluster DNA-binding motif	5'-CGGN8CGG-3'	[194,-196]
AraR	Zn(2)Cys(6) binuclear cluster domain		[197,198]
PacC	Three Cys2His2 zinc fingers	5'- GCCARG	[22]
XlnR	Zinc binuclear cluster protein	5'-GGCTAAA	[48]
ClbR	Zn(II)2Cys6-binuclear cluster DNA-binding motif	CGG or CCG triplets	[174]
AreA	Highly conserved DNA binding motif comprising a Cys(4) zinc finger followed by a basic domain	5'-GATA (core sequence)	[182]
Negative regulators			
Name	**Structure**	**Consensus region**	**References**
CREA	Cys2His2 type transcription factor	5'-SYGGRG	[183-187]
CREB			
CREC			

ACE1 contains three Cys2His2-type zinc fingers and it was shown to bind *in vitro* to eight sites containing the core sequence 5'-AGGCA scattered along the 1.15-kb *cel7a* promoter [86]. Deletion of *ace1* resulted in an increase in the expression of all the main cellulase and hemicellulase genes in sophorose- and cellulose-induced cultures, indicating that ACE1 acts as a repressor of cellulase and xylanase expression [87] and of *xyr1* during growth on D-xylose [72]. A strain bearing a deletion of both the *ace1* gene and *ace2* gene expressed cellulases and xylanases similar to the Δ*ace1* strain, probably due to the remaining activity of XYR1 [87].

All together the above data suggest that the substrate-unspecific activator XYR1 is fine-tuned by more specific transcriptional regulators such as ACE1 and ACE2 (Fig. **1**). This working model concurs with the findings that XYR1 binds to an inverted repeat either as a homo- or a heterodimer, respectively, thereby providing the opportunity for specific regulatory proteins to interact with the accordant promoter and/or XYR1. The role of the HAP2/ 3/5 complex in this regulation may be that of a general transcriptional enhancer raising the accessibility of the other factors to the cellulase promoters.

The putative methyltransferase LaeA is a global regulator that affects the expression of multiple secondary metabolite gene clusters in several fungi, and it can modify heterochromatin structure in Aspergillus nidulans. Seiboth *et al.* [88] showed that the expression of genes for lignocellulose degradation are controlled by the orthologous *T. reesei* LAE1: the protein methyltransferase LAE1. In a *lae1* deletion mutant a complete loss of expression of all seven cellulases was observed, auxiliary factors for cellulose degradation, β-glucosidases and xylanases were no longer expressed. Conversely, enhanced expression of *lae1* resulted in significantly increased cellulase gene transcription. Lae1- modulated cellulase gene expression was dependent on the function of the general cellulase regulator XYR1, but also *xyr1* expression

was LAE1-dependent. Chromatin immunoprecipitation followed by highthroughput sequencing ('ChIP-seq') showed that *lae1* expression was not obviously correlated with H3K4 dior trimethylation (indicative of active transcription) or H3K9 trimethylation (typical for heterochromatin regions) in CAZyme coding regions, suggesting that LAE1 does not affect CAZyme gene expression by directly modulating H3K4 or H3K9 methylation. These data demonstrate that the putative protein methyltransferase LAE1 is essential for cellulase gene expression in *T. reesei* through mechanisms that remain to be identified.

To learn more about the function of LAE1 in T. reesei, Karimi-Aghcheh *et al.* [89] further assessed the effect of deletion and overexpression of lae1 on genome-wide gene expression. They found that in addition to positively regulating 7 of 17 polyketide or nonribosomal peptide synthases, genes encoding ankyrinproteins, iron uptake, heterokaryon incompatibility proteins, PTH11-receptors, and oxidases/monoxygenases are major gene categories also regulated by LAE1. Chromatin immunoprecipitation sequencing with antibodies against histone modifications known to be associated with transcriptionally active (H3K4me2 and -me3) or silent (H3K9me3) chromatin detected 4089 genes bearing one or more of these methylation marks, of which 75 exhibited a correlation between either H3K4me2 or H3K4 me3 and regulation by LAE1.

CRE1 is the main transcription factor mediating CCR [90, 91], a mechanism promoting the assimilation of high-energy yielding carbon sources over that of sources yielding less energy, described in more details below.

2.2. Carbon Catabolite Repression of Cellulase and Hemicellulase Genes' Expression in *T. reesei*

Expression of most of *T. reesei* cellulase and hemicellulases genes does not occur in the presence of glucose in culture medium. Two mechanisms are responsible for this phenomenon: inducer exclusion (that is, inhibition of inducer [= sophorose] uptake by D-glucose [53] and glucose repression [59, 84, 92]. The latter specifies a transcriptional regulation controlling the preferential use of substrates such as D-glucose or other monosaccharides whose catabolism provides a high yield of ATP namely CCR.

Consequently, one of the earliest attempts for engineering cellulase production was removal of CCR. Classical mutagenesis combined with selection for 2-desoxyglucose resistance (an agent believed primarily to enrich carbon catabolite-resistant mutants [93] has led to increased cellulase producers such as *T. reesei* RUT C30 [94], RL-P37 [95] and CL847 [96], thus supporting the possible importance of CCR in cellulase formation.

In *Trichoderma spp.*, the key player in this glucose repression is the Cys2His2 type transcription factor CREI [90, 97]. *cre1* is missing in the cellulase hyperproducer strain RUT C30 [90] and importance of its deletion for the increase of cellulase production has been highlighted recently [98]. The *cre1* gene is located on scaffold 2: 786955-789433 (ID 120117), and the mutant is characterized by a loss of a 2478-base pair fragment, which starts downstream of the region encoding the CRE1 zinc finger and reaches into the 3'-non-coding region [99]. Le Crom *et al.* [100] 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.* [101], 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.

In vivo functionality of the CRE1 binding sites has been shown for the *cbh1* and *xyn1* promoters of *T. reesei* where mutations in the binding sequences led to constitutive expression of these genes in the presence of D-glucose [92, 102]. Functional CREI binding sites have been shown to consist of two closely spaced 5'-SYGGRG motifs, and it has been suggested that direct CREI repression would occur only through such double binding sites. Phosphorylation of a serine in a conserved short stretch within an acidic region of *T. reesei* CREI has been demonstrated to regulate its DNA binding [103]. Phosphorylation of this serine may involve a casein kinase 2. Casein kinases of this class are known from various other organisms to play a role in the regulation of a large number of transcription factors [104]. However, the SNF1 kinase, which plays a central role in the regulation of CCR in yeasts [105], appears not to be involved in the phosphorylation of CRE1 in *T. reesei* [106].

Another gene whose product is involved in CCR in *T. reesei* is represented by *cre2* whose disruption led to deregulation of genes normally subject to CCR [107]. Interestingly, the E3 ubiquitin ligase LIM1 also responds to cellulase inducing conditions and binds to the *cbh2*-promoter [108].

The way in which the presence of glucose triggers CCR is still only poorly understood in filamentous fungi. In *S. cerevisiae*, the D-glucose and D-fructose phosphorylating enzymes are also involved in D-glucose and carbon catabolite sensing, due to the presence of three hexose-6-phosphorylating enzymes including two hexokinases and one glucokinase. Each of them enables *S. cerevisiae* to grow on D-glucose, but the hexokinase Hxk2p is responsible for the main enzymatic activity and glucose repression mediated by the carbon catabolite repressor Mig1p (whose DNA-binding domain is highly similar to that of CRE1) [109-111]. The mechanism by which Hxk2p contributes to glucose repression has not yet been fully elucidated, but its catalytic activity seems to be dispensable and thus signal transmission may rather depend on substrate binding-induced conformational changes in the Hxk2p protein or a direct regulatory role of the Hxk2p in the nucleus (discussed, for example, in Linhoff *et al.* [80]).

Portnoy *et al.* [112] investigated how *xyr1*, *ace1* and *ace2* are regulated in cellulase induction conditions and how this regulation relates to carbon catabolite repression in the low cellulase producer strain *T. reesei* strain QM 9414, the high-producer strain RUT C30 [94, 113] and the hyperproducer strain *T. reesei* CL847 [96]. They demonstrated that in QM

9414 all three genes are induced by lactose and *xyr1* is also induced by D-galactose. Moreover, *ace1* is carbon catabolite repressed, whereas full induction of *xyr1* and *ace2* requires CRE1. These regulatory patterns showed significant differences in RUT C30 and CL847 strains. Rate of cellulase production by strain CL847 on lactose was around 15-fold higher than that for strain QM 9414, consistently with the 15-fold-increase of the *cbh1* transcript level. These data indicate that gene expression is a major limiting step for cellulase biosynthesis. Consistent with its role as the major transcriptional regulator of cellulase gene expression, a strongly increased basal expression of *xyr1* was observed in strain CL847, which was further induced by lactose. This increase indicates an improved function of the transcriptional machinery required for *xyr1* expression in strain CL847. The basal expression of *ace2* was not significantly altered in strain CL847, and the inducible level was the same as that in strain QM 9414. This indicates that the lack of CRE1 function, which seems to be required for *ace2* gene expression, as indicated by the lower expression levels in the Δcre1 mutant, has been overcome during the breeding of CL847. While these data suggest that *ace2* expression is not limiting for cellulase induction on lactose, they nevertheless show that wild-type expression levels appear to be necessary for the formation of high levels of cellulase. Expression of *ace1*—even though it is a repressor of cellulase formation—was also increased in the mutant strain CL847. However, *ace1* is subject to CRE1-dependent CCR. The comparison reveals that the basal expression level of *ace1* in CL847 is lower than that in the Δcre1 strain and decreases during the glucose feed. The approximate doubling of this level during the lactose feed is conserved, however. It has therefore been concluded that carbon catabolite derepression of *ace1* has partially reverted in CL847, leading to a lower concentration of this repressor under cellulase-producing conditions. The present findings of reduced *xyr1* but increased *cbh1* transcription in the Δcre1 strain would be consistent with the operation of post-translational modification of XYR1. Nevertheless, these data show clearly that the expression of *xyr1*, *ace1*, and *ace2* has been significantly altered in the hyperproducer CL847, suggesting that their wild-type expression was insufficient for hyperproduction. Identification of the proteins and genes responsible for the mechanisms observed may result in a major breakthrough in the understanding of cellulase formation and may offer a straightforward means for its improvement. These observations suggest that a strongly elevated basal transcription level of *xyr1* and reduced upregulation of *ace1* by lactose may have been important for generating the hyperproducer strain and that thus, these genes are major control elements of cellulose production.

3. REGULATION OF PRODUCTION OF CELLULASES AND HEMICELLULASES IN *NEUROSPOSRA CRASSA*

Neurospora crassa, a non pathogenic filamentous fungus of the class ascomycetes, is a well-known model organism that has been used for 90 years to study genetics, biochemistry, and fungal biology [114]. It was genetically characterized [115] and it has been shown to be able to degrade cellulose since 30 years ago [116]. *N. crassa* is able to synthesize and secrete high levels of all three enzyme types involved in cellulose degradation [117-121], as well as endoxylanase and β-xylosidase activities [122, 123]. There are 23 predicted cellulase genes and 19 predicted hemicellulase genes in the genome of *N. Crassa* (*http://www.broadinstitute.org/ annotation/genome/neurospora/MultiHome.html*). In addition, *N. crassa* is a well-known ethanol producing microorganism that has been used for fermentation of agricultural residues [124].

The cellulase complex in *N. crassa* is composed by four endoglucanases, three exoglucanases and one β-glucosidase [125]. A summary of inducibility of cellulases, hemicellulases and related enzymes in *N. crassa* is reported in the (Table **1**). In 1964, Eberhart *et al.* [126] showed the presence of two β-glucosidases, including an aryl-β-glucosidase and a cellobiase, acting complementarily in *N. crassa*. Based on their production in response to specific inducers or various conditions of growth, these enzymes represent two fundamentally different classes of disaccharidases. Results of Eberhart *et al.* [127] on the induction of β-glucosidases (EC 3.2.1.21) in *N. crassa*, showed that the aryl-β-glucosidase can be induced either by disaccharides, that are usually used as substrates by this class of enzyme, or by monosaccharides that are not their usual substrates [120, 128-132]. Induction in the presence of monosaccharides and spontaneous production associated with conidiation is reversals of catabolite repression [133-135] because enzymes are not produced when a significant level of glucose is present in the induction medium. Both cellobiase and aryl-β-glucosidase seem to be exceptions to the general situation that disaccharide substrates are not the best inducers of specific disaccharidases in *Neurospora* [120, 130, 132], being production of both enzymes induced by cellobiose. Aryl-β-glucosidase production is semiconstitutive at late stages of culture growth prior to conidiation. At early stages, aryl-β-glucosidase is induced by cellobiose, laminaribiose, and gentiobiose, and in small part induced by galactose, amino sugars, and aryl-β-glucosides. Among the monosaccharides, xylose and galactose induce aryl-β-glucosidase. Cellobiase is induced by cellobiose, but other inducers have little effect on this enzyme such as galactose and maltose. Cellobiase activity is very low in all stages of the vegetative life cycle in the absence of β-glucoside inducer. Experimental results showed that a mixture of xylose and cellobiose induced increase of cellobiase, while added xylose did not change significantly the induction of aryl-β-glucosidase. Cellobiose is clearly the best inducer, with an optimum effect from 0.05 to 1 mM. The induction of β-glucosidases was inhibited by glucose, 2-deoxy-D-glucose, and sodium acetate. Sodium phosphate concentrations between 0.01 and 0.1 M stimulated induction of both enzymes, while concentrations above 0.1 M were inhibitory. The optimal condition for induction of both β-glucosidases was pH 6.0. Cellobiase induction was relatively more inhibited than aryl-β-glucosidase in the range of pH 6.0 to 8.0. The time required for the induction of these enzymes by cellobiose is 6 hours. As described below, fatty acids and surfactants have positive effects on the cellulases production [121], however, experimental results showed that oleic acid had no effect on production of β-glucosidase, while Tween 80 decreased its production [121]. This is probably due to some difference in the cellulase and β-glucosidase released.

In fact in most organisms studied, β-glucosidase is an intracellular enzyme, released only by autolysis [136]. A recent study [137] demonstrated that a *N. crassa* mutant carrying deletions of two genes encoding extracellular β-glucosidase enzymes and one intracellular β-glucosidase lacks β-glucosidase activity, but its cellulase gene expression is efficiently induced in the presence of cellobiose, cellotriose, or cellotetraose as a sole carbon source while sophorose does not act as an inducer. Furthermore, the inclusion of a deletion of the catabolite repressor gene, *cre-1*, in the triple β-glucosidase mutant resulted in a strain that produces higher concentrations of secreted active cellulases on cellobiose. So cellobiose is an inducer of β-glucosidases but carbon catabolite repression (CCR, see the following paragraph) masks this inducing activity.

Eberhart *et al.* [116] studied the extracellular endocellulase (EC 3.2.1.4) production in mycelia and ungerminated conidia of *N. crassa*. They demonstrated a simple induction system of cellobiose and potassium phosphate buffer (pH 6.0) of extracellular cellulase to provide energy and substrates for protein synthesis. Yazdi *et al.* [125, 121, 138] have shown for the first time that *N. crassa* is capable of synthesizing and secreting high levels of the cellulase complex enzymes growing on microcrystalline cellulose, and other carbon sources, as an inducer for the enzymes. They also studied the role of surfactants and fatty acids on the production of the cellulases, since in other species it has been reported that surfactants and fatty acids stimulate production of the cellulase complex [139-141]. Yazdi *et al.* [121] demonstrated that the presence of C18 fatty acids and surfactants, such as Tween 80, increases production of both endoglucanase and exoglucanase in the medium. It is probably due to an increase in the permeability of the cell membrane, thus permitting more of the enzymes to be secreted, as postulated for other species by Reese & Maguire [139] and Demain & Birnbaum [142].

Analysis of the effects of different carbon sources, such as glucose, xylan and cellulose, on the production of extracellular cellulases and xylanases by *N. crassa*, reported by Mishra *et al.* [122], showed that the extracellular activities were very poor when the fermentation was carried out with glucose, while the maximum xylanase production was observed when *N. crassa* was grown on commercial xylan. However, significant amounts of xylanase were produced when cellulose powder was used as a carbon source.

In 2012, Sun *et al.* [143] showed that *N. crassa* responds to the presence of cellulose (Avicel) by inducing both cellulase and hemicellulase gene expression, while the exposure to xylan only induces hemicellulase gene expression. In addition, exposure to Avicel induces some hemicellulase genes to a much higher expression level than exposure to xylan. These data suggest crosstalk between inducer molecules and regulatory pathways that are involved in deconstruction of plant cell walls in filamentous fungi. In this work, 353 genes have been identified that were significantly induced by xylan; in particular three genes, *gh51-1* (arabinofuranosidase), *gh10-2* (endoxylanase) and *gh43-5* (β-xylosidase) showed increased expression levels of over 200 fold. Among 353 identified genes only 30 genes were induced by exposure to xylose. Although none of the xylanolytic-related genes was essential for growth of *N. crassa* on xylan, in fact mutations in a number of them affected xylanase activity. These observations indicate some redundancy among enzymes associated with hemicellulose degradation, similar to those identified with cellulose degradation [48].

3.1. Transcriptional Factors Involved in Regulation of Cellulase and Hemicellulase Genes Expression in *N. crassa*

The knowledge of *N. crassa* genome sequence [144] has allowed the identification of the proteins involved in regulation of cellulase ad hemicellulase genes expression (Table **2**, Fig. **1**).

Two zinc binuclear cluster transcription factors (CLR-1 and CLR-2) are important regulators of genes encoding both cellulases and hemicellulases in the presence of cellulose as carbon source, but they are not required for growth or hemicellulase activity production in the presence of xylan as reported by Coradetti *et al.* [145]. In particular, Coradetti *et al.* [145] demonstrated that CLR-1 is a crucial element in cellobiose sensing mechanism of *N. crassa* during its growth on avicel. CLR-1 promotes expression of several genes necessary for cellobiose utilization, as well as that of *clr-2*. CLR-2, maybe in a complex with CLR-1 directly induces cellulase and hemicellulase gene expression, when *N. crassa* is grown on avicel. Phylogenetic analyses of CLR-1 and CLR-2 protein sequences performed by the same group, showed that these factors are conserved in the genomes of most filamentous ascomycete fungi degrading cellulose suggesting that homologs of CLR-1 and CLR-2 play an important role in plant cell-wall degradation.

In nature, the enzymatic breakdown of plant cell wall polymers can occur in different surrounding pHs and there is a regulatory mechanism controlling pH-dependent transcriptional regulation. *pacc* gene in *N. crassa* (ORF NCU00090) is the *pacc*/RIM101 orthologue, extensively studied in *A. nidulans* and *S. cerevisiae* [146]. The transcription factor PacC responds to changes in extracellular pH by activating specific alkaline genes and repressing specific acid genes [147, 148].

A xylan degradation regulator-1 (*xlr-1* NCU06971) is essential for hemicellulose degradation in *N. crassa*. *xlr-1* encodes a member of a TF family containing conserved fungal Zn(2)-Cys(6) binuclear cluster domain with significant amino acid homology to *xyr1* in *Trichoderma* species [148], sharing 57.6% identity with homolog in *T. reesei*. Recently, Sun *et al.* [143] demonstrated that a deletion of *xlr-1*gene abolishes growth of *N. crassa* in both xylan and xylose containing media, but it slightly affects the growth on Avicel and the production of cellulase activity in the presence of this substrate. To determine regulatory mechanisms for hemicellulose degradation, the authors explored the transcriptional regulation of XLR-1 under xylose, xylanolytic and cellulolytic conditions. Their results showed that XLR-1 regulated only some predicted hemicellulase genes in *N. crassa* and was required for a full induction of several cellulase genes. Moreover, among the genes induced by xylan there are 19 permease/transporter genes and their full induction requires a functional *xlr-1*. Of these 19 transporters, five have been functionally tested for transport of D-glucose and

D-xylose. Another transcription factor identified in *N. crassa* was NIT2 protein (AreA in *A. nidulans*), a member of the GATA factors family, characterized in *N. crassa* as a positive regulator of genes encoding enzymes for nitrogen source catabolism under nitrogen limiting conditions [149, 150]. Goncalves *et al.* [148] suggested that *nit-2* also acts as a repressor of carbon metabolism. They analyzed *cis* elements present in the gene encoding glycogen synthase (*gsn*) promoter and showed that *nit-2* is able to bind these *cis* elements. Moreover, the knocked-out nit-2 strain showed loss of glycogen accumulation despite having low *gsn* gene expression as compared to the wild-type strain, suggesting they may have a role in glycogen metabolism regulation. A link between carbon and nitrogen regulation was already reported by Lockington *et al.* [151] in *A. nidulans*. Although the result was preliminary, the authors suggested the existence of a link in the regulation of the carbon and nitrogen utilization pathways in filamentous fungi.

3.2. Carbon Catabolite Repression of Cellulase and Hemicellulase Genes Expression in *N. crassa*

Sun *et al.* [152] investigated CCR of cellulase expression in *N. crassa* and they showed that, under cellulolytic conditions, CRE-1 regulates genes involved in plant cell wall utilization by directly binding to adjacent motifs in promoter regions and also may compete for binding with positive regulatory factors. They demonstrated that deletion of *cre-1* caused constant expression of cellulase genes, resulting in higher cellulolytic enzyme activity. Moreover, *cre-1* caused the repression of cellulolytic genes during growth on Avicel. Some genes known to be directly regulated by CRE-1 homologs in other systems (such as *cbh*-1 of *T.reesei* and XlnA of *A. nidulans*) and also a large number of other target genes of predicted or unknown function in *N. crassa* were identified. These genes may be regulated directly or indirectly by CRE-1. For example, CRE-1 binds to the promoter region of *cbh-1* in *N. crassa* and may compete for binding with pathway specific cellulolytic regulator required for induction; the identity of cellulolytic regulators in *N. crassa* is currently unknown. Among CRE-1 targets identified in *N. crassa* there are a hypothetical protein of unknown function (NCU 03181), an additional xylanase (NCU07225) and *gh6-3* (NCU07190). It is worth of note that a MFS monosaccharide transporter (NCU04963) was identified as a direct target of CRE-1 in *Neurospora*. These results suggest that CRE-1 may directly regulate genes involved in sugar transport, in addition to regulating genes encoding regulatory/enzymes associated with utilization of alternative carbon sources. In summary, CRE-1 functions as a global transcription factor in *N. crassa* and affects both gene repression and activation, both directly and indirectly.

The same authors recently [143] studied the *cre-1* regulation in hemicellulase expression. As described above, in *N. crassa*, transcription of most hemicellulase genes is via induction by xylanolytic molecules and is regulated via *xlr-1* and/or other transcription factors. However, the hemicellulolytic system is also responsive to CCR. CRE-1 mediated CCR regulates the expression level of some, but not all hemicellulase genes in *N. crassa* under Avicel conditions. *xlr-1* is regulated by a combination of induction and derepression and it is also subject to non-CRE-1 mediated CCR. These observations imply that other mechanisms regulate CCR in filamentous fungi in addition to CRE-1, similar to what has been described for *S. cerevisiae* [153].

4. REGULATION OF PRODUCTION OF CELLULASES AND HEMICELLULASES IN *ASPERGILLUS* SPP

The genomes of four *Aspergillus spp.*, *A. nidulans*, *A. oryzae*, *A. niger* and *A. fumigatus*, have been recently sequenced (*http://www.aspergillusgenome.org/*), and shown to contain around 200 genes -out of 14,600- involved in polysaccharides' degradation [154]. *Aspergillus spp.* have been so far described as high cellulases' producers and many genes coding for cellulase, endoxylanases, β-xylosidases and pectinases have been cloned and characterized from *Aspergillus* spp. strains [24]. A summary of inducibility of cellulases, hemicellulases and related enzymes in *Aspergillus spp.* is reported in the (Table **1**).

The influence of carbon and nitrogen sources on the production of cellulases has been so far investigated showing that the enzyme production is strongly variable according to the carbon source.

For instance, Hanif *et al.* [155] showed that even low concentrations of glucose negatively affect β-cellobiohyd rolase (CBH) production in *Aspergillus niger*, whilst cellulose and wheat bran stimulate β-cellobiohydro lase and filter paperase (FPase) activities, respectively. It was shown that addition of glucose inhibited cellulase production, even in cultures of *A. niger* gowing on wheat bran, showed to be a good inducer.

In several manuscripts, lactose has been defined as the best inducer for cellulase production in *Aspergillus* spp. Mrudula and Murugammal [156] confirmed that lactose is the best inducer of cellulase activity production by *Aspergillus niger*. In fact, lactose was shown the best carbon source to obtain high level of both CMCase and FPase activities, in both liquid and solid state fermentation.

As shown by Ali and Sayed [157], xylose is the best carbon source for induction of both endo- and exo-cellulase activity production in *A. terreus*, whilst the production of β-glucosidase is positively affected by both glucose and xylose. Lignocellulosic substrates, like agroindustrial wastes, have been so far described as good substrate for cellulase and xylanase production by filamentous fungi, as *Aspergillus* spp.

Among the several examples, Ghori *et al.* [158] recently demonstated the properties of corn-stover as an inducer of cellulase activity production by *A. niger*. Moreover, they demonstated that addition of cane molasses and yeast sludge to the fermentation medium leads to an increase of cellulase production. However, the induction mechanism involved in solid state fermentation have been showed to be more complex [159].

The effect of several carbon sources on glycosyl hydrolases gene expression has been studied by Nazir *et al.* [160] who reported differential expression of endoglucanase and beta-glucosidase isoforms of *A. terreus*, in both solid and liquid cultures. Maximal expression of four endoglucanase isoforms was observed in presence of rice straw and corn

cobs, in solid state and liquid fermentation respecivetly. Addition of fructose and cellobiose to corn cobs containing medium caused the up-regulation of endoglucanase activity, whereas addition of mannitol, ethanol and glycerol selectively repressed the expression of at least three endoglucanase isoforms. As far as the beta-glucosidase profiling is concerned, addition of glucose, fructose, sucrose, cellobiose, mannitol and glycerol resulted in down-regulation of most of the isoforms.

Many manuscripts have been reported concerning induction of xylanase production in *Aspergillus* spp.. It is well known that xylose, xylan and crude xylan-containing substrates mainly induce xylanolytic enzymes production in *Aspergilli spp.*. There are rare cases where other monomeric or polymeric substrates, such as glucose and cellulose, induce xylanolytic expression. For instance, Hrmova *et al.* [161] observed the induction of xylanolytic enzymes by cellulose, cellobiose and even by a heterodysaccharide consisting of glucose and xylose in *A. terreus*. The regulation of xyalonlytic enzymes is not identical in all *Aspergillus* spp.. As a matter of fact, Kimura *et al.* [162] cloned a xylose-inducible, glucose repressed endoxylanase gene from *A. oryzae* in *A. nidulans*, where its expression was instead increased by adding glucose.

Pinaga *et al.* [163] studied the effect of several compounds on xylanase production by *A. nidulans*. Xylooligosaccharides such as xylobiose, xylotriose and xylotetraose induced xylanase activity production, their efficiency being directly related to their chain length. However, xylans such as wheat arabinoxylan, oat spelt xylan, birchwood xylan and 4-O-methyl-D-glucorono-D-xylan were found to be the most powerful inducers. Xylose, on the contrary, was not shown to be a good inducer.

Xylanases production by *A. phoenicis* was shown positively affected by xylan, xylose and *b*-methylxyloside, similarly to the cases of other fungi belonging to the *Aspergillus* genus such as *Aspergillus sydowii* and *A. tubingensis,* as studied and discussed by Rizzatti *et al.* [164]. This study also demonstrated that the levels of production of xylanase by *A. phoenics*, decreased when glucose was added to the inducers xyalna or xylose, similarly to *A. sydowii* whose xyalanase production is inhibited by glucose [165].

Methyl β-d-xyloside was shown a more effective inducer than xylan, for both extracellular xylanase and intracellular β-xylosidase by Simao *et al.* [166]. The same group also demonstated that both glucose and cycloheximide inhibit the positive effect of methyl β-d-xyloside on xylanase production. However, not much is known about the uptake system for the inducers xylose and xylobiose in *Aspergillus spp,*. but more is known about the formation of the inducing compounds. For instance, the *A.niger* β-xylosidase, encoded by *xlnD*, has been shown to have an important role in xylanolytic inducer formation, being active towards xylan and xylooligosaccharides for the formation of D-xylose [167].

Galacturonic acid is the main inducer of several pectinolytic enzymes encoding genes such as *pelA, plyA, pgaX, rglA* and *pmeA* [168]. As reported by de Vries *et al.* [169], galacturonic acid positively affects even the expression of several genes encoding enzymes which act on the pectin side chains such as arabinofuranosidases (*abfA* and *abfB*), endoarabinase (*abnA*), endogalactanase (*galA*) and galactosidase (*lacA*).

As far as the induction of extracellular arabinases is concernid, pentose sugars and polyols generated by the metabolic pathway of L-arabinose and D-xylose catabolism were shown to be invooved in *Aspergillus niger* arabinases production. Particularly, induction occurred with L-arabinose and L-arabitol but not with D-xylose or xylitol, L-arabitol being the best inducer for a-L-arabinofuranosidase and endoarabinase activities [170].

4.1. Transcriptional Factors Involved in Regulation of Cellulase and Hemicellulase Genes Expression in *Aspergillus* spp

Transcription factors involved in the regulation of *Aspergillus* spp. (hemi)cellulolytic enzymes encoding genes, mostly belong to Zn(II)2Cys6 binuclear cluster DNA-binding motif family (Table **2**, Fig. **1**).

XlnR is the main transcriptional activator which has been largely studied for its involvement in the regulation of cellulases, hemicellulases and accessory enzyme genes for xylan degradation in *Aspergillus* spp.. It is an orthologue of the *xyR1* gene of *T. reesei* [18].

van Peiji *et al.* [171] finely described the role of XlnR transcriptional activator. It has been demonstrated that XlnR regulates the transcription of the *xlnB, xlnC* and *xlnD* genes encoding endoxylanases B, endoxylanase C and β-xylosi dase, respectively. It is also involved in the activation of cellulase genes transcription, such as those coding for the two endoglucanase *eglA* and *eglB*. In addition, XlnR has been shown to positively affect the transcription of several accessory enzymes gene involved in hemicelluloses degradation, including glucuronidase A, acetylxylan esterase A, arabinoxylan arabinofuranohydrolase A and feruloyl esterase A.

Several northern blot analyses have been performed on *A. niger* strains, in order to demonstrate the important role of XlnR on the activation of different glycosyl hydrolases genes transcription. These analyses allowed the comparison of level of expression of genes of interest in an *A. niger* wild-type strain, a *xlnR* loss-offunction mutated strain and a multiple-copy strain [171, 172].

More recently, similar studies have been performed by Tani *et al.* [173] who demonstrated that cellulose affects positively both cellulase and hemicellulase activities production in *A. aculeatus*, through two different pathways, namely XlnR-dependent and XlnR-independent pathways. Real-time PCR experiments have been performed to identify the genes controlled by the XlnR-independent pathway. Particularly, both cellobiose and cellulose were shown to induce the expression of the gene regulated by XlnR-independent signaling pathway, the latter stimulating expression of FIII-avicelase (*cbhI*), FII-carboxymethyl cellulase (*cmc2*), and FIa-xylanase (*xynIa*).

Recently, further analyses on (hemi)cellulase genes regulation have been performed by Kunitake *et al.* [174]. ClbR, a new activator with a Zn(II)2Cys6 binuclear cluster DNA-binding motif specific for fungi, has been identified. It has been shown to control the cellobiose and cellulose respon-

sive induction of cellulase and xylanase genes which are regulated by both XlnR-dependent and XlnR-independent signaling pathways. For instance, disruption of *clbR* gene caused the decrease of the cellobiose- and cellulose-responsive induction of the *cbhI*, *cmc2*, and *xynIa* genes and the cellulose-responsive induction of the *cmc1* and *xynIb* genes Kunitake *et al.* [174].

Differently from *T. reesei*, fine-tuning transcription factors like Ace1 and Ace2 cannot be found in *Aspergillus* spp. The putative ACE1 proteins of *A. nidulans*, stzA (AF202995), is deposited into the database as a gene encoding a protein that alleviates sensitivity to salt and DNA damaging agents. Interestingly, *stzA* has been identified as an orthologue of the *T. Reesei* ACE1 gene [175]. The authors provided evidence of competition, or interaction, between the ACE1/StzA and AreA binding sites in promoters of *stzA* and its orthologs, and in genes involved in the metabolism of amino acids. The *A. nidulans* and *A. fumigatus cpcA* (cross pathway control regulator of amino acid biosynthesis) promoters have seven potential ACE1/StzA binding sites, six of which are highly conserved in position. The presence of potential CPC1 binding sites (5'-TGAC/GTCA) in the *stzA* and *ace1* promoters suggests an intriguing link between intracellular amino acid availability and cellulase gene expression. In accordance with these findings a recent study by Gremel *et al.* [108] indeed revealed that cellulase gene expression can be enhanced by the addition of methionine.

PacC is the major factor involved in pH-dependent expression in *Aspergillus spp.*. pII regulation of genes encoding cell wall-degrading enzymes has not been studied in detail in *Aspergillus*. However, indications for pH-dependent expression of xylanolytic and pectinolytic genes have been obtained [176]. Kojma *et al.* [176] demonstrated that *A. kawachii* produces different polygalacturonases using culture media with different pHs whilst *A. nidulans* PacC mutant strain does not produce arabinofuranosidase activity [177] and two endoxylanase, *xlnA* and *xlnB* [178]. Even cellulase production in *A. fumigatus* is affected by pH [179]. Indeed, two and one PacC *consensus* sites have been revealed in the promoter regions of *xlnA, xlnB* [180] and *xlnD* [181], respectively. The role of the transcriptional activator AreA on total cellulase production has been studied by Lockington *et al.* [182]. *areA* gene product is known to control the expression of genes encoding the enzymes involved in nitrogen metabolism in ammonium derepressing conditions [183].

The homologous of AreA was identified in *N. crassa* as NIT2 protein, a member of the GATA factors family, characterized in *N. crassa* as a positive regulator of genes encoding enzymes for nitrogen source catabolism under nitrogen limiting conditions [149, 150]. Goncalves *et al.* [148] suggested that *nit-2* also acts as a repressor of carbon metabolism. They analyzed *cis* elements present in the gene encoding glycogen synthase (*gsn*) promoter and showed that *nit-2* is able to bind these *cis* elements. Moreover, the knocked-out nit-2 strain showed loss of glycogen accumulation despite having low *gsn* gene expression as compared to the wild-type strain, suggesting they may have a role in glycogen metabolism regulation. A link between carbon and nitrogen regulation was already reported by Lockington *et al.* [151].

It has been showed that the amount of total secreted cellulase activity increased in a strain containing the constitutively activating *areA* allele, xprD1, and decreased in a strain containing the loss of function allele, areA217. To deepen AreA role in cellulase genes regulation, two genes encoding exocellulases, and one gene encoding an endocellulase were cloned. The putative regulatory regions of all the genes contain potential binding sites for the global carbon and nitrogen regulatory proteins, CreA and AreA, potential *consensus* binding sites for XlnR, whilst the AceII DNA binding *consensus* sequence involved in induction in *T. reesei*, misses in all the genes Lockington *et al.* [182]. Real-time PCR techniques were used to assess the relative expression levels of genes encoding hydrolase activities and of the genes encoding regulatory elements such as AreA, PacC and CreA in an effort to identify possible transcriptional regulation mechanisms in *A. oryzae* solid state fermentation [159]. This study showed the complexity of the regulation of genes coding for hydrolytic enzymes under solid state fermentation, as other factors such as post-transcriptional regulation appeared to be involved.

4.2. Carbon Catabolite Repression of Cellulase and Hemicellulase Genes Expression in *Aspergillus* spp

It has been reported that *creA*, *cre B* and *creC* genes products are involved in the regulatory mechanism of carbon catabolite repression in *Aspergillus spp.* [183-187]. CreA-mediated repression in *Aspergillus* has been demonstrated for genes encoding cellulase, arabinases, several endoxylanases and other xylanolytic activities such as xylosidase, feruloyl esterase and some pectinases [22]. The binding *consensus* motif for *A. nidulans* CreA was determined to be 5'-SYGGRG [188]. Besides glucose, other monomeric carbon sources result in CreA-mediated repression of gene expression, such as xylose. For instance, high concentrations of xylose have been shown to activate the CreA-mediated repression, by down-regulating the expression levels of several xylanolytic and cellulolytic genes in *A. niger* [169] and *A. terreus* [157].

Interestingly, Flipphi *et al.* [189] showed that in *A. nidulans*, mutations in both the single glucokinase and the single hexokinase genes belonging to the fungus, lead to a CreA-mediated carbon catabolite derepression, similarly to *T. reesei* which also features only one glucokinase and one hexokinase.

CreB encodes a deubiquitinating enzyme and it is a functional member of a novel subfamily of the *ubp* family defined by the human homolog UBH1 [186]. It forms a complex with a WD40-repeat protein encoded by *creC* [182], which is required to prevent the proteolysis of CreB in the absence of CCR [187]. Interestingly, the E3 ubiquitin ligase LIM1 also responds to cellulase inducing conditions and binds to the *cbh2*-promotor [108].

In addition, CreD has been reported to be involved in CCR of *Aspergillus* sp.. Mutations in *creD* suppress the phenotypic effects of mutations in *creC* and *creB* [190]. CreD contains arrestin domains and PY motifs and is highly similar to *Saccharomyces cerevisiae* Rod1p and Rog3p, which interact with the ubiquitin ligase Rsp5p [191].

5. CONCLUSIONS

The regulation of (hemi)cellulolytic genes appears to be basically the same among filamentous fungi such as *T. reesei, N. crassa, Asperigillus* spp., although their regulatory mechanisms are quite complex and present some differences. In particular, cross talks between expression of cellulolytic and hemicellulolytic genes make the regulatory mechanisms more complicated. Xyr homologs (Xyr1 of *T.reesei*, XlR of *N. crassa*, and XlnR of *Aspergillus* spp) mediate expression of both xylanolytic and cellulolytic genes in response to xylan. Moreover, Xyr homologs mediate cellulose-inductive expression of the xylanolytic genes as well as the cellulolytic genes. In addition to Xyr homologues, AceI and AceII in *T. reesei,* and ClbR in *A. aculeatus*, are suggested to be involved in the regulation of the expression of these genes. This further makes the regulatory mechanisms of (hemi) cellulolytic genes complicated. Unfortunately, the roles of AceI and AceII in the regulation of cellulolytic genes remain ambiguous and need to be investigated further. Carbon catabolite repression of cellulase expression appears to be essentially the same among fungi. As described above, major inductive signals for fungi to degrade plant cell wall are derived from cellulose and xylan. Xyr homologs receive inducing signals from these two different polysaccharides and activate transcription of many (hemi)cellulolytic genes. Xyr homologues could recognize two distinct inductive signals prior to induction of the target genes due to their differential conformational changes.

As a main difference among the cellulase and hemicellulase regulatory systems from different fungi, these systems appear to be more specialized in *T. reesei* than in the other fungi, considering that *T. reesei* fine-tuning transcription factors like Ace1 and Ace2 cannot be found in *Aspergillus* and *Neurospora* spp.

However, more research is needed to completely disclose the molecular mechanisms of regulation of cellulase and hemicellulase gene expression in *T. reesei, Aspergillus* and *Neurospora* spp. Their elucidation could provide a basis for the rational application of transcriptional regulators for biotechnological processes in filamentous fungi, leading to efficient biocthanol production from lignocellulosic biomass.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application

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The aims of this study were to evaluate the microbial diversity of different lignocellulosic biomasses during degradation under natural conditions and to isolate, select, characterise new well-adapted bacterial strains to detect potentially improved enzyme-producing bacteria. The microbiota of biomass piles of *Arundo donax*, *Eucalyptus camaldulensis* and *Populus nigra* were evaluated by high-throughput sequencing. A highly complex bacterial community was found, composed of ubiquitous bacteria, with the highest representation by the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* phyla. The abundances of the major and minor taxa retrieved during the process were determined by the selective pressure produced by the lignocellulosic plant species and degradation conditions. Moreover, cellulolytic bacteria were isolated using differential substrates and screened for cellulase, cellobiase, xylanase, pectinase and ligninase activities. Forty strains that showed multienzymatic activity were selected and identified. The highest endo-cellulase activity was seen in *Promicromonospora sukumoe* CE86 and *Isoptericola variabilis* CA84, which were able to degrade cellulose, cellobiose and xylan. Sixty-two percent of bacterial strains tested exhibited high extracellular *endo*-1,4-ß-glucanase activity in liquid media. These approaches show that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the 'greener' technology of second-generation biofuels.

Lignocellulosic biomass includes dedicated energy crops, such as miscanthus, switchgrass, *Arundo donax*, *Populus nigra* and *Eucalyptus camaldulensis*. These plants can easily grow in farmland not suitable for food crops or in soils subjected to accelerated erosion, which affects most of the Mediterranean hilly croplands[1–3]. Vegetable biomasses represent an inexpensive alternative to fossil sources of fermentable sugars that can be utilised in several industrial applications, including second-generation biofuels and biopolymer production[4].

The enzymatic hydrolysis of plant carbohydrates has emerged as the most prominent eco-technology for the degradation of such biomasses. From the microbiological point of view, lignocellulosic biomass represents a complex ecosystem in which environmental conditions influence living organisms. In particular, geochemical (pH and salinity) and physical (temperature, pressure and radiation) factors can have a selective pressure on the biodiversity of microorganisms[5]. As a consequence, autochthonous microbial communities may prevail over other microorganisms because they possess enzymes that are able to degrade complex molecules such as cellulose and hemicellulose, forming the lignocellulosic biomasses that are the most abundant renewable energy source on Earth[6].

Generally, at an industrial level, the bioconversion of pretreated cellulose-based materials into fermentable sugars is performed by using a reaction mixture composed of multiple enzymes for complete hydrolysis. However, because the biorefining process is still economically unfeasible, novel biocatalysts from bacteria could

help overcome costly hurdles due to the operative steps of cooling, oxygen pumping, stirring and neutralisation, as well as the intrinsically high cost of hydrolytic enzyme production[7]. Different microorganisms producing hemicellulolytic enzymes that are potentially usable as new biocatalysts for hemicellulose hydrolysis have been isolated from different natural environments such as compost[8,9]. They belong to specific groups of microorganisms that are able to synthesise cellulase, xylanases and other biocatalysts necessary to allow a complete hydrolysis of the recalcitrant components of the lignocellulosic biomass[10]. Cellulolytic microorganisms can synthesise distinct enzymes such as endoglucanases, exoglucanases, including d-cellodextrinases, cellobiohydrolases and ß-glycosidase, which cooperate in cellulose degradation. Indeed, the hemicellulolytic microorganisms produce xylanases for degrading xylan into xylose that include endo-β-1,4-xylanases and β-xylosidases as well as auxiliary enzymes such as α-glucuronidases, α-arabinofuranosidases, acetylesterases and acetyl xylan esterases[11].

The use of culture-independent high-throughput sequencing can potentially reveal uncultivable microbiota and enables the study of the microbial ecology and taxonomic diversity at a high resolution. A thorough determination of the microbial diversity in biomass degradation can be fundamental to evaluating potential sources of novel enzymes and activities[12,13].

In the present work, the changes in the microbiota during the natural biodegradation of lignocellulosic biomasses of *A. donax*, *E. camaldulensis* and *P. nigra* were studied. In addition, new well-adapted bacterial strains from the three lignocellulose biomasses were isolated, identified and characterised. This study shows that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the 'greener' technology of second-generation biofuels.

Results

Physicochemical measurement. The temperature values observed in the piles were approximately 24°C during the first 45 days. This value increased up to about 29°C after 135 days of biodegradation, before declining up to 25°C at the end of the experiment (180 days). The values of a_w ranged from to 0.91 to 0.99. The environmental temperature increased from April (20.5°C on the average) to August (34.2°C on the average) and declined in September (29.2°C).

Microbial Diversity of Lignocellulosic Biomasses by High-Throughput Sequencing. The microbiota of three different lignocellulosic biomasses (*A. donax*, *E. camaldulensis* and *P. nigra*) was characterised by partial 16S rRNA gene sequencing obtained from DNA directly extracted from environmental samples. A total of 238,450 number of reads were obtained by high-throughput sequencing. However, following the removal of short, ambiguous and/or low-quality pyrotag reads, the final data set consisted of 138,336 high-quality reads with an average sequence length of 456 bp. The alpha-diversity was determined by calculating the Shannon diversity index and the Chao1 richness index based on OTUs of 97% identity (Table 1). The results showed that the highest diversity indices were observed in the *E. camaldulensis* and *P. nigra* biomasses after 180 days of biodegradation in the underwood condition ($P < 0.05$) but were quite variable in the *A. donax* biomass (Table 1). Good's coverage indicated that more than 90% of the microbial diversity was described in most of the samples.

The relative abundances of bacterial taxa were examined at the level of phyla and class to determine whether there were any significant shifts in the composition of the bacterial communities according to the plant species, degradation conditions and sampling time.

In total, twenty-six different phyla were detected in the biomass samples, but only *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were detected in all samples (Fig. 1). These phyla together

Table 1 | Number of sequences analysed, observed diversity and estimated sample coverage for 16S rRNA amplification from DNA extracted from the chipped lingo-cellulosic biomasses

Sample	No. reads	No. OTUs	Chao1	Shannon index[a]	Good's coverage (%)
At0	12,021	322	432.68	4.28^{AB}	99.24
At1OF	6,235	477	682.92	6.59^{DE}	97.45
At1UW	9,713	506	713.56	5.51^{BC}	98.38
At2OF	4,158	592	955.73	7.09^{DEF}	93.70
At2UW	2,564	286	443.81	6.09^{C}	95.44
At3OF	1,699	404	618.70	7.33^{F}	88.99
At3UW	5,451	441	561.42	6.36^{CDE}	97.54
At4OF	5,160	620	1111.02	6.94^{DEF}	94.46
At4UW	4,293	501	671.50	6.61^{DE}	95.64
Et0	5,582	340	555.28	6.05^{C}	97.76
Et1OF	5,271	322	485.11	3.92^{A}	97.67
Et1UW	3,207	286	430.38	4.65^{B}	96.35
Et2OF	3,983	453	656.84	6.65^{DE}	95.81
Et2UW	1,069	228	405.60	6.52^{DE}	89.5
Et3OF	4,520	470	671.11	6.52^{DE}	96.00
Et3UW	7,091	623	978.51	6.46^{CDE}	96.63
Et4OF	4,271	571	882.78	7.49^{FG}	94.66
Et4UW	5,309	887	1367.25	8.37^{GH}	93.60
Pt0	10,116	471	623.72	5.94^{C}	98.65
Pt1OF	4,292	501	865.63	6.18^{CD}	94.43
Pt1UW	10,117	845	1128.45	8.24^{G}	97.68
Pt2OF	3,524	728	1097.07	7.89^{FG}	91.06
Pt2UW	1,226	367	727.57	7.58^{FG}	84.01
Pt3OF	5,031	743	1086.64	6.63^{DE}	93.76
Pt3UW	2,188	583	1169.58	8.08^{FG}	86.15
Pt4OF	2,576	654	975.19	7.82^{FG}	88.94
Pt4UW	7,669	1,629	3122.43	9.48^{H}	89.79

[a]Different letters after Shannon index values indicate significant differences ($P < 0.05$).
Abbreviations. A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

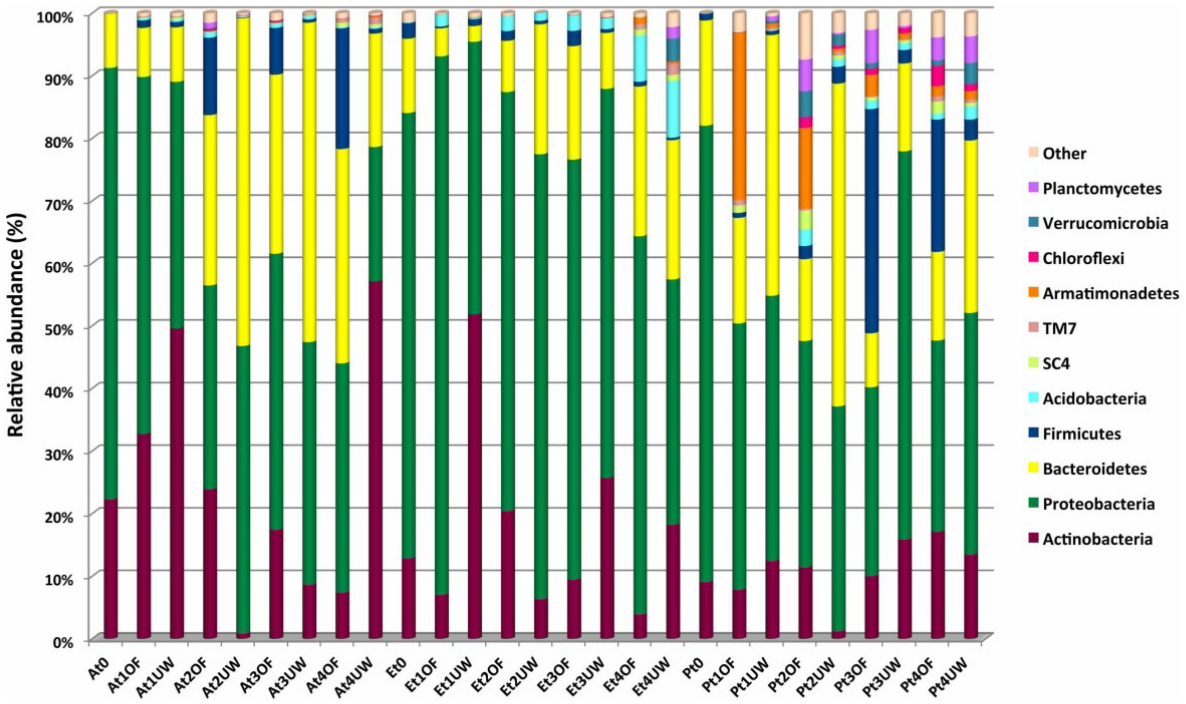

Figure 1 | Abundance of bacterial phyla in lignocellulosic biomasses during the biodegradation process. Only OTUs with an incidence >1% in at least two samples are shown. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

accounted for approximately 98%, 99% and 91% of the total biodiversity in *A. donax*, *E. camaldulensis* and *P. nigra*, respectively.

The native composition of the microbial community in the *A. donax* biomass was strongly dominated by *Proteobacteria* (69.03%), followed by *Actinobacteria* (22.23%) and *Bacteroidetes* (8.61%). During the biodegradation process, the microbial composition remained the same in terms of diversity but varied in terms of abundance. This pattern was primarily observed in the piles processed under the open field condition (Fig. 1). Interestingly, in this environment, an inverse correlation was observed between *Actinobacteria* and *Bacteroidetes*. In particular, *Actinobacteria* decreased from 32.70% after 45 days of degradation under the open field condition to 7.31% after 180 days ($P < 0.05$), whereas *Bacteroidetes* increased from 7.84% after 45 days to 34.30% after 180 days ($P < 0.05$). *Firmicutes* was very low at the initial time (0.02%) and remained roughly constant in the underwood pile (0.77% after 180 days of biodegradation). By contrast, in the open field pile, the relative abundance of this taxon gradually increased from 1.20% after 45 days of biodegradation to 19.28% after 180 days ($P < 0.05$). Minor phyla included *Acidobacteria* and candidate phyla SC4 and TM7 were recovered in all *A. donax* biomass samples, although at an incidence ≤ 1% (Fig. 1).

As in the *A. donax* biomass, *Proteobacteria* was the taxa that heavily dominated the native microbial community in *E. camaldulensis* and *P. nigra* (71.26% and 73.01%, respectively) ($P < 0.05$), remaining high during all degradation processes, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria* (Fig. 1). In the *E. camaldulensis* pile, *Acidobacteria* increased to 7.35% and 9.02% after 180 days of degradation under the open field and underwood conditions, respectively, whereas a slight variation was detected in the *P. nigra* samples. By contrast, the abundance of *Firmicutes* increased in the *P. nigra* pile after 135 (35.88%, $P < 0.05$) and 180 days (21.20%, $P < 0.05$) of degradation under the open field condition and was relatively stable in the *E. camaldulensis* pile (Fig. 1). Other taxa, including *Armatimonadetes*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, SC4 and TM7, were also detected. Interestingly, *Armatimonadetes* increased in the *P. nigra* pile after 45 days under the open field condition (26.70%, $P < 0.05$) and then gradually decreased during the degradation process to 1.63% (Fig. 1).

The microbial diversity was also analysed at a deeper taxonomic level. The identification of OTUs at the class level is reported in the heatmap shown in Fig. 2. *Actinobacteria*, *γ-Proteobacteria*, *β-Proteobacteria*, *α-Proteobacteria*, *Acidobacteria*, *Sphingobacteria*, *Flavobacteria* and *Bacilli* were recovered in all samples, with the exception of *Acidobacteria*, which was not detected in the *A. donax* biomass at the initial time. The abundance of *α-Proteobacteria* was high and exhibited the same variations during the biodegradation experiment in all three lignocellulosic biomasses. Interestingly, *γ-Proteobacteria* was the dominant taxa at the beginning of the experiment, with an abundance of 58.32%, 42.64% and 67.48% in the *A. donax*, *E. camaldulensis* and *P. nigra* samples, respectively; however, a dramatic reduction was recorded in all plant biomasses after 180 days (1.07%, 8.12% and 11.49%, respectively) (Fig. 2). A steady reduction in class *Actinobacteria* during degradation of the *A. donax* biomass under the open field condition was observed. By contrast, *Bacilli* showed an opposite trend, increasing from 0.02% at time zero to 19.26% after 180 days. Under the underwood condition, the middle phase of the biodegradation process was primarily dominated by *Sphingobacteria*, which showed an abundance of approximately 50%. Ever-increasing *Sphingobacteria* and *β-Proteobacteria* taxa abundance was recorded in the *E. camaldulensis* pile during the degradation process under the open field experiment. *Acidobacteria* showed a similar trend under both the open field and underwood conditions. The *P. nigra* biomass was characterised by an increase in the incidence of *Bacilli* and *β-Proteobacteria* during the biodegradation process under the open field conditions (Fig. 2). Other taxa were present at a very low incidence and with a great variability in the different

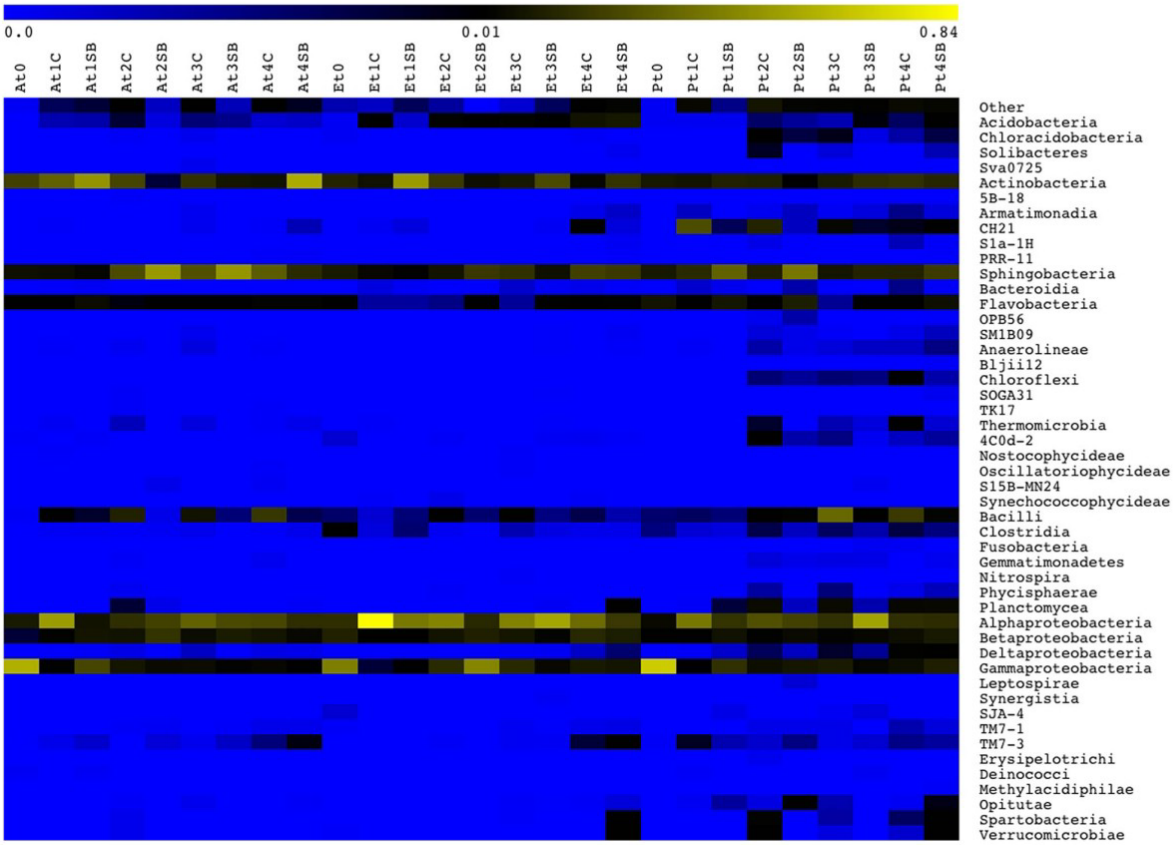

Figure 2 | Distribution of bacterial classes in lignocellulosic biomasses during the biodegradation process. Colour scale indicates the relative abundance of each OTU within the samples. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

samples. However, as shown in Fig. 2, the highest bacterial diversity was recorded in the *P. nigra* biomass. An interesting finding was the high incidence of the uncultured bacterium CH21 (26.45%) in the *P. nigra* pile during the first phase (after 45 days) and its constant decrease during the other phases of the degradation process in the open field. *Chloroflexi* was detected only in the *P. nigra* biomass and from 90 days to 180 days of biodegradation, showing an abundance ranging from 0.33% to 1.55%.

However, the PCoA of the weighted UniFrac community distances showed a marked difference between the native microbiota of the lignocellulosic biomasses and the microbiota in the analysed samples during the biodegradation process. The three samples of the chipped biomasses of *A. donax*, *E. camaldulensis* and *P. nigra* at harvest time clustered separately (Fig. 3). However, the microbial diversity seemed to be influenced by a correlation between the degradation condition and the biomass type. In fact, no difference was observed among the samples during the biodegradation process under the underwood condition (Fig. 3 panel a). By contrast, under the open field condition, *P. nigra* samples at different time points clustered separately from the other biomasses (Fig. 3, panel b). A similar trend was observed in the *A. donax* biomass, except for the samples collected after 45 days of degradation (Fig. 3, panel b). Moreover, the statistical ADONIS test showed that the composition of bacterial community in the different piles during biodegradation process was significantly influenced by lignocellulosic plant species ($P < 0.001$), by the degradation conditions ($P < 0.05$) and by sampling time ($P < 0.01$). This difference increased combining the three factors. In fact, the analysis performed with ADONIS found significant difference combining *lignocellulosic plant species* x *sampling time* ($P < 0.001$), *lignocellulosic plant species* x *degradation condition* ($P < 0.001$) and *degradation condition* x *sampling time* ($P < 0.01$).

Multienzymatic Screening and Identification of Isolated Cellulolytic Bacterial Strains. A culture-dependent approach enables the isolation of putative cellulolytic strains. A total of 366 aerobic endo- and exo-cellulolytic bacteria were isolated from samples of the *A. donax*, *E. camaldulensis*, and *P. nigra* biomasses. In particular, more than one hundred isolates were obtained from each pile, although the *P. nigra* biomass produced the highest number (127 isolates). In total, 95.9% of the isolates that were obtained from Avicel agar plates, exhibited exo-cellulase activity, whereas isolates with endo-cellulase activity on the CMC agar plates were detected at values less than 50%. To establish the number of putative multifunctional-degrading bacteria, all isolates were assayed for different enzymatic activities using the methods described below. Forty aerobic endo- and exo-cellulolytic strains that showed higher I_{CMC} values (> 10) were chosen for further characterisation. The polyphasic approach of identification resulted in bacterial isolates with different shapes, dimensions and, in some cases, spore presence (data not shown) and great biodiversity, as twenty genera and twenty-seven different species were found (Table 2). *Curtobacterium* spp. and *Bacillus* spp. were the most representative genera with the species *Curtobacterium flaccumfaciens* and

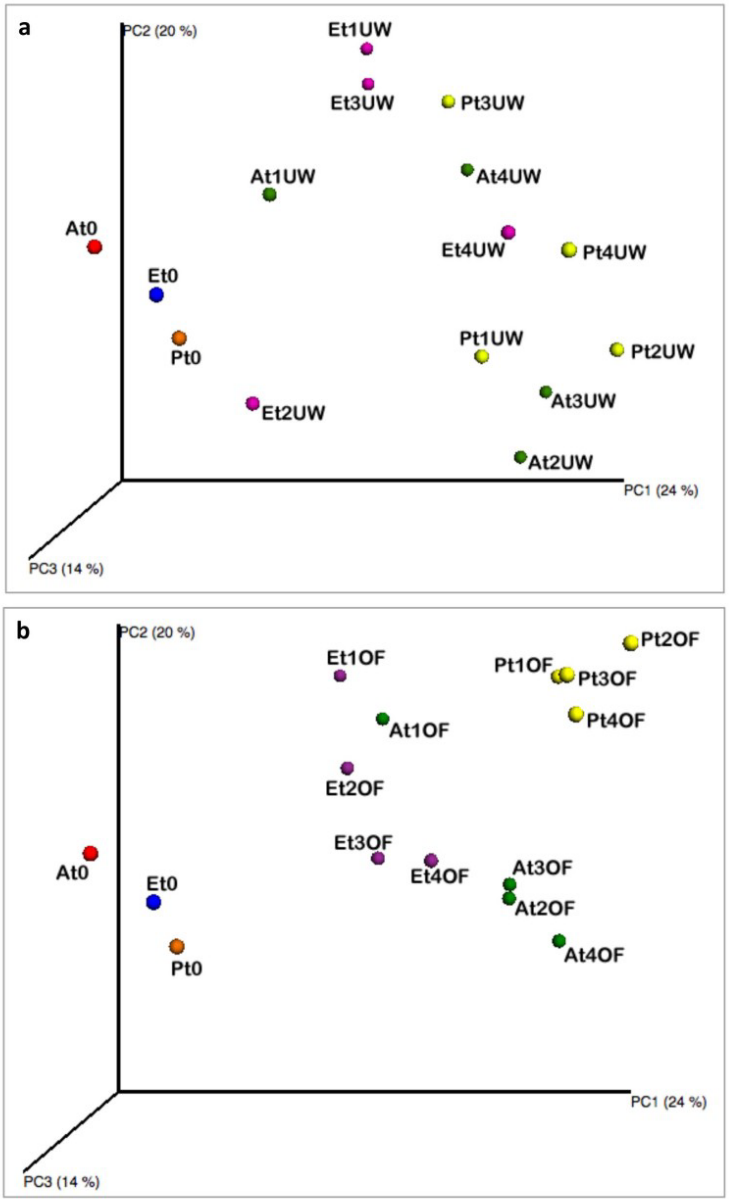

Figure 3 | Principal Coordinates Analysis of weighted UniFrac distances for 16S rRNA gene sequence data of lignocellulosic biomasses during the biodegradation process under the underwood (panel a) and open field (panel b) conditions. *Colour label.* red: *A. donax* at harvest time; blue: *E. camaldulensis* at harvest time; orange: *P. nigra* at harvest time; green: *A. donax*; violet: *E. camaldulensis*; yellow: *P. nigra*. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

Curtobacterium citreum as well as *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis*, respectively. The bacterial strains showed activities for three to six different enzymes. All strains showed endo- and exo-cellulolytic activities and, at same time, expressed cellobiase, xylanase and pectinase (Table 2). The highest endo-cellulase activity was observed in the CE86 strain, identified as *Promicromonospora sukumoe* (I_{CMC} = 34, $P < 0.05$), in *Isoptericola variabilis* CA84b (I_{CMC} = 32, $P < 0.05$) and *Staphylococcus warneri* CE83 (I_{CMC} = 32, $P < 0.05$). The first two strains were also able to degrade exo-cellulose, cellobiose and xylan. Moreover, *Isoptericola variabilis* CA84b produced the highest pectinase activity (I_{PEC} = 22, $P < 0.05$). *Curtobacterium citreum* CE711 and *Bacillus amyloliquefaciens* CA81 represented the most versatile strains because they possessed six different enzymatic activities. Thirty-two bacterial strains showed, in addition to endo- and exo-cellulases, cellobiase and xylanase activities that were missed in only 10% of the screened strains (*Staphylococcus warneri* CE83, *Lysobacter enzymogenes* CE710, *Lysobacter gummosus* CP72, *Mycobacterium frederiksbergense*

Table 2 | Identification and enzymatic activities of bacterial strains isolated from different lignocellulosic biomasses

Strain	Source	C[b]	A[b]	CE[c]	X[c]	P[a]	AZ[c]	AB[c]	L[c]	AD[c]	Identification (% identity)	Accession Number
CE86	*E. camaldulensis*	34 ± 1.0^{N}	+	++	+	0.0 ± 0.0^{A}	-	-	-	-	*Promicromonospora sukumoe* (99%)	KF057947
CA84b	*A. donax*	32 ± 0.0^{N}	+	++	+	22 ± 0.5^{FG}	-	-	-	-	*Isoptericola variabilis* (98%)	KF057948
CE83	*E. camaldulensis*	32 ± 1.0^{N}	+	+	-	0.0 ± 0.0^{A}	-	-	-	-	*Staphylococcus warneri* (100%)	KF057949
CP72	*P. nigra*	28 ± 1.0^{M}	+	-	++	0.0 ± 0.0^{A}	-	-	-	-	*Lysobacter gummosus* (99%)	KF040972
CP710b	*P. nigra*	28 ± 0.5^{M}	+	-	+	12 ± 0.5^{E}	-	-	-	-	*Mycobacterium frederiksbergense* (99%)	KF057950
CA817	*A. donax*	27 ± 0.5^{M}	+	+	+	2 ± 0.2^{B}	-	-	-	-	*Cellulosimicrobium cellulans* (99%)	KF040973
CA812	*A. donax*	27 ± 1.0^{M}	+	+++	+++	0.0 ± 0.0^{A}	-	-	-	-	*Bacillus amyloliquefaciens* (99%)	KF040974
SBP79	*P. nigra*	26 ± 0.5^{LM}	+	++	+	12 ± 0.0^{E}	-	-	-	-	*Promicromonospora citrea* (99%)	KF057951
SBA88	*A. donax*	26 ± 0.3^{LM}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Microbacterium lacus* (99%)	KF057952
SBE74	*E. camaldulensis*	24 ± 1.0^{II}	+	±	+	0.0 ± 0.0^{A}	-	-	-	-	*Enterobacter aerogenes/Kluyvera cryocrescens* (98%)	KF057960
CE77	*E. camaldulensis*	22 ± 1.0^{HI}	+	-	-	0.0 ± 0.0^{A}	++	-	-	-	*Novosphingobium resinovorum* (100%)	KF040976
CP81	*P. nigra*	22 ± 0.5^{HI}	+	+	+	3 ± 0.0^{C}	-	-	-	-	*Xanthomonas campestris* (100%)	KF040971
CP77	*P. nigra*	22 ± 0.9^{HI}	+	+	+	4 ± 0.0^{D}	-	-	-	-	*Xanthomonas orizae* (98%)	KF040975
CE75b	*E. camaldulensis*	22 ± 0.2^{HI}	+	++	+	0.0 ± 0.0^{A}	-	-	-	-	*Pediococcus acidilactici* (99%)	KF057953
CP77b	*P. nigra*	22 ± 0.5^{HI}	+	++	±	0.0 ± 0.0^{A}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF057954
SBA76	*A. donax*	22 ± 1.0^{HI}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Pantoea ananatis* (99%)	KF057955
SBP71	*P. nigra*	22 ± 1.0^{HI}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Schumannella luteola* (97%)	KF057956
CP78b	*P. nigra*	22 ± 0.0^{HI}	+	±	+	12 ± 0.0^{E}	-	-	-	-	*Mycobacterium frederiksbergense* (99%)	KF057957
CA816	*A. donax*	21 ± 0.5^{I}	+	+++	-	20 ± 1.0^{F}	-	-	-	-	*Bacillus subtilis subsp. subtilis* (99%)	KF040977
CA81	*A. donax*	20 ± 1.0^{GH}	+	+++	+++	0.0 ± 0.0^{A}	+	+	-	-	*Bacillus amyloliquefaciens* (99%)	KF040978
CA82	*A. donax*	20 ± 0.0^{GH}	+	+++	++	4 ± 0.2^{D}	-	-	-	-	*Bacillus licheniformis* (99%)	KF040979
CE84	*E. camaldulensis*	20 ± 0.5^{GH}	+	+	+++	0.0 ± 0.0^{A}	++	-	-	-	*Novosphingobium resinovorum* (99%)	KF040980
CA81b	*A. donax*	20 ± 1.0^{GH}	+	+++	++	4 ± 0.1^{D}	-	-	-	-	*Bacillus licheniformis* (99%)	KF040981
CE73b	*E. camaldulensis*	20 ± 0.9^{GH}	+	++	+	0.0 ± 0.0^{A}	-	-	-	-	*Pediococcus acidilactici* (99%)	KF057958
SBP73	*P. nigra*	20 ± 0.0^{GH}	+	-	±	0.0 ± 0.0^{A}	-	-	-	-	*Aurantimonas altamirensis* (99%)	KF057959
CE85	*E. camaldulensis*	18 ± 1.0^{FG}	+	++	++	2 ± 0.0^{B}	-	-	-	-	*Isoptericola variabilis* (98%)	KF040982
CE710	*E. camaldulensis*	18 ± 0.5^{FG}	+	-	++	2 ± 0.1^{B}	-	++	-	-	*Lysobacter enzymogenes* (99%)	KF040983
CP81b	*P. nigra*	18 ± 1.0^{FG}	+	++	++	0.0 ± 0.0^{A}	-	-	-	-	*Promicromonospora sukumoe* (99%)	KF040984
CA83	*A. donax*	17 ± 1.0^{EF}	+	++	+	2 ± 0.1^{B}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040985
CA77	*A. donax*	16 ± 0.5^{DEF}	+	+	+++	2 ± 0.0^{B}	-	-	-	-	*Sphingobacterium multivorum* (99%)	KF040986
CE75	*E. camaldulensis*	16 ± 1.0^{DEF}	+	++	+	2 ± 0.1^{B}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040987
CE73	*E. camaldulensis*	16 ± 0.5^{DEF}	+	+	-	0.0 ± 0.0^{A}	-	-	-	-	*Promicromonospora citrea* (99%)	KF040988
CA83b	*A. donax*	15 ± 0.3^{CDE}	+	++	++	0.0 ± 0.0^{A}	-	-	-	-	*Curtobacterium flaccumfaciens* (99%)	KF040989
CA84	*A. donax*	14 ± 0.5^{BCD}	+	+++	+	0.0 ± 0.0^{A}	-	-	-	-	*Curtobacterium citreum* (99%)	KF040990
CP713	*P. nigra*	14 ± 0.3^{BCD}	+	+++	++	20 ± 0.3^{F}	-	-	-	-	*Cellulomonas flavigena* (98%)	KF040991
CA818	*A. donax*	13 ± 1.0^{BC}	+	++	+	2 ± 0.0^{B}	-	-	-	-	*Microbacterium testaceum* (99%)	KF040992
CE78	*E. camaldulensis*	12 ± 0.0^{AB}	+	++	+	2 ± 0.3^{B}	-	-	-	-	*Curtobacterium citreum* (99%)	KF040993
CP710	*P. nigra*	12 ± 0.5^{AB}	+	+	+	0.0 ± 0.0^{A}	-	-	-	-	*Labedella gwakjiensis* (99%)	KF040994
CE711	*E. camaldulensis*	10 ± 0.9^{A}	+	++	+	2 ± 0.0^{B}	+	-	-	-	*Curtobacterium citreum* (99%)	KF040995
CP78	*P. nigra*	10 ± 0.5^{A}	+	+++	+	0.0 ± 0.0^{A}	-	-	-	-	*Raoultella terrigena* (99%)	KF040996

Enzymatic activities: C = endo-cellulase; A = exo-cellulase; CE = cellobiase; X = xylanase; P = pectinase; AZ = peroxidase; AB = laccase; L = ligninase with guaiacol and lignin alkali; AD = ligninase with guaiacol and *Arundo donax*; [a]I_{CMC} or I_{PEC} index, values represent the means ± SD of three replicates. Different letters after values indicate significant differences ($P<0.05$); [b]growth; [c]– negative; + low intensity; ++ middle intensity; +++ high intensity.

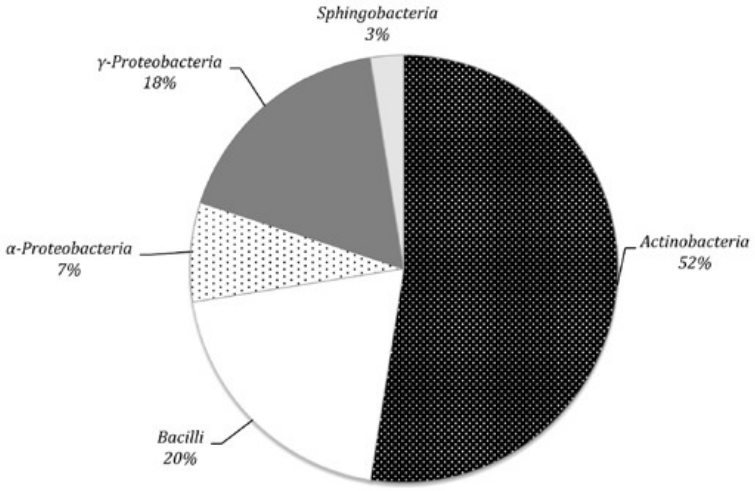

Figure 4 | Percentage composition of different phyla of eso- and endo-cellulolytic bacteria isolated from lignocellulosic biomasses on the basis of 16S rRNA gene sequence similarity.

CP710b, *Promicromonospora citrea* CE73, *Novosphingobium resinovorum* CE77, *Bacillus subtilis* subsp. *subtilis* CA816 and *Aurantimonas altamirensis* SBP73). All the strains belonging to the different *Bacillus* spp. and *Curtobacterium* spp. had the highest cellobiase activities. In particular, the genus *Curtobacterium*, represented by the species *Curtobacterium citreum* and *Curtobacterium flaccumfaciens*, was able to grow at least on four/five different substrates and, in one case, also showed peroxidase activity. In total, 87.5% of the bacterial strains produced xylanase (Table 2). Similar behaviour was observed for pectinase activity production as only 15% of the strains possessed an I_{PEC} from 12 to 22. Laccase and peroxidase activities were detected in five strains belonging to the species *Curtobacterium citreum* CE711, *Novosphingobium resinovorum* CE77 and CE84, *Lysobacter enzymogenes* CE710 and *Bacillus amyloliquefaciens* CA81. Lignin hydrolysis was not detected in any of the strains tested.

Phylogenetic Analysis of Selected Cellulolytic Bacteria. 16S rRNA gene sequences of the forty cellulolytic bacteria identified as described below were grouped by phylogenetic analysis into five different clusters, generating a *consensus* tree. The clusters were *Actinobacteria* (n=21; 52.5%), *Bacilli* (n=8; 20.0%), *α-Proteobacteria* (n=3; 7.5%), *γ-Proteobacteria* (n=7; 17.5%), *Sphingobacteria* (n=1; 4%) (Fig. 4). A phylogenetic tree was generated from the distance data using the Neighbour-Joining method with the Maximum Composite Likelihood model in a MEGA4 Program (Fig. 5). The nucleotide sequences of related type strains of different genera were included in the data set. High bootstrap values were observed and indicated significant branching points in the phylogenetic tree. Strains representative of the dominant class of *Actinobacteria* were placed in a cluster with bootstrap values higher than 52% and could be differentiated into nine subclusters of different genera. The bacterial strains primarily belonged to *Curtobacterium* (33.3%) and *Promicromonospora* (19.0%), whereas other genera (*Schumannella*, *Labedella*, *Cellulomonas*, *Cellulosimicrobium*, *Microbacterium*, *Isoptericola*, and *Mycobacterium*) were represented by one or two strains. The strains *Curtobacterium citreum* CE711, *Curtobacterium citreum* CA84 and *Curtobacterium citreum* CE78 showed a sequence similarity of 100%. Representatives of the *Bacilli* class were placed in three clusters of the genera *Bacillus* (62.5%), *Staphylococcus* (12.5%) and *Pediococcus* (25.0%). The strains belonging to *Bacillus licheniformis* CA82 and CA81b showed a similarity level of 100%, as did *Bacillus amyloliquefaciens* CA812 and CA81 and *Pediococcus acidilactici* CE75b and CE73b (Fig. 5). The *α-Proteobacteria* strains were placed in two different genera, in which two strains of *Novosphingobium resivorum* (CE77 and CE84) exhibited a sequence similarity of 100% with 100% of the bootstrap analysis. The other strain, belonging to *Aurantimonas altamirensis* (SBP73), formed a phylogenetically distinct cluster with a low 16S rRNA gene sequence similarity (60%) (Fig. 5). The representative strains of the *γ-Proteobacteria* cluster belonged to the genera *Xanthomonas* and *Lysobacter* and included the species *Xanthomonas campestris*, *Xanthomonas oryzae*, *Lysobacter gummosus*, and *Lysobacter enzymogenes*. The other genera, identified as the *Enterobacter aerogenes/Klebsiella cryocrescens* group, *Raoultella* and *Pantoea*, were represented by one strain. The strain *Sphingobacterium multivorum* CA77 formed a phylogenetically distinct cluster with the type strain of the respective species with 100% of the bootstrap analysis (Fig. 5).

Screening of Cellulolytic Microorganisms in Liquid Medium. Sixteen bacterial strains were selected based on at least one of these characteristics: higher halo dimension on CMC agar, multienzymatic activities, high pectinase activity or high peroxidase activity, and no pathogenicity for humans; these strains were submitted to a quantitative CMCase assay. All the analysed strains achieving the maximum AZO-CMCase level of production between the 8th and 26th hours, corresponding to the exponential and stationary phases of growth, respectively (data not shown). In Table 3, the values of the maximum AZO-CMCase activity measured for each strain and the corresponding time of production are reported. *Schumannella luteola* SBP71 showed the highest AZO-CMCase activity, at 0.33 ± 0.09 U mL^{-1}, although no significant differences were found with other strains. In particular, high activity levels ranging from 0.20 ± 0.06 to 0.32 ± 0.08 U mL^{-1} were also found for *Pediococcus acidilactici* CE75b, *Curtobacterium citreum* CA84, *Curtobacterium flaccumfaciens* CA83, *Cellulomonas flavigena* CP713 and *Pantoea ananatis* SBA76a (Table 3). *Mycobacterium frederiksbergense* CP78b, *Curtobacterium flaccumfaciens* CP77b and *Promicromonospora citrea* SBP79 (with a maximum value of 0.18 ± 0.02 U mL^{-1} and 0.16 ± 0.02 U mL^{-1}) exhibited less AZO-CMCase activity than the microorganisms mentioned above but more than *Cellulosimicrobium cellulans* CA817 (0.15 ± 0.01 U mL^{-1}) and *Isoptericola variabilis* CA84b (0.13 ± 0.01 U mL^{-1}). All other bacterial strains tested showed values lower than 0.10 U mL^{-1}.

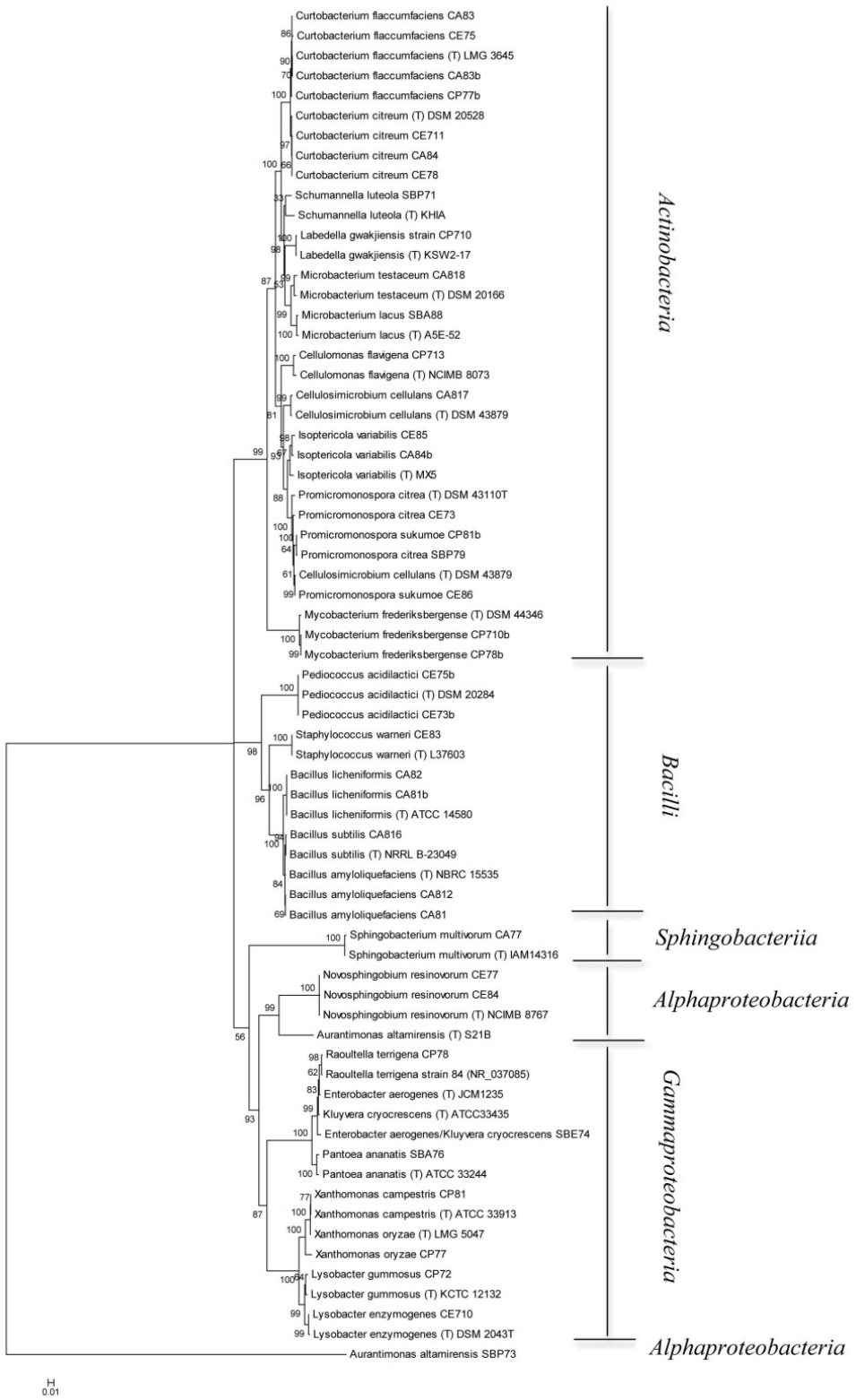

Figure 5 | Neighbour-Joining tree based on the comparison of 16S rRNA gene sequences showing the relationships among cellulolytic strains. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. Strains marked with "(T)" represent type strains. The *scale bar* estimates the number of substitutions per site.

Table 3 | Maximum value of Azo-CMCase activity measured for each strain and the corresponding time of production

Bacterial strains	Maximum value of AZO-CMCase activity[a] (U mL^{-1})	Time (h)
Schumannella luteola SBP71	0.33 ± 0.09^{F}	12
Pediococcus acidilactici CE75b	0.32 ± 0.10^{EF}	12
Curtobacterium citreum CA84	$0.29 \pm 0.01^{D-F}$	17
Curtobacterium flaccumfaciens CA83	$0.24 \pm 0.03^{C-F}$	15
Cellulomonas flavigena CP713	$0.22 \pm 0.10^{B-F}$	20
Pantoea ananatis SBA76a	$0.20 \pm 0.06^{A-F}$	8
Mycobacterium frederiksbergense CP78b	$0.18 \pm 0.02^{A-E}$	26
Curtobacterium flaccumfaciens CP77b	$0.16 \pm 0.02^{A-D}$	20
Promicromonospora citrea SBP79	$0.16 \pm 0.02^{A-D}$	12
Cellulosimicrobium cellulans CA817	$0.15 \pm 0.01^{A-D}$	20
Isoptericola variabilis CA84b	$0.13 \pm 0.01^{A-C}$	12
Bacillus subtilis subsp. *subtilis* CA816	0.09 ± 0.00^{AB}	12
Microbacterium testaceum CA818	0.09 ± 0.01^{AB}	17
Curtobacterium flaccumfaciens CA83b	0.08 ± 0.03^{AB}	12
Promicromonospora sukumoe CE86	0.07 ± 0.02^{A}	14
Bacillus licheniformis CA82	0.07 ± 0.00^{A}	15

[a]The values represent the means ± SD of three replicates of three independent experiments. Different letters after the values indicate significant differences ($P < 0.05$).

Discussion

In recent years, the competitive production of alternative renewable biofuels has stimulated research into new bacteria as a source of highly active and specific cellulases. They exhibit several advantages such as a fast growth rate, production of enzymes that are often more effective catalysts due to less feedback inhibition, and secretion of a complete multi-enzyme system for an efficient conversion of lignocelluloses into fermentable sugars[11,14]. In this context, particular attention must be given to exploring the biodiversity of natural niches so that cellulase-producing bacteria can be isolated and characterised. For these reasons, in this work, the microbial diversity of natural ecosystems, represented by lignocellulosic biomasses of *A. donax*, *E. camaldulensis* and *P. nigra*, was evaluated by culture-independent and culture-dependent approaches.

A highly complex bacterial community was found, in which the most frequently occurring bacteria were those belonging to the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* phyla. *Proteobacteria* was the most abundant taxa recovered in the *E. camaldulensis* and *P. nigra* piles, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria*. These taxa are related to microorganisms previously characterised as biomass degraders. The biodiversity of the microbial community in our study corresponded well with a previous study in which *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, along with members of the class *Proteobacteria*, comprised 83% of the microbial richness and heavily dominated switchgrass-adapted communities[15]. Moreover, bacterial species belonging to *Proteobacteria* and *Acidobacteria*, together with *Firmicutes* (*Clostridium* and *Bacillus* genera) and followed by *Bacteroidetes*, *Chlamydiae/Verrucomicrobia* and *Actinobacteria* (mainly Streptomyces), are known as the major plant biomass-degrading microbes in peat swamp forests[16]. During the natural biodegradation process of plant substrates, the indigenous bacterial community would initially have grown by utilising the more accessible cellulose and hemicellulose and only later would use the more resilient lignin component[17]. With regard to lignin decomposition, *Actinobacteria*, *Firmicutes* and *Acidobacteria* are the major taxa involved in this process[18]. *Acidobacteria* was recovered in the late stage of our experiment and its abundance increased during the biodegradation process, especially in the *E. camaldulensis* piles. *Firmicutes* showed a similar trend in the *A. donax* and *P. nigra* biomasses, with the relative abundance of this taxon gradually increasing in the piles processed under the open field condition. Wu and He[19] reported that *Firmicutes* could be the main microbes for lignin depolymerisation since a dominance of this phylum was recovered in enriched microbial consortia using a medium with lignin. Moreover, this phylum is common in natural processes such as rice straw compost[20] and decaying wood[21], suggesting its importance in the degradation of lignocellulolytic materials.

Analysing the microbial diversity more deeply, *Actinobacteria*, *γ-Proteobacteria*, *β-Proteobacteria*, *α-Proteobacteria*, *Acidobacteria*, *Sphingobacteria*, *Flavobacteria*, *Bacilli* and *Acidobacteria* were recovered in all samples. In particular, *Actinobacteria*, *α-Proteobacteria*, *Sphingobacteria* and *β-Proteobacteria*, all potent plant polysaccharide-degrading microbes that play an important role in plant biomass degradation in the tropical peat swamp forest ecosystem[16], were the most abundant taxa during the biodegradation process in all lignocellulosic piles.

Different bacteria belonging to the *Actinobacteria* class are involved in complex glycoside degradation such as chitin and cellulose and are fundamental in lignin and polyphenol degradation[22]. Martins and coworkers[23] reported that biomass degradation in the composting process, including the deconstruction of recalcitrant lignocellulose, is fully performed by bacterial enzymes, most likely by members of the *Clostridiales* and *Actinomycetales* orders. *β-Proteobacteria* and *α-Proteobacteria* were also recovered in our study. According to Castillo et al.[22], the dominance of members of the phylum *Proteobacteria* such as *γ-Proteobacteria* is observed only at the beginning of the biodegradation process, and its strong reduction during the experiment could be due to its involvement in lignocellulosic waste declining during the early stages of the process. In the open field experiment, *Bacilli* increased in both the *A. donax* and *P. nigra* biomasses. Members belonging to this taxon are known to have specific genes encoding enzymes involved in cellulose and hemicellulose degradation[24,25].

The constant increase in the relative abundance of *Sphingobacteria* and *Acidobacteria* recorded in the *E. camaldulensis* pile during the degradation process in the open field experiment suggests that these species play a role in the decomposition of lignocellulosic material. Kanokratana et al.[26], using complementary shotgun pyrosequencing, identified different genes encoding glycosyl hydrolases targeting cellulose and hemicellulose degradation in a bagasse pile, most of which were found in orders *Clostridiales*, *Bacteroidales*, *Sphingobacteriales* and *Cytophagales*. Moreover, the *Acidobactria* taxon is able to a use a diversity of carbon sources, from simple sugars to complex plant biomass substrates[26].

Another adapted-lignocellulosic taxon was *β-Proteobacteria*, which increased in the *E. camaldulensis* and *P. nigra* piles. In recent work, Stursova et al.[27] identified *β-Proteobacteria*, *Bacteroidetes* and *Acidobacteria* as the primary cellulose decomposers in forest litter.

An interesting finding was the high incidence of the uncultured bacterium CH21 in the *P. nigra* pile during the first phase of the

degradation process in the open field. CH21 is a member of the phylum *Armatimonadetes*, formerly called candidate division OP10[28]. The phylum *Armatimonadetes* is very poorly studied and its phylogeny is still poorly defined[29]. Members of this phylum are detected in different ecosystems and they are phylogenetically different. This phylum includes species with a wide variety of metabolic potentials[30]. Wang and co-workers[31] reported that their prevalence in plant-fed anaerobic bioreactors indicates a role in degradation of plant material. In our research, CH21 is the only recovered member of this phylum. Since it's relative abundance increased in the *Populus nigra* pile after 45 days and decreased during the other phases of the degradation process in the open field, it is possible its involving in the biodegradation of this specific lignocellulosic biomass during the first phase of the process.

The differing trends observed in this study in terms of taxa abundance during the biodegradation process and between the vegetable species used demonstrated a local selective pressure in the lignocellulosic ecosystems.

The culture-dependent methodology used here, which was based on a functional approach of detection and isolation to find new lignocellulose-degrading bacterial strains, provides us with key insight. Special attention to the methodology was required to determine the optimal culture and assay conditions. A comparison with another study[11] revealed that differential substrates containing CMC and Avicel are effective for the enumeration and isolation of putative colonies of cellulolytic microorganisms[32]. According to Soares et al.[6], exo- and endo-cellulolytic bacterial isolates are found at different frequencies, and the number of microorganisms that were able to grow on Avicel as the sole carbon source was high. The cellulolytic strains isolated from the biomasses showed multienzymatic activities useful to perform the hydrolysis of a complex substrate such as lignocellulose, an important initial step in many technological applications[33] that require the action of different specific enzymes. All forty bacterial strains submitted to the multienzymatic screening showed both endo- and exo-glucanase activities, confirming that these enzymes act synergistically during the saccharification of celluloses. These observations were reinforced by the fact that many of these bacterial strains also possessed β-cellobiase as well as cellulolytic activity. Xylanase activity was also commonly observed, which is unsurprising because a close correlation between cellulase and xylanase activities has been demonstrated and is due to their coexpression in the same operon.

The Neighbour-Joining phylogenetic method generated a consensus tree that grouped all 16S rRNA gene sequences of the isolated strains into five different clusters at the class level. Culture-dependent data showed similar predominant bacterial classes detected by high-throughput sequencing in the lignocellulosic biomasses, with an abundance of *Actinobacteria*, *Bacilli*, *α-Proteobacteria*, *γ-Proteobacteria* and *Sphingobacteria*. Moreover, the bacteria isolated are dominant players since these microbial strains were isolated from a high serial decimal dilutions (10^{-6}–10^{-7}). According to phylogenetic research on cellulose-decomposing bacteria isolated from soil carried out by Ulrich et al.[34], *Actinobacteria* are the most prevalent bacterial group based on 16S rRNA gene sequences. This cluster included the species *Curtobacterium citreum*, which is able to use up to six different lignocellulose components and that is phylogenetically related to *Microbacterium testaceum* and *Microbacterium lacus*. In particular, previous studies reported the production of enzymes involved in cellulose and xylan degradation by *Microbacterium* species[35]. The *Actinobacteria* cluster included other genera involved in lignocellulosic biomass degradation, such as *Cellulomonas*. Akasaka et al.[36] reported that more than 60% of isolates from rice plant residues was closely related to *Cellulomonas* and involved in their degradation. Our study revealed the production of cellobiase and pectinase activities in *Actinobacteria* members, such as *Cellulosimicrobium cellulans*, *Isoptericola variabilis*, *Promicromonospora sukumoe* and *Promicromonospora citrea*, which belong to the suborder *Micrococcineae*. Many of the representatives of the *Promicrosporaceae* and *Corinebacteriaceae* families can degrade polysaccharides such as cellulose and xylan[37,38]. The *Bacilli* cluster was primarily represented by the *Pediococcus* and *Bacillus* genera on the basis of 16S rRNA gene sequence analysis. Interesting, Zhao and co-workers[39] reported the use of a strain of *Pediococcus acidilactici* with high tolerance to temperature and a lignocellulose-derived inhibitor in simultaneous saccharification and fermentation (SSF) for high lignocellulosic lactic acid production. The strains CA812, CA81, CA816, CA82 and CA81b, isolated from the *A. donax* biomass and phylogenetically correlated to the *Bacillus* genus, showed high endo-cellulolytic and multi-enzymatic activities. The capacity of *Bacillus* strains to produce large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers isolated from compost, soil and several other natural habitats[40]. In particular, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus licheniformis*, isolated from soil and compost, are able to hydrolyse cellulosic waste-material[8] by both cellulolytic activities[41] and multi-enzyme complexes[42]. The selected bacterial strains could have cross-specificity facilitated by specific or non-specific active sites and also distinct catalytic domains binding to different substrates[33]. *Xanthomonas campestris*, known as a phytopathogenic bacterium, and *Lysobacter gummosus* and *Lysobacter enzymogenes*, potent biocontrol agents that release cellulolytic enzymes such as glucanase[43], were identified in the phylogenetic group of *γ-Proteobacteria*. Other strains of this class, such as *Enterobacter* sp. and *Pantoea* sp., although are known as insect-associated bacteria that are able to produce bioactive compounds and digestive enzymes that are responsible for lignocellulose degradation[44], they are less attractive for possible biotechnological application since human disease has been reported to be caused by these bacteria as well as *Raoultella terrigena* and *Sphingobacterium multivorum*.

The two strains CE77 and CE84, included in the *α-Proteobacteria* cluster and characterised for their peroxidase activity, were closely related to the species *Novosphingobium resinovorum*, previously isolated from soil and studied for its capacity to degrade oil resins[45]. Interestingly, the strain *Aurantimonas altamirensis* SBP73 showed multienzymatic activity. To our knowledge, this study is the first to report the multienzymatic activity of this specie.

The bacterial strains screened in liquid medium showed enzymatic activity levels similar to the values reported in previous studies[46]. For example, Ekperigin[47] reported that the maximum activity of the cellulose-degrading enzyme determined for CMC in the culture supernatant of *Branhamella* spp. was 0.34 U mL^{-1}. Even though comparing cellulase production across studies is difficult because there are too many differences in the production of extracellular enzymes (e.g., media composition, fermentation conditions and raw materials), the results demonstrated that the bacterial strains isolated and characterised in this study could represent a very interesting biological source for the conversion of lignocellulose carbohydrates into products of commercial significance. Moreover, their biotechnological performance could be enhanced by modifying the biotechnological parameters of the fermentation process such as the temperature[33].

In conclusion, in this work, pyrosequencing-based technology increased our knowledge of lignocellulosic-adapted microbiota and the microbial dynamic during the degradation of three different biomasses under natural conditions. The dominant taxa found during the biodegradation process were members of classes *Actinobacteria*, *γ-Proteobacteria*, *α-Proteobacteria* and *Sphingobacteria*. However, the abundance of the major and minor taxa retrieved during the process was determined by selective pressure employed by the lignocellulosic plant species and the degradation conditions.

In addition, new multifunctional degrading bacteria have been selected and identified that are potential producers of multiple enzymes that have synergistic actions on cellulose and hemicellulose.

This step is fundamental for biotechnological applications of interest to industry because they constitute a microbial source of new multi-functional enzymes that can increase the efficiency of the hydrolysis of lignocellulosic biomass into fermentable sugars for biofuel eco-technology.

Methods

Lignocellulosic Biomasses and Sampling. Chipped wood from *A. donax*, *E. camaldulensis* and *P. nigra* was processed for biodegradation under natural conditions for 180 days. The chipped wood was used to form two piles of approximately 30 kg (length 0.85 m, width 0.85 m and height 0.70 m) for each plant species; on March 22, 2012, the piles were placed under two different environmental conditions in order to increase microbial biodiversity: open field conditions, in which the biomass piles were established on agronomic soil without any coverage shady trees, in the experimental station of Department of Agriculture (Naples, Italy; 40°48′50.1″N, 14°20′48.2″E); the underwood conditions performed reproducing the natural environment that usually arise in a forest since the piles were placed under oak trees in the woodland at the Department of Agriculture (Naples, Italy; 40°48′47.8″N, 14°20′50.4″E). Samples of 0.5 kg were collected from the external part (right and left side of the pile) and the internal central part of the biomass immediately after preparation (T0) and at 45, 90, 135 and 180 days (T1, T2, T3 and T4, respectively) of biodegradation. During the process, temperature (°C) was measured by using specific temperature sensors (VWR International PBI, Milan, Italy) placed directly in the core of the piles at a depth of 30–40 cm. The water activity (a_w, water readily available for microbial metabolic activities) was also monitored using a HygroPalm23-AW (Rotronic AG, Basserdorf, Germany).

Analysis of the Microbiota by High-Throughput Sequencing of the 16S rRNA Gene. Total DNA recovery from microorganisms adherent to the plant biomass was performed as previously described[48]. The bacterial diversity was evaluated by pyrosequencing using the primers Gray28F (5′-TTTGATCNTGGCTCAG-3′) and Gray519r (5′-GTNTTACNGCGGCKGCTG-3′) spanning the V1-V3 region of the 16S rDNA of *E. coli*[58]. 454-adaptors were included in the forward primer followed by a 10-bp sample-specific Multiplex Identifier (MID). The PCR mixture (50-µl total volume) included 20 ng of target DNA, 1 x *Taq* DNA polymerase buffer (Invitrogen, Milano, Italy), 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 µM of each primer and 2.5 U of *Taq* DNA polymerase (Invitrogen). PCR amplification was performed as previously reported[59]. After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and quantified using the QuantiFluor™ (Promega, Milano, Italy), and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions using Titanium chemistry.

Bioinformatics and Data Analysis. The pyrosequencing data were filtered using the following quality check parameters using the QIIME software package[51]: a minimum sequence length of 200 bp, maximum homopolymer number of 5, minimum quality score of 25, and maximum number of ambiguous bases and primer mismatches of 0. The QIIME software was also used to denoise, split sequences into the proper samples, and pick operational taxonomy units (OTUs) at 97% sequence identity using UCLUST[52]. The representative sequences were submitted to the RDPII classifier[53] to obtain a taxonomy assignment using the Greengenes 16S rRNA gene database[54].

Alpha diversity was evaluated by rarefaction curves, Good's coverage, Chao1 richness[55] and Shannon diversity indices[56]. To test for significant differences in alpha diversity, QIIME's compare_alpha_diversity.py script was used to run nonparametric two-sample *t*-tests. The default number of Monte Carlo permutations (999) was used to calculate *P*-values in the nonparametric *t*-tests, and a significance threshold of $P < 0.05$ was used. Statistical tests for calculating the influence of sample variables on microbial ecology were applied using QIIME's group_significance.py (ANOVA) and compare_categories.py (ADONIS) scripts. The OTU taxonomy tables generated by QIIME were used to produce heatmaps using the software TMeV v 4.8[57]. Beta diversity was also evaluated by UniFrac[58], and principal coordinate analysis (PCoA) was generated by QIIME. Unweighted UniFrac distances were also calculated, but we only report weighted distances here because sample clustering was more informative.

Isolation of Cellulolytic Functional Groups in the Lignocellulosic Biomasses. Different samples were collected from the chipped vegetable biomass piles of *A. donax*, *E. camaldulensis* and *P. nigra* for the isolation of aerobic cellulolytic bacteria. Serial dilutions (0.1 mL) of the samples were spread on the surface of plates containing basal medium (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 element solution and 15 g L^{-1} agar bacteriological, pH 7.0) with 5 g L^{-1}Avicel (Sigma-Aldrich, Milan, Italy) or 5 g L^{-1} carboxymethylcellulose (CMC) with 0.1% Congo red (Sigma-Aldrich) for exo-cellulolytic or endo-cellulolytic differential isolation, respectively. After 4 days under aerobic conditions at 28°C, the endo-cellulolytic colonies were differentially visualised by a clear halo on CMC. All colonies grown on the substrate Avicel as only carbon source were considered as exo-cellulolytic bacteria. Morphologically different exo- and endo-cellulolytic colonies were isolated, purified again on the same isolation media and stored at 4°C until their characterisation.

Screening for Functional Activities on Solid Media. The endo-cellulolytic activity of all isolates was measured on CMC by a semi-quantitative agar spot method. After adjusting the turbidity of the bacterial suspensions (0.5 of McFarland Turbidity Standard corresponding to approximately 1.5×10^8 CFU mL^{-1}), the cells were spotted on CMC agar medium in triplicate. After a 4-day incubation at 28°C, the plates were stained with 0.1% Congo red solution for 30 min followed by washing with 5 M NaCl[8,16]. Cellulase activities on screening media were recorded as the "Indices of Relative Enzyme Activity, I_{CMC}= diameter of clearing or halo zone/colony diameter"[11]. The exo-cellulase activity was similarly estimated in a semi-quantitative way by inoculating all the isolates by spotting on the Avicel agar and observing the development of bacterial colonies after incubation at 28°C for 10 days.

All isolates were also assayed for cellobiase, xylanase, pectinase and ligninase activities. In particular, cellobiase activity was assessed with cellobiose agar (10 g L^{-1} bacteriological peptone, 15 g L^{-1} agar bacteriological [Oxoid, Milan, Italy], 7.5 g L^{-1} cellobiose and 0.06 g L^{-1} bromothymol blue [Sigma-Aldrich]). After incubation at 28°C for 3 days, the cellobiase activity was detected by observing yellow haloes around the colonies. For xylanase detection, the method described by Ko et al.[59] was applied, adding 0.05% Remazol brilliant blue-R and a 0.5% solution of sonicated (FALC Instruments, HK3300) xylan (Sigma-Aldrich) to the LB Agar medium (Oxoid). After 3–5 days of incubation at 28°C, the hydrolysis of xylan was detected by observing a clear zone around the colonies. Pectinase activity was measured following the method described by Pepe et al.[60] and the results were recorded as the "Indices of Relative Enzyme Activity, (I_{PEC}) = diameter of clearing or halo zone/colony diameter".

Ligninase activity was detected by using the CGA culture medium described by Okino et al.[61], modified by replacing the sugarcane bagasse lignin with vegetable biomass powder from *A. donax* or 2 g L^{-1} lignin alkali (Sigma-Aldrich). A reddish-brown halo around a colony indicates the successful oxidation of guaiacol by lignin-olytic enzymes. Laccase activity was determined for all the isolated strains by growth on agar medium composed of liquid basal medium (LBM) supplemented with 0.1% 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid [ABTS], Sigma-Aldrich) as described by Toutella et al.[62]. After 10 days of incubation at 25°C in darkness, a green colour in the medium indicated the presence of laccase activity. Azure-B (Sigma-Aldrich) agar was used to evaluate the production of lignin peroxidase and man-ganese-dependent peroxidase by the formation of a clear halo around a colony after 10–30 days of incubation at 25°C in darkness[63].

Identification and Phylogenetic Analysis of Cellulolytic Strains. Bacterial isolates were identified by molecular method (16S rRNA gene sequencing). Total genomic DNA was extracted and purified by InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's recommendations. Approximately 50 ng of DNA was used as the template for PCR assays. Synthetic oligonucleotide primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) were used to amplify the 16S rRNA gene. The PCR mixture was prepared as reported by Alfonzo et al.[64]. The PCR conditions were performed as described by Pepe et al.[65]. The PCR products, after visualisation by agarose (1.5% wt/vol) gel electrophoresis at 100 V for 1 h, were purified by using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequences were analysed as previously reported[61] and compared with the GenBank nucleotide data library using Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Multiple nucleotide alignments of nearly full-length 16S rRNA sequences of isolated strains and type strains within each of the defined species were performed using the Clustal W program[66] from MEGA version 4.0[67]. The nucleotide sequences of the type strains were retrieved from the Ribosomal Database Project (RDP - http://rdp.cme.msu.edu/). The phylogenetic tree was inferred by using the Neighbour-Joining method with the Maximum Composite Likelihood model in MEGA4 program with bootstrap values based on 1,000 replications.

Screening of Cellulolytic Activity in Liquid Media. The bacterial strains were pre-inoculated by dissolving a single colony in 3 mL of liquid medium containing 0.5% CMC, 0.7% yeast extract, 4 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 0.004 g L^{-1} $FeSO_4 \cdot 7H_2O$. After overnight incubation at 37°C, a volume of the broth culture corresponding to 0.1 O.D. was used to inoculate 100-mL plugged Erlenmeyer flasks, each containing 20 mL of the same medium. During incubation at 37°C on a rotary shaker at 225 rpm, samples of the liquid culture were withdrawn and used to measure the optical density ($O.D._{600nm}$) and the extracellular *endo*-1,4-ß-glucanase activity by AZO-CMCase assay (Megazyme, Ireland), following the supplier's instructions. The analytical determinations correspond to the mean value of three replicates.

Accession Numbers. The 16S rRNA gene sequences obtained were deposited in the GenBank nucleotide database under accession numbers from KF040971 to KF040996 and from KF057947 to KF057960 (http://www.ncbi.nlm.nih.gov/Blast.cgi).

The pyrosequencing data are available in the Sequence Read Archive database of the National Center of Biotechnology Information (PRJNA248067).

Statistical Analyses. One-way ANOVA followed by Tukey's HSD *post hoc* for pair-wise comparison of means (at $P < 0.05$) was used to assess the difference in the

enzymatic activities of isolated strains such as I_{CMC}, I_{PEC} and Azo-CMCase. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

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Author contributions

V.V. wrote the main manuscript text and, in particular, microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing and Phylogenetic analysis of selected cellulolytic bacteria. She prepared Figure 1, 2, 3, 4, 5 and table 1. A.Aliberti and A.R. wrote the isolation and identification of cellulolytic bacterial strains by culture-dependent approach and selection by multienzymatic screening. They prepared Table 2. V.F., S.G. and A.Amore wrote the screening of cellulolytic microorganisms in liquid medium and prepared Table 3. D.E. wrote the microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing. M.F. wrote lignocellulosic biomasses and sampling. O.P. is the corresponding author. All authors reviewed the manuscript.

Additional information

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