DEVELOPMENT OF NEW BIOCATALYSTS FOR THE HYDROLYSIS OF LIGNOCELLULOSIC SUBSTRATES

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To Sarah

TABLE OF CONTENTS

Abstract

The industrial conversion of lignocellulosic biomasses into second-generation biofuels or other high-added-value products includes a saccharification step to hydrolyze the polysaccharides into fermentable sugars carried out by means of an enzymatic cocktail including cellulases. The production costs of these enzymes represent the main contributor to the expense of the overall process and a huge amount of enzymes is required for the hydrolysis. Therefore, the production of new cellulolytic enzymes more efficient in lignocellulose conversion represents one of the main routes to contribute to the cost reduction of biomass conversion. In this context the aim of this PhD project has been to develop biocatalysts for the enhanced hydrolysis of cellulose into monosaccharides, particularly for spent mushroom substrate supplemented with wheat straw. Two new GH5 family cellulases (Cel1 and Cel2) have been cloned, recombinantly expressed in *Escherichia coli*, characterized and tested in the enzymatic hydrolysis of three pretreated biomasses *Populus nigra, Panicum virgatum* and spent mushroom substrate (SMS) supplemented with wheat straw (WS). Based on these results, Cel2 was selected for the development of improved biocatalysts by directed evolution. A library of 30,000 random mutants was generated and screened for their activity. The diversity was introduced into the *cel2* gene by error-prone Polymerase Chain Reaction and the screening on solid and liquid medium was set up and applied for all the mutants. The improved variants were cultured in a flask and were characterized. The thermoresistant mutants were then tested in the enzymatic hydrolysis of pretreated SMS/WS in conjunction with a commercial enzymatic hydrolysis performance booster mixture (MetZyme® SUNO™ BOOSTER 144), allowing us to obtain improved biocatalysts compared to the wild type Cel2. Moreover, the selection of a lytic polysaccharide monooxygenase was performed in order to set up a recombinant expression system, considering the capability of these enzymes to enhance the bioconversion of lignocellulosic substrates when added to the enzymatic cocktail.

Riassunto

Nel corso del XX secolo, lo sviluppo tecnologico ha apportato da un lato grandi benefici nella vita di tutti i giorni e dall'altro effetti dannosi sulla salute umana e sull'ambiente oltre che una riduzione delle fonti fossili disponibili (Jones, K. 2017). In questo contesto nasce una nuova area della chimica, la "chimica verde". La United States Environmental Protection Agency (EPA) ha definito la chimica verde come "*la progettazione di prodotti e processi chimici che riducono o eliminano l'uso o la generazione di sostanze pericolose*" (http://www.epa.gov/laws-regulations/ summary - pollution-prevenzione-act). Più in generale, la chimica verde può essere considerata un'area di chimica e ingegneria chimica focalizzata su approcci tecnologici per prevenire l'inquinamento e ridurre il consumo di risorse non rinnovabili (Cernansky, 2015). Questo può essere ottenuto con lo sviluppo di processi ad alta efficienza energetica che siano coerenti con i dodici principi della chimica verde introdotti da Anastas e Warner nel 1998 (Anastas, 1998) e l'utilizzo di materie prime rinnovabili, sostenibili, efficienti, economiche, convenienti e sicure, riducendo così la dipendenza dai combustibili fossili (Prasad et al. 2007).
Le biotecnologie assumono un ruolo fondamentale nello sviluppo di questi processi attraverso biotrasformazioni e fermentazioni utilizzando rispettivamente enzimi e microrganismi (Liguori et al. 2013).

Biomasse lignocellulosiche

La biomassa lignocellulosica, derivata dalle pareti delle cellule vegetali, è la materia prima più abbondante sulla Terra ed è composta principalmente da zuccheri, quindi è una fonte rinnovabile ed economica per la produzione di bioprodotti, come etanolo, additivi alimentari, zuccheri fermentabili, biopolimeri, enzimi (Maitan-Alfenas et al.2015; Capolupo & Faraco, 2016; Mezule et al.2015). Queste biomasse possono essere fornite da colture energetiche dedicate, che non competono con la coltivazione di alimenti e mangimi (Brethauer & Studer, 2015; Liguori et al. 2016; Ventorino et al. 2015) o da rifiuti agricoli, agroindustriali e alimentari (Balan, 2014; Welker et al., 2015). L'accumulo di questi rifiuti può causare problemi ambientali ed economici (Maitan-Alfenas et al. 2015), per cui è importante trovare nuove applicazioni per il loro riciclo e riutilizzo. Un'ampia

varietà di biomasse lignocellulosiche, con rapporti di cellulosa e lignina variabili, è stata utilizzata per la produzione di zuccheri (*Populus nigra, Panicum virgatum*, paglia di grano). Tra i materiali lignocellulosici derivanti da rifiuti agricoli o alimentari, assume una grande rilevanza il sottoprodotto della coltivazione dei funghi (spent mushroom substrate, SMS), considerato che 1 kg di funghi genera 5-6 kg di SMS e la produzione globale di funghi è aumentata considerevolmente negli ultimi anni. Questa biomassa può quindi rappresentare una risorsa come fonte di monosaccaridi per la produzione di materiali a valore aggiunto.

Cellulasi come biocatalizzatori per la conversione di substrati lignocellulosici

Al giorno d'oggi, la produzione di bioprodotti a livelli competitivi è ostacolata dai costi elevati dei principali enzimi necessari per la conversione della biomassa lignocellulosica (cellulasi ed emicellulasi) (Liguori et al. 2016, Montella et al. 2016).

Per ridurre i costi enzimatici di saccarificazione, le attività di ricerca e sviluppo sia nei settori accademici che industriali si sono concentrate sullo sviluppo di biocatalizzatori più efficienti per l'idrolisi della lignocellulosa, attraverso l'isolamento di nuovi microorganismi da diversi *habitat*, la scoperta di nuovi enzimi mediante approcci metagenomici o il miglioramento di quelli esistenti mediante l'ingegneria genetica.

Nell'ambito del progetto BIOrescue "Enhanced bioconversion of agricultural residues through cascading use" (720708), questo progetto di dottorato ha previsto lo sviluppo mediante evoluzione guidata di biocatalizzatori basati su cellulasi per la conversione di substrato di fungo esaurito.

- **Clonaggio, espressione ricombinante e caratterizzazione di due nuove cellulasi, Cel1 e Cel2**

  Due nuove cellulasi della famiglia GH5 (Cel1 e Cel2), precedentemente identificate come responsabili dell'attività cellulasica di *Streptomyces argenteolus* AE58P (Ventorino et al. 2016), sono state clonate ed espresse in forma ricombinante in *Escherichia. coli,* al fine di selezionare una cellulasi per lo sviluppo di biocatalizzatori migliorati mediante evoluzione guidata. Cel1 e Cel2 ricombinanti sono state quindi caratterizzate, determinando la temperatura e il pH ottimali per l'attività cellulasica, la specificità di substrato, la cinetica, la loro termoresistenza (da 37°C a 60°C) e

pH resistenza (da pH acido a pH neutro). Inoltre, entrambe le cellulasi sono state testate nell'idrolisi enzimatica delle biomasse pretrattate *P. nigra*, *P. virgatum* e SMS implementato con paglia di grano (Wheat Straw, WS) e paragonate ad una mix commerciale. È risultato che entrambi gli enzimi mostrano delle rese di glucosio e xilosio paragonabili agli enzimi commerciali nell'idrolisi di *P. nigra* e *P. virgatum.* Le rese ottenute nell'idrolisi di SMS/WS sono risultate tre volte superiori a quelle ottenute con la mix commerciale, ma molto più basse rispetto a quelle ottenute con le altre due biomasse, sottolineando l'importanza di migliorare i biocatalizzatori nell'idrolisi di questo substrato.
Considerando la maggiore termoresistenza e le rese di idrolisi di poco superiori paragonate a quelle di Cel1, Cel2 è stata selezionata per la costruzione di una libreria di 30,000 mutanti.

- **Sviluppo di biocatalizzatori basati su cellulasi mediante evoluzione guidata**

È stata messa a punto una strategia per generare la libreria di 30,000 mutanti random di Cel2 mediante evoluzione guidata, e un metodo automatizzato per selezionare le varianti più attive. In seguito a saggio di attività su mezzo solido sono stati selezionati 943 mutanti, successivamente sottoposti a saggio in mezzo liquido in microscala, che ha permesso la selezione di 63 mutanti per la produzione in beuta. Tra questi, 21 mutanti hanno mostrato un'attività di almeno 2 volte maggiore rispetto al *wild type* Cel2 e sono stati caratterizzati determinando la temperatura e il pH ottimali e la termoresistenza. 13 mutanti mostravano una maggiore termoresistenza rispetto all'enzima *wild type* dopo 72 ore di incubazione per cui sono stati testati nell'idrolisi enzimatica della biomassa pretrattata SMS/ WS, dopo essere stati aggiunti alla mix commerciale SUNO^{TM} BOOSTER 144, che migliora la capacità delle cellulasi di idrolizzare i substrati cellulosici. Tutti i mutanti hanno mostrato una resa di glucosio 2 o 4 volte superiore rispetto al *wild type* Cel2, anch'esso aggiunto al SUNO^{TM} BOOSTER 144.

Monossigenasi litiche dei polisaccaridi: enzimi per aumentare la conversione di substrati lignocellulosici

Tra le classi di enzimi coinvolti nella conversione della lignocellulosa, le monossigenasi litiche dei polisaccaridi (LPMOs) (Chabbert et al.2017; Muller et al.2017) si distinguono come la classe più promettente per la loro capacità di migliorare l'efficienza

della depolimerizzazione della cellulosa (Chylenski et al. 2017; Hemsworth et al. 2015; Johansen 2016). Infatti, l'introduzione delle LPMOs nei cocktail enzimatici potrebbe comportare una riduzione significativa dei costi del processo di idrolisi (Johansen 2016).
Un recente lavoro svolto nel laboratorio in cui è stata condotta l'attività di tesi, ha permesso di scoprire geni codificanti per enzimi coinvolti nella sintesi, nel metabolismo e nel riconoscimento di carboidrati complessi (CAZy), mediante analisi metagenomica di biomasse lignocellulosiche e di sviluppare una banca dati di sequenze (Montella et al.2017) che codificano per circa 5.000 enzimi di differenti classi. Al fine di identificare sequenze codificanti per le LPMOs all'interno della banca dati, sono state eseguite analisi bioinformatiche da Bernard Henrissat del Centre National de la Recherche Scientifique (CNRS) di Marsiglia, che hanno rivelato la presenza di nove LPMOs batteriche appartenenti al T3PSB (campione di biomassa utilizzato per le analisi metagenomiche proveniente da *P. nigra* dopo 135 giorni di biodegradazione naturale nel sottobosco) (Montella et al. 2017).

- **Selezione di una monossigenasi litica dei polisaccaridi**

Le nove sequenze delle monossigenasi litiche dei polisaccaridi, precedentemente identificate mediante metagenomica, sono state analizzate allineandole con le sequenze riportate in letteratura e valutando le caratteristiche dei microrganismi che le producono.
È risultato che tutte le sequenze appartengono al genere degli *Streptomyces*, batteri gram positivi noti per la loro capacità di degradare una varietà di polisaccaridi complessi e recalcitranti. Inoltre, i genomi di *Streptomyces* sono un'eccezione rispetto alla maggior parte dei genomi di batteri che degradano biomassa, perché hanno fino a sette geni (*S. coelicolor*) codificanti le LPMOs.
La proteina codificata dal gene_T3PSB_53014 di *Streptomyces coelicolor* A3 (2) è stata selezionata per la messa a punto del sistema di espressione ricombinante, poiché il coinvolgimento nella degradazione della cellulosa era già stato precedentemente verificato, a differenza di altre LPMOs. Inoltre, tale enzima risulta particolarmente interessante grazie alla presenza di un dominio CBM2, cruciale per il legame del substrato.

- **Messa a punto di un sistema di espressione ricombinante di una monossigenasi litica dei polisaccaridi**

Il gene codificante per la LPMO da *Streptomyces coelicolor* A3 (2) è stato sintetizzato, clonato nel vettore di espressione pET22b ed espresso in forma ricombinante in *E. coli.* Per l'espressione della proteina sono state valutate diverse condizioni ed è stata effettuata un'analisi qualitativa mediante SDS-PAGE. Solo una piccola differenza nell'intensità della banda è stata osservata quando l'induzione è stata effettuata utilizzando 0,5 mM isopropil β-d-1-tiogalactopiranoside (IPTG) per 3 ore a 30 °C. In Courtade et al. 2017 è riportato che l'induzione con IPTG può comportare una miscela di LPMO nativo e prematuro (con peptide segnale non scisso) e lisi cellulare. In ogni caso, al fine di utilizzare l'LPMO in una miscela di enzimi per la degradazione della cellulosa, sarebbe necessario effettuare la purificazione dell'enzima.

Conclusioni

Questo lavoro ha previsto:

I) Il clonaggio e l'espressione ricombinante in *E. coli* di due nuove cellulasi della famiglia GH5 prodotte da *Streptomyces argenteolus* AE58P, chiamate Cel1 e Cel2, le cui sequenze sono state depositate in banca dati NCBI (MN845760 e MN845759)

II) la caratterizzazione di Cel1 e Cel2 espressi in forma ricombinante in *E. coli*, mediante la determinazione di specificità di substrato, cinetica, optimum di temperatura e pH, termoresistenza e resistenza al pH, idrolisi di substrati lignocellulosici. Sulla base dell'elevata termoresistenza e delle migliori rese di idrolisi, selezione di Cel2 per la costruzione di una libreria di mutanti mediante evoluzione guidata.

III) Messa a punto della strategia per la costruzione e la selezione automatizzata di collezioni di varianti evolute e sua applicazione per la produzione di 30,000 mutanti

IV) L'ottenimento di varianti di Cel2 con attività cellulasica e rese di glucosio migliorate rispetto all'enzima *wild type*.

V) La selezione di una monossigenasi litica dei polisaccaridi (*Lytic polysaccharide monooxigenase* - LPMO) e la messa a punto di un sistema di espressione ricombinante

List of abbreviations

AZO-CMC: Azo-CarbossiMetilCellulosa/Azo-CarboxyMethylCellulose

BG: β-glucosidases

CAZY: Carbohydrate-Active EnZYmes

CBM: Carbohydrate- Binding Module

cDNA: complementary DNA

CE: Carbohydrate Esterases

CMC: CarbossiMetilCellulosa/CarboxyMethylCellulose

EG: Endoglucanase

epPCR: error prone Polymerase Chain Reaction

FOLy: Fungal Oxidative Lignin Enzymes

FW: forward

GH: Glycoside Hydrolases

HPLC: high-performance liquid chromatography

IPTG: Isopropil-β-D-1-tiogalattopiranoside

LB: Luria Bertani

LDA: Lignin Degrading Auxiliary enzymes

LO: Lignin Oxidases

LPMO: Lytic Polysaccharide Monooxigenase

PCR: Polymerase Chain Reaction

PL: Polysaccharide Lyases

pNPG: 4-Nitrophenyl β-D glucopyranoside

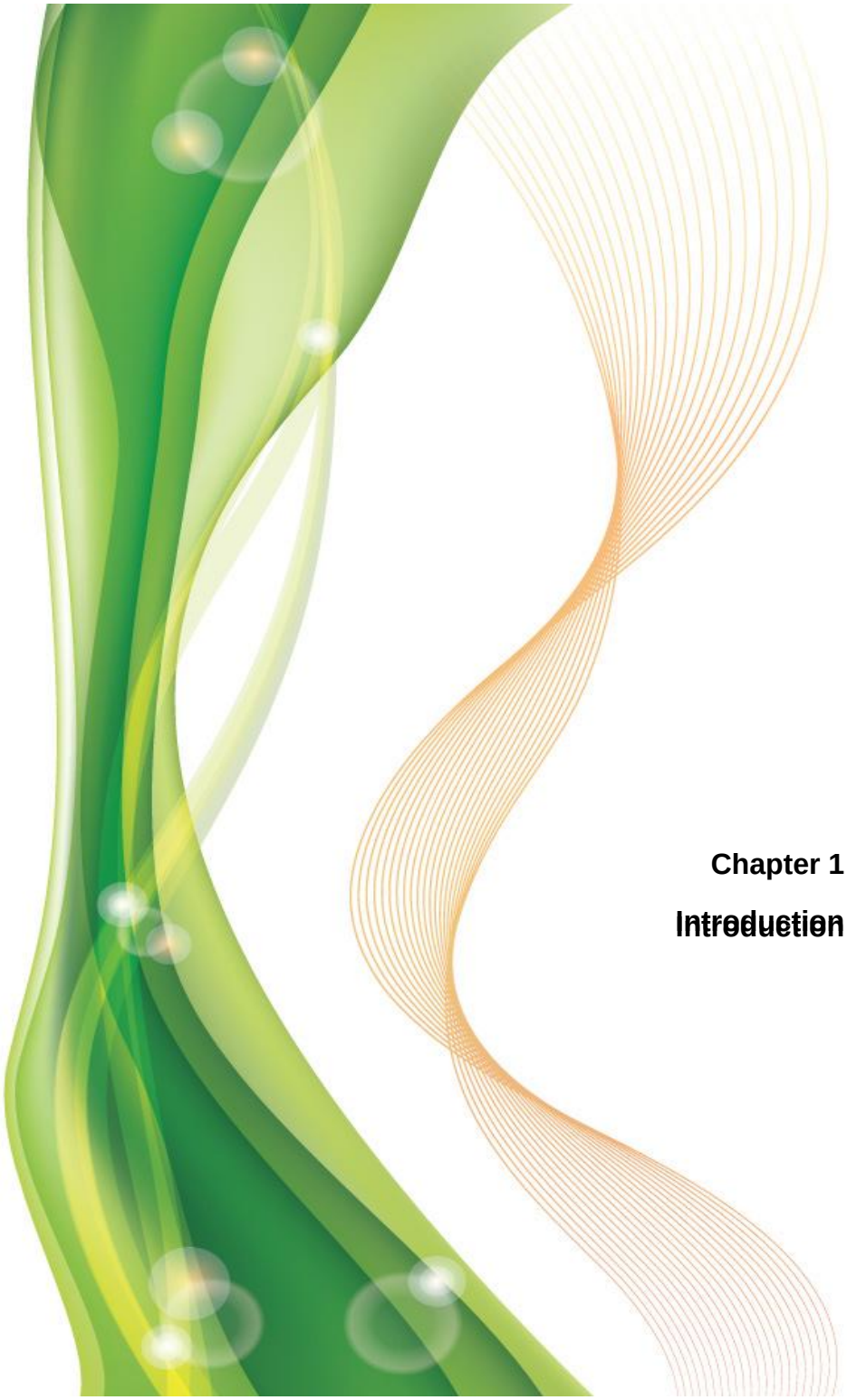
REV: reverse

SDS-PAGE: Sodium Dodecyl Sulphate- PolyAcrylamide Gel Electrophoresis

SMS: Spent Mushroom Substrate

WS: Wheat Straw

WT: wild-type



Chapter 1

Introduction

Chapter 1: Introduction

1.1 Green chemistry

During the 20th century, technological development changed the fortunes of humanity and the planet (Jones, K. 2017). At the end of the 1980s, the concept of sustainable development was emerging as a driving principle since humanity's impact on the environment has been greatly accelerated with rapidly increasing population and the concomitant decrease of natural resources. In this context, attention on the problems of chemical pollution and resource depletion was increased and a new area of chemistry was emerging, "green chemistry". Green chemistry can be considered as an area of chemistry and chemical engineering focused on the designing of products and processes that minimize the use and generation of hazardous substances (http://www.epa.gov/laws-regulations/ summary-pollution-prevention-act). Whereas environmental chemistry focuses on the effects of polluting chemicals on nature, green chemistry focuses on technological approaches to prevent pollution and reducing consumption of non-renewable resources (Cernansky, 2015).

Alternative sources need to be renewable, sustainable, efficient, cost effective, convenient and safe (Prasad et al. 2007); also, new bio-based processes need to be coherent with the 12 principles of green chemistry introduced by Anastas and Warner in 1998 (Anastas, 1998). In this context, we can consider the major role played by biotechnology. This is defined by the Organization for Economic Development and Cooperation (OECD) as "*The application of science and technology to living organisms as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services*". In particular, white biotechnologies are important in the development of new bio based processes, through biotransformations and fermentations, using enzymes and living cells from yeast, moulds, bacteria and plants, respectively (Liguori et al. 2013; Llevot & Meier, 2016; Adrio & Demain, 2014).

Biotechnology has been widely used in the industry for several years, for example in agro-food industries, for the antibiotics production, in paper pulp industry or to reduce artificial surfactants in powder detergents. The use of living organisms to obtain natural products and processes has many advantages because it allows to

reduce the use of fossil sources and the environmental impact, as wastes are biologically degradable (Glaser J. A. 2005).
Bio-based products can be produced in biorefineries, which can be considered as an integral unit that can accept different biological feedstocks and convert them to a range of useful products including chemicals, energy and materials (Clark et al., 2006).
Moreover, every day different types of waste are generated in large quantities (agricultural, food or industrial), causing significant problems in management and disposal. These wastes are rich in sugars, which are easily assimilated by microorganisms, making them suitable for use as a raw material in the production of industrially relevant compounds, thereby reducing production costs and the environmental impact (Amore et al. 2013; Pennacchio et al. 2018). In addition due to legislation and environmental reasons, the reutilization of biological wastes is of great interest since the industry is increasingly being forced to find an alternative use for its residual matter. Therefore, investigation and development of potential value-added processes for biological wastes is highly attractive (Couto S.R., 2008).

1.2 Lignocellulosic biomasses

Lignocellulosic biomass, derived from plant cell walls, is the raw material most abundant on the Earth. It is composed predominantly of sugars and is, therefore, a renewable and inexpensive source for the production of bioproducts, such as ethanol, food additives, fermentable sugars, biopolymers and enzymes(Maitan-Alfenas et al. 2015; Capolupo & Faraco, 2016; Mezule et al. 2015). Moreover, the price of lignocellulose biomass is significantly lower than that of vegetable oil, corn and sugarcane.
High yields of lignocellulosic biomasses can be provided by dedicated energy crops (e.g. switchgrass and hybrid poplars), that do not compete with food and feed cultivation (Brethauer & Studer, 2015; Liguori et al. 2016; Ventorino et al. 2015) or by forestry, agricultural, agro-industrial and food wastes (Figure 1) (Balan, 2014; Welker et al., 2015). However, the environmental and economic problems (Maitan-Alfenas et al. 2015) caused by the accumulation of lignocellulose wastes has triggered the interest in the search for new applications for the recycling and reuse of these wastes.

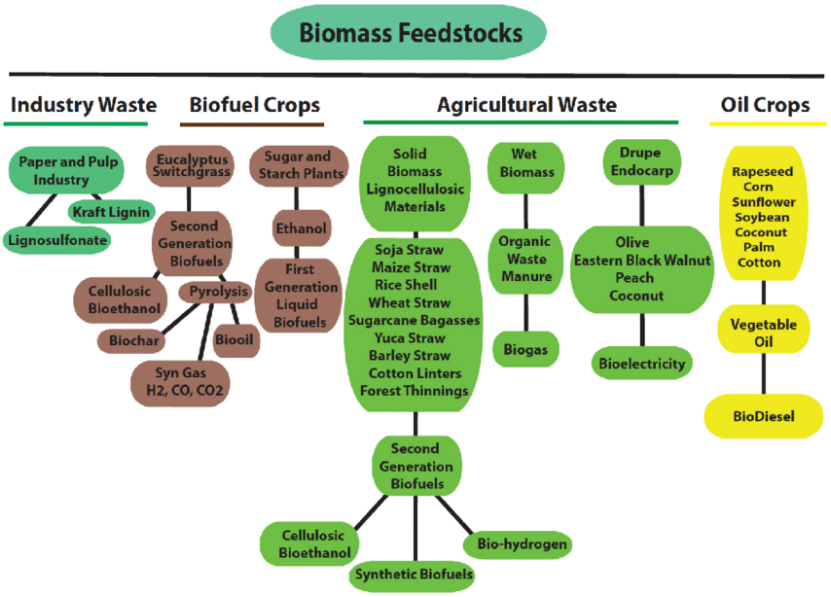


Figure 1: Biomass feedstocks and their utilization in the production of biofuels, bioenergy and bioproducts (Welker et al. 2015)

The three major components of lignocellulose (Figure 2) are cellulose (40–50%), hemicellulose (25– 30%), and lignin (15–20%), that together form a complex macromolecular structure (Brethauer & Studer, 2015).

Cellulose is the major component of plant cell walls. It is a linear polysaccharide composed of thousands of glucose (C6) units linked with β-1,4-glycosidic bonds. These chains, generally 36, are connected through van der Waals interactions and hydrogen bonds, forming the microfibrils, regular and non-soluble crystalline structures, containing amorphous regions (Guerriero et al. 2016; Galkin & Samec, 2016; Brethauer & Studer, 2015; Menendez et al. 2015). The amorphous regions are generally more accessible for degradation by enzymes than the crystalline ones. However, a pre-treatment step is often necessary. These microfibrils, together with hemicelluloses and lignin form the plant cell wall (Bettache et al. 2018).

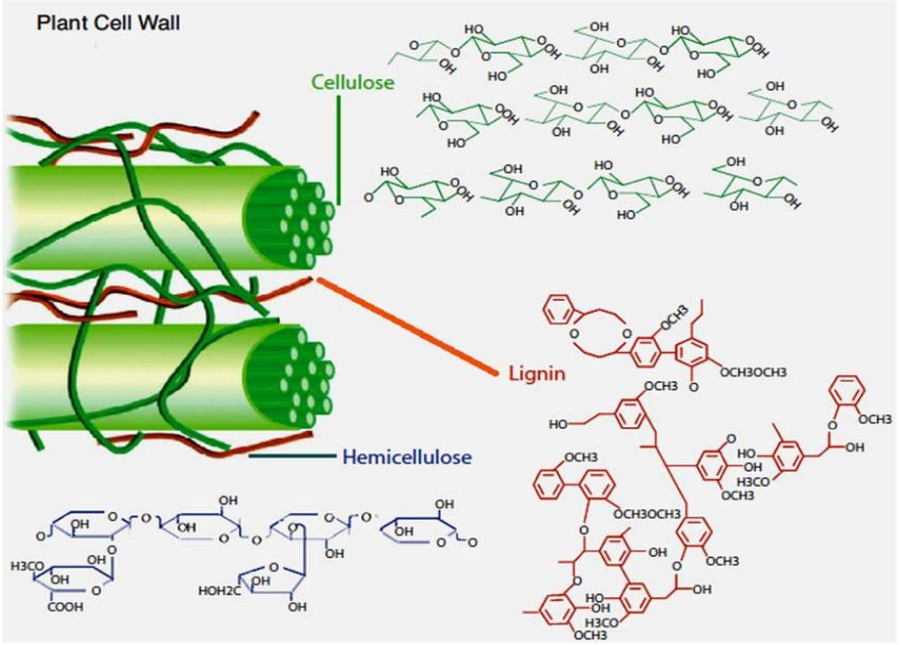


Figure 2: Cell wall of a lignocellulose biomass (Bamdad et al. 2018)

Hemicellulose is a low molecular weight polysaccharide, present in the primary and secondary plant cell wall. Its structure can change, depending on the tissue and type of plant with an average degree of polymerization of 150 units (Sorieul et al. 2016). These are organized in a branched structure, all linked together by glycosidic bonds (Guerriero et al. 2016; Galkin & Samec, 2016; Brethauer & Studer, 2015; Zhou et al. 2017; López-Mondéjar et al. 2019). Hemicelluloses are classified according to the main sugar residue in the backbone as xylans, mannans, and xyloglucans. Almost all plants contain xylan. Xylan hemicellulose is the glucan with a backbone of 1,4-β-D-xylopyranose and with a branch chain of 4-oxymethyl-glucuronic acid. Depending on plants, several branch chains are linked to the backbone, consisting of xylose, or L-arabinofuranoside, in most of cases linked to the C2 or C3 of the main chain, 4-O-methyl glucuronic acid, mannose, galactose.
Mannan hemicellulose is an inhomogeneous polymer with the main chain composed by mannose and glucose linked together by β(1→4) bonds. Branch chains can be formed by galactosyl, linked to the main chain by α(1→6) bonds and acetyl on the C2 and C3 of mannose. Xyloglucans are composed by D-glucopyranose linked with β(1→4) bonds. The branch chains are linked to the backbone

by the xylose that is at O-6 of the main chain. Xyloglucans mainly contain xylose, glucose and galactose and in plants they can contain fucose and arabinose.
The hemicellulose is linked to the microfibrils by hydrogen bonds and to lignin by covalent ester bonds.

Lignin is the third most abundant biopolymer of the lignocellulosic material and approximately 5×10^6 metric tons of lignin is produced industrially, every year (Pollegioni et al. 2015). It plays an important role in the plant cell wall, since it gives a structural support and also protects the hydrophilic cellulose and hemicellulose (Sorieul et al., 2016; Welker et al., 2015). Structurally, lignin is a high molecular weight etheropolymer, containing monolignol units linked by carbon-carbon (C-C) and aryl-ether (C-O-C) bonds. The main components are p-coumaryl, coniferyl (4-hydroxy-3-methoxyinnamyl alcohol) and synapyl (4-hydroxy-3,5-dimethoxyinnamyl alcohol) alcohols (Pollegioni et al., 2015; López-Mondéjar et al. 2019). These monomeric units give a three-dimensional amorphous structure with random sequences and bonds, depending on the plant family, the biological tissue and the species (Galkin & Samec, 2016).

The chemical components and physical structure of lignocellulosic biomasses make the bioconversion of these materials difficult (Akimkulova et al. 2016). Therefore, lignin and hemicellulose need to be removed before the enzymatic hydrolysis of cellulose (Pei et al. 2016).
A wide variety of different lignocellulose biomasses have been tested and used to produce fermentable sugars (Table 1) and the amounts of carbohydrates and lignin are highly variable from one biomass to another, depending on different conditions (such as the age of the plant and the stage of growth) (Mussatto and Teixeira, 2010; Yadav et al. 2018; Merino et al. 2007; Grover et al. 2015; Grimm and Wösten 2018)

Table 1: Cellulose, hemicellulose and lignin content in some lignocellulosic biomasses (Mussatto and Teixeira, 2010; Yadav et al. 2018; Merino et al. 2007; Grover et al. 2015; Grimm and Wösten 2018)

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
P. nigra	45	21	24
P. virgatum	31-38	26-34	18-22
Wheat straw	34-39	21-34	22-25
Spent mushroom substrate	36-40	20-22	12

1.2.1 Poplar (*Populus nigra*)

Poplars (*Populus spp*.) are trees, functional in agriculture and agricultural landscapes, used worldwide as one of the most important families of woody plants for industry, particularly in relation to fiber or pulp and paper production (Berguson et al. 2010). The wide cultivation of these plants for human use is strictly connected to their fast growth and composition. In fact, poplars can produce a significant amount of biomass in a short period of time, with high cellulose and low lignin contents, becoming a source of fermentable sugars for the production of added-value chemicals and materials. Today, the use of poplar is expanding to contribute to the wood, veneer, and bioenergy industries, simultaneously providing environmental benefits. Moreover, poplars have some advantages over other bioenergy crops such as grasses because the wood does not need to be stored, which allows harvesting to occur throughout the year. Black poplar (*P. nigra*) is a pioneer deciduous wind-pollinated tree species, widely distributed across Europe, Asia and northern Africa (Rigo et al. 2016). Macromolecular composition of *P. nigra* in terms of the percentage of cellulose, hemicellulose and lignin, is 45%, 21% and 24%, respectively (Merino et al. 2007).

1.2.2 Switchgrass (*Panicum virgatum*)

Switchgrass (*Panicum virgatum*) is a perennial native grass mainly found in the Great Plains region of the United States. However, it is able to adapt to many other climates and soil types. It can grow up to nine feet tall and produces a dense, fibrous root system that grows as deep as the plant is tall and is composed mainly of cellulose and hemicellulose with a low content of lignin. These properties make this lignocellulosic material very appealing in relation to its use for the production of added-value products. Switchgrass has been identified by the U.S. Department of Energy as the most important biomass feedstock for the generation of energy. In fact, it produces 40% more energy than is needed to grow, harvest and process it into ethanol (Anderson et al., 2016).

1.2.3 Spent mushroom substrate (SMS)

SMS is a by-product of edible mushroom cultivation (Xiao et al., 2018; Mohd Hanafi et al., 2018). Global mushroom production and consumption *per capita* have increased significantly during the last few years. Indeed, in 1978 the world's mushroom production was about 1 billion of kg, rising to about 35 billion of kg in 2013 although during these years, the global population has increased just 1.7- fold (Royse et al. 2017).

In parallel, with the increase of mushroom production and consumption, the generation of SMS has also increased, considering that 1 kg of fresh mushroom produces 5-6 kg of SMS (Rinker, 2017; Xiao et al. 2018; Mohd Hanafi et al., 2018).

SMS is stored, temporarily, before disposal in a municipal landfill site or use on land as a soil conditioner product. Therefore, this biomass represents a residual material, whose disposal entails ecological and economic problems, linked to the large amounts released. It would be more sustainable and economically expedient if it were recycled and reused (Phan & Sabaratnam, 2012). SMS is a lignocellulosic material containing mainly sawdust, sugarcane bagasse, oil palm empty fruit bunch, wheat straw-bedded horse manure, hay, poultry manure, ground corncobs, cottonseed meal, cocoa shells, gypsum and other substances (Phan & Sabaratnam, 2012). This biomass can therefore represent a source of monosaccharides to be fermented and can produce added-value

chemicals and materials. This has triggered the interest in the search for new treatment technologies and new uses for this by-product (Rinker 2017), considering its great relevance from the perspective of the circular economy (Figure 3) (Liguori & Faraco, 2016).

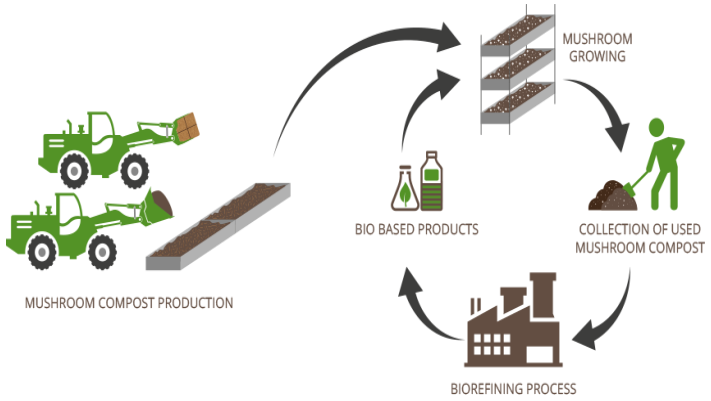


Figure 3: Spent mushroom compost valorisation for bioproduct generation in the circular economy (BIOrescue project funded by EU H2020/BBI JU)

1.2.4 Wheat Straw (WS)

Wheat Straw (WS), the farm by-product or residue of wheat production, is another of the lignocellulose biomasses useful for the generation of added-value materials. Wheat is one of the most commonly produced crops around the world, totalling globally 730 million tons in 2014 (Zheng et al., 2018). Considering that the average yield of WS is 1.3 kg per kg^{-1} of wheat grain (Schnitzer et al. 2014), large amounts of WS is generated every year. However, its monetary value remains low. As for SMS, the disposal of this residual material impacts negatively on the environment and economy, since the usual disposal methods include discarding it in the field or burning (Talebnia et al. 2010). This lignocellulose material is composed, mainly, of cellulose and hemicellulose and its conversion into valuable products (fine chemicals or monomeric sugars usable for the production of biofuels such as bioethanol and methane) has economic and market potential. However, the cell wall of WS naturally resists enzyme attack due to the hemicellulose and lignin content, and therefore a pretreatment step is necessary (Sun et al. 2018).

1.3 Enzymes involved in lignocellulose conversion

The bioconversion of lignocellulose materials into fermentable sugars (such as glucose, xylose, and arabinose), usable for the generation of valuable products, requires a multistep process in order to increase the accessibility of the substrate for the enzymatic hydrolysis (Jiang et al. 2017). This is a bottleneck in the process because the cost is still extremely high, and a large amount of enzymes is required (Isikgor & Becer, 2015). Today, the major enzymes in all biomass conversion cocktails are cellulases and hemicellulases. In several studies it has been demonstrated that a mixture of cellulases and hemicellulases for lignocellulose conversion can improve the rate and the yield of glucan, since the removal of hemicellulose increases the accessibility of the cellulose fibrils and, consequently, of the substrate (Maitan-Alfenas et al., 2015). However, in order to obtain a complete enzymatic hydrolysis of the lignocellulose substrates, the synergistic action of several enzymes like xylanases, arabinofuranosidases, mannanases, besides cellulases and hemicellulases, is necessary.

Generally, the enzymes involved in the conversion of lignocellulosic biomasses belong to the classes of glycosyl hydrolases, endo-glucanases, cello-biohydrolases and β–glucosidases, and they act together, producing monomers of glucose (Guerriero et al., 2016; Coimbra et al., 2016). According to the Carbohydrate-Active EnZYmes (CAZY) (www.cazy.org) database, lignocellulose-degrading enzymes belong to the Glycoside Hydrolases (GH), Polysaccharide Lyases (PL), Carbohydrate Esterases (CE) Auxiliary Activities (AA) which includes LPMOs and redox enzymes involved in lignin breakdown (Sun et al. 2014).

Focusing our attention on the GH (EC 3.2.1.-GHs), these are a group of enzymes which can catalyze the hydrolysis of the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate. Generally, the hydrolysis of the glycosidic bond occurs through an acid catalysis by two amino acid residues of the enzyme: a proton donor and a nucleophile/base (Davies & Henrissat, 1995). This hydrolysis takes place by means of one of two mechanisms, depending on the orientation of the catalytic residues, and entails a retention or an

inversion of the anomeric configuration. Moreover, another mechanism has been demonstrated for two families of glycosidases utilizing NAD^+ as a cofactor (Rajan et al., 2004). Glycoside hydrolysis with an inversion of the anomeric configuration is achieved through a single-displacement step, which involves an oxocarbenium ion-like transition state. The reaction takes place with general acid and general base assistance from two amino acid side chains, normally glutamic or aspartic acids. The retention is characterized by a double-displacement mechanism which involves a covalent glycosyl enzyme intermediate and the formation of an oxocarbenium ion-like transition state in each step. In this case also, the reaction is mediated by an acid/base amino acid and a nucleophilic one, typically glutamate or aspartate. Furthermore, in order to increase the cellulose degradation, some bacterial and fungal glycoside hydrolases show a multimodular architecture comprising a carbohydrate binding module (CBM), which is a linker, and a catalytic domain, which increases the interaction of the enzyme with the recalcitrant substrate (Guerriero et al., 2016).
One of the largest of glycoside hydrolase families is the family 5 (GH5). Family GH5 enzymes are found widely distributed across Archae, bacteria and eukaryotes, notably fungi and plants. Several enzymes belong to this family, mainly endoglucanase (cellulase) and endomannanase, as well as exoglucanases, exomannanases, β-glucosidase and β-mannosidase. Other activities include 1,6-galactanase, 1,3-mannanase, 1,4-xylanase, endoglycoceramidase, as well as high specificity xyloglucanases. The GH5 enzymes use for hydrolysis a double-displacement mechanism with a retention of configuration.

1.3.1 Cellulases

Cellulases are involved in the hydrolytic scission of the β(1→4) glucosidic bond in cellulose, leading to the formation of glucose and short cellodextrins. In order to obtain a complete degradation of the cellulose the synergistic action of three cellulase enzymes is necessary, as described below. Endo-1,4-β-glucanases (EG) (EC 3.2.1.4) hydrolyse randomly the internal bonds of the glucose chains, releasing oligosaccharides with several polymerization degrees; cellobiohydrolases (EC 3.2.1.176) remove the cellobiose

units and can act on the crystalline portion of the cellulose, attacking from the reducing and non-reducing ends of the glucose chain; β-glucosidases (BG) (EC 3.2.1.21) catalyze the hydrolysis mainly of cellobiose to glucose and also remove glucose units from the non-reducing ends of the small cyclodextrins (Guerriero et al., 2016; Horn et al. 2012; Maitan-Alfenas et al., 2015).

1.3.2 Hemicellulases

Hemicellulases are enzymes involved in the hydrolysis of the hemicellulose fraction of the lignocellulose biomass. Considering the complex structure of hemicellulose, the action of several enzymes is required: endo-enzymes to break the main chain, exo-enzymes to release monomeric sugars and accessory enzymes to break the side chain of the polymers or oligosaccharides depending on the hemicellulose type.
In detail, the enzymatic hydrolysis of xylan, the major polymer of hemicellulose, occurs due the action of the endo-β-1,4- xylanase (EC 3.2.1.8) that breaks randomly the internal bond of xylan to release xylo-oligosaccharides; the β-Xylosidases (EC 3.2.1.37) that carry out the hydrolysis of the β(1→4) hydrolysing the non-reducing ends of the xylose chains to release the xylose. The mannan degradation occurs due to the action of the β-endomannanases (EC 3.2.1.78), that break the main chain of galacto(gluco)mannans and release mannooligosaccharides (mainly mannobiose and mannotriose); β-mannosidases (EC 3.2.1.25), that act on the non-reducing ends of mannooligosaccharides, releasing mannose. The xyloglucan degradation requires endoglucanases and β-glucosidases (de Souza, W. G. 2013)

1.3.3 Lignin degrading enzymes

The enzymes involved in lignin degradation can be divided in lignin-modifying enzymes (LME) and lignin degrading auxiliary enzymes (LDA). Moreover, another group of enzymes was recently discovered to have a role in lignin degradation, heme-thiolate haloperoxidases. These enzymes show the same catalytic properties of other three classes of oxidoreductases containing heme group (classic plant and fungal peroxidases, cytochrome P450 monooxygenases, and catalases). The LME includes

laccases (EC 1.10.3.2), lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13) and versatile peroxidases (EC 1.11.1.16). The laccases are multi-copper enzymes with low specificity. They can oxidize o- and p-quinols, and often acting also on aminophenols and phenylenediamine. The lignin peroxidases oxidize non-phenolic units of lignin by removing one electron and creating cation radicals. The manganese peroxidases oxidize a bound Mn(II) ion to Mn(III) using H_2O_2, so the ion Mn(III) can diffuse into the lignified cell wall, where it oxidizes phenolic components of lignin and other organic substrates.
Auxiliary enzymes allow the lignin degradation process through the sequential action of several proteins which may include oxidative generation of H_2O_2. This group includes glyoxal oxidase (GLOX; EC 1.2.3.5), aryl alcohol oxidases (AAO; EC 1.1.3.7), pyranose 2-oxidase (POX; EC 1.1.3.10), cellobiose dehydrogenase (CDH; EC 1.1.99.18) and glucose oxidase (EC 1.1.3.4) (.de Souza 2013 W. R; Janusz et al. 2017).

1.3.4 Accessory enzymes

In order to increase the sugar yields during the biomass hydrolysis, accessory enzymes are considered essential in enzymatic cocktails. Such enzymes include α-L-arabinofuranosidase (EC 3.2.1.55), that removes the arabinose substituent, α -glucuronidase (EC 3.2.1.139) that catalyze the synthesis of D-glucuronate, reacting with H_2O, α -galactosidase (EC 3.2.1.22), which hydrolyse the terminal non-reducing α-D-galactose residues in α-D-galactosides, acetylxylan esterase (EC 3.1.1.72) which catalyzes the hydrolysis of acetyl groups of xylans and xylo-oligosaccharides and ferulic acid esterase (EC 3.1.1.73) which catalyzes the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar. The synergistic action of the enzymes is a widely observed phenomen in biomass hydrolysis and it depends on several factors such as the nature of the substrate and the source of the enzymes. The design of GH mixtures with small amounts of synergistic proteins to release the sugars from the biomass has proven to be an effective strategy (Maitan-Alfenas et al., 2015). Moreover, it was demonstrated in the last years that the utilization of synergistic proteins, lacking of hydrolytic activity as expansin proteins or expansin –like proteins (swollenin), can facilitate the release of sugars from lignocellulosic biomasses. In

fact, the expansins disrupt hydrogen bonding between cell wall polysaccharides without hydrolyzing them (Saloheimo et al. 2002; Maitan-Alfenas et al., 2015).
Among the classes of enzymes involved in the biological conversion of lignocellulose, through a synergistic action of hydrolytic and oxidative activities, the lytic polysaccharide mono-oxygenases LPMOs (Chabbert et al., 2017; Muller et al. 2017) stand out as the most promising class due to their capability to enhance the efficiency of the cellulose depolymerisation (Chylenski et al. 2017; Hemsworth et al. 2015; Johansen 2016). As a matter of fact, these enzymes perform an oxidative cleavage of the scissile β-1,4 glycosidic bonds of the highly ordered crystalline cellulose, chitin, xylan, xyloglucan, glucomannan, lichenan, β-glucan, and starch, making the substrate more accessible to the GH. However, even if the involvement of oxygen-dependent enzymes in cellulose degradation was presented in 1970, only in 2010 were LPMOs were described for the first time (Hemsworth 2015). LPMOs belong to the carbohydrate-active enzyme (CAZy) families, in particular the auxiliary activity (AA) families 9, 10, 11, and 13 (Johansen 2016). Their activity requires an enzymatic (e.g. cellobiose dehydrogenase) or non-enzymatic (e.g. ascorbic acid) external electron source and molecular oxygen for the hydroxylation at the C1, C4 or C6 carbon and the release of oxidized soluble oligosaccharides (Chylenski et al. 2017, Montella et al. 2015). LPMOs contain surface-exposed catalytic copper involved in the regioselective hydroxylation of crystalline cellulose. This regioselectivity has been demonstrated for some enzymes belonging to the LPMOs (Hemsworth 2015; Montella et al. 2015).

1.4 Novel enzyme development

The production costs of the enzymes involved in the conversion of lignocellulose biomasses, are still very high impacting negatively on the total costs of the biomass conversion process. Moreover, they prevent the development of biochemical at competitive levels, although lignocellulose materials are abundant and usually low-priced (Isikgor & Becer, 2015; Kumar et al. 2016). Therefore, research has been focused on the development of biocatalysts which are more efficient in the lignocellulose hydrolysis, through the isolation of new microbes producing cellulolytic enzymes or

discovery of new enzymes by metagenomic approaches or the improvement of the existing biocatalysts by genetic engineering (Montella, et al. 2016; Faraco, 2013; Liguori et al., 2015; Amore et al., 2013; Pennacchio et al., 2018; Ventorino et al., 2015; López-Mondéjar et al., 2019). Several studies on the isolation of new cellulolytic microorganisms have been reported, including bacteria and fungi (Ventorino et al. 2016). These include filamentous bacteria belonging to the class of Actinobacteria (Berlemont, 2013; Vetrovský et al., 2014), which are Gram-positive prokaryotes with a Chargaff coefficient (G+C%) between 60 and 75%. It has been reported that enzymes are the second most important products of Actinobacteria, after natural antibiotics, and are mainly produced by strains belonging to the *Streptomyces* genus. These microorganisms are excellent producers of enzymes, such as chitinases, amylases, cellulases, xylanases and lipases, for the efficient degradation of lignocellulose or for other industrial uses (Bettache et al., 2018). On the other hand, together with the discovery of new microorganisms, the development of new/improved biocatalysts is a crucial aspect. There are two major ways in which enzymes can be modified in order to obtain an improvement of their function: the rational/semi-rational design of existing biocatalysts and directed evolution (Adrio & Demain, 2014).

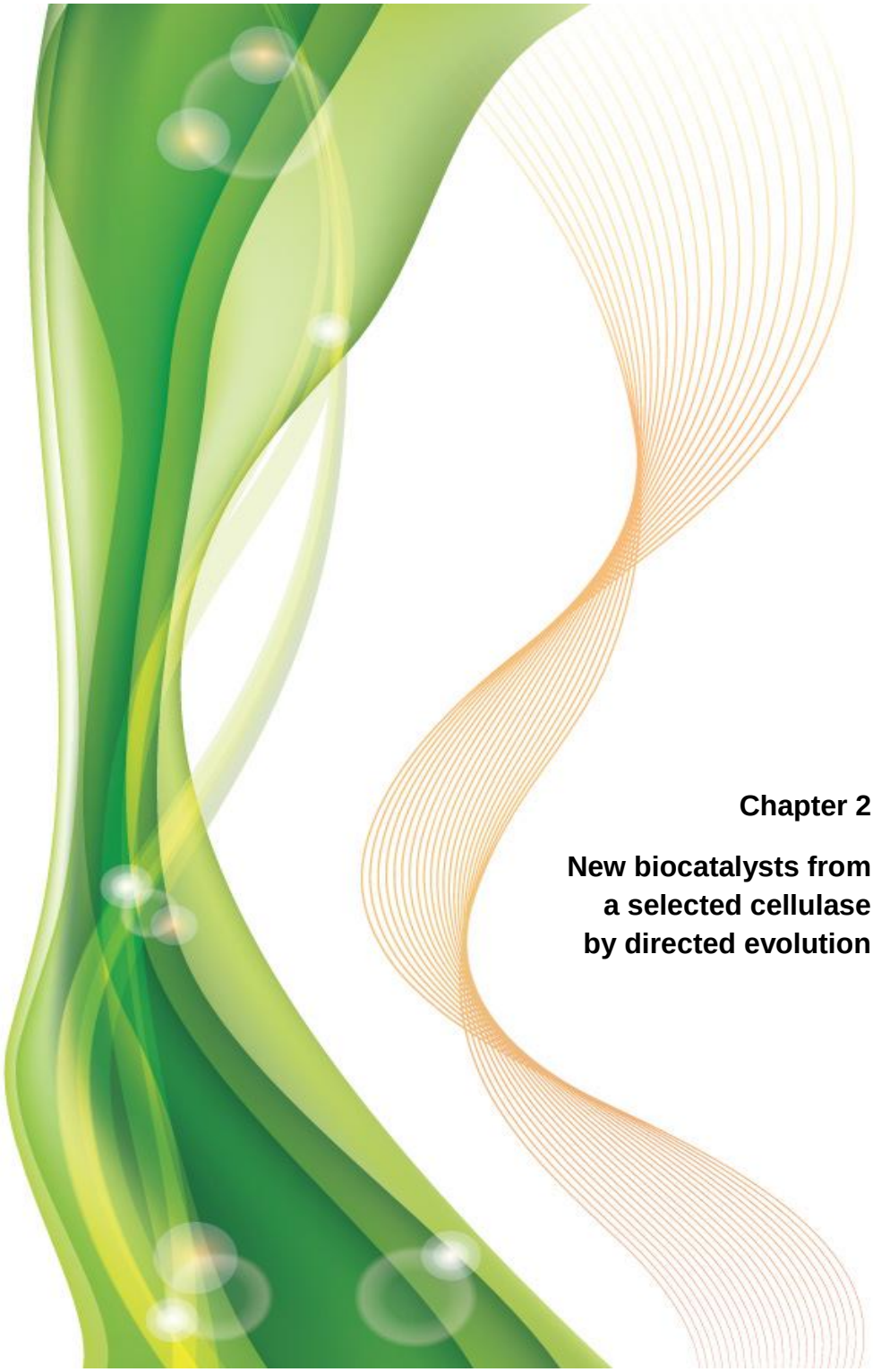
The first approach, including site directed mutagenesis, requires knowledge of the 3-dimensional structure and chemical mechanism of the enzymatic reaction. On the contrary, directed evolution does not require any extensive knowledge about the enzyme and it allows the creation of a large number of variants screened for activity, enantioselectivity, catalytic efficiency, solubility, specificity and enzyme stability.

1.5 Aims of the thesis

The overall aims of this project are the development of novel improved enzyme variants with an increased efficiency to hydrolyse the carbohydrates present in SMS, combined with WS into fermentable sugars, and the selection of a bacterial LPMO, to set up a recombinant expression system, considering the capability of this class of enzymes to enhance the yield of cellulose conversion.

The main outlines of this work are reported below.

I) The cloning and recombinant expression in *E. coli* of two new cellulases of the family GH5 produced by *S. argenteolus* AE58P (named Cel1 and Cel2)
II) The characterization of the recombinantly expressed enzymes, Cel1 and Cel2, in order to select the best one, for the construction and screening of the directed evolution library
III) The setting up of a strategy to create and screen cellulase mutants for their activity
IV) The creation and screening of a library of 30,000 cellulase mutants
V) The scaling up of the production of the best mutants and their characterization
VI) The testing of the selected variants in a pretreated SMS/WS (40:60) hydrolysis.
VII) The selection of a LPMO and the setting up of a recombinant expression system



Chapter 2

New biocatalysts from a selected cellulase by directed evolution

Chapter 2: New biocatalysts from a selected cellulase by directed evolution

2.1 Introduction

The development of biocatalysts with new or improved properties can be achieved by adopting two main approaches: rational design or directed evolution (Adrio & Demain, 2014).
Directed evolution has one significant advantage compared to rational design since no knowledge of the protein structure or the effects of specific amino acid substitutions is required.
Directed evolution mimics natural evolution and consists in an iterative two-step process including: 1) the generation of a library of several variants of a protein of interest; and 2) high throughput screening for activity, enantioselectivity, catalytic efficiency, solubility, specificity and enzyme stability, to identify the best performing mutants. The best performing mutants selected through the screening could be used as templates for further rounds of directed evolution or could be subjected to a scale up of production and characterization.
This chapter deals with the results concerning the cloning and recombinant expression of two cellulases and the selection of one of these for the development of improved biocatalysts through directed evolution in order to obtain improved variants in terms of activity.
In detail, in section 2.2.1 the saccharification experiments on the lignocellulosic biomasses *P. nigra*, and *P. virgatum*, are described for two cellulases of the family GH5, named Cel1 and Cel2, cloned and recombinantly expressed in *E. coli*. During a previous work performed in the same laboratory where the PhD thesis research was carried out (Ventorino et al., 2016), these enzymes were identified as responsible for the cellulase activity of *Streptomyces argenteolus* AE58P, selected as the best cellulase producer among 24 Actinobacteria.
In section 2.2.2, the manuscript “Development of improved cellulase variants for SMS conversion by directed evolution” describes, in more detail, the cloning and recombinant expression of Cel1 and Cel2, their characterization and the selection of the better performing enzyme to be subjected to directed evolution for the development of improved biocatalysts for the conversion of SMS/WS.

2.2 Results

2.2.1 Saccharification of lignocellulosic biomasses

As reported in Pennacchio et al. 2018, *P. nigra* and *P. virgatum* were successfully hydrolysed using the extracellular enzymatic mixture of *S. argenteolus* AE58P. Therefore, these biomasses were selected for the saccharification experiments, in order to compare the hydrolysis yields of recombinant Cel1 and Cel2 to those of wild type *S. argenteolus* AE58P. The lignocellulosic biomasses were pretreated according to Garbero et al. (2010) were used as substrate slurry in saccharification experiments, carried out in capped tubes, on the rotary shaker ThermoMixer C (Eppendorf, Milan, Italy). were successfully previously
The experiments were carried out by adopting the enzymatic extracts of Cel1, Cel2 and *S. argenteolus* AE58P wild type (Ventorino et al. 2016), at a concentration of pretreated biomass of 10% (w/v) in a total volume of 2.5 mL of 50 mM sodium citrate buffer (pH 5.0). The components of the enzymatic commercial cocktail adopted as the benchmark were Accelerase1500, Accelerase BG and Accelerase XY, provided by Genencor. The enzyme mixtures were prepared at the amounts expressed as units per gram of pretreated biomass: 5.4, 145 and 4,000, respectively.
In order to test the saccharification yields of the enzymatic extracts, Accelerase 1500 was replaced with the same amount of enzymatic crude extract of Cel1, Cel2 or WT. The reactions were incubated at 600 rpm and 50°C. The samples were collected at different time intervals until 72 hours and centrifuged at 6,000 rpm for 30 min at 4°C. The supernatants were analysed to quantify the amount of sugars released by high-performance liquid chromatography (HPLC; Dionex, Sunnyvale, California, USA), using the protocol described in Ventorino et al. (2016).

Considering that the lignocellulosic substrates bioconversions generally take 72 hours and that *P. nigra* and *P. virgatum* were successfully hydrolysed using the extracellular enzymatic mixture of *S. argenteolus* AE58P, after 72 hours of incubation (Pennacchio et al. 2018), the glucose and xylose yields for Cel1 and Cel2 were evaluated after 72 hours of incubation. Cel1 and Cel2 exhibited glucose and xylose yields comparable to the yields of the commercial cocktail and the wild type enzyme on pretreated *P.*

nigra and *P. virgatum* (Table 2). The glucose and xylose yields in *P. nigra* were higher than those in *P. virgatum,* considering the macromolecular composition in terms of glucans and xylans of these biomasses (Pennacchio et al. 2018).

Table 2: Saccharification experiments on pretreated *P. nigra* and *P. virgatum* using culture supernatants of *E. coli* expressing Cel1 or Cel2, enzymatic commercial cocktail and a culture supernatant of *S. argenteolus* AE58P.

	Populus nigra		***Panicum virgatum***	
	Glucose yield (g L^{-1})	**Xylose yield (g L^{-1})**	**Glucose yield (g L^{-1})**	**Xylose yield (g L^{-1})**
	72 h	**72 h**	**72 h**	**72 h**
Enzymatic commercial cocktail	23.90±0.22	6.96±0.02	4.69±0.02	1.7±0.01
***S. argenteolus* AE58P**	21.62±0.21	3.840.06	4.06±0.03	1.92±0.02
Cel1	25.10±0.12	7.11±0.003	5.26±0.04	3.52±0.01
Cel2	25.28±0.08	7.29±0.04	5.38±0.05	3.85±0.07

2.2.2 Development of improved cellulase variants for spent mushroom substrate conversion by directed evolution *(manuscript submitted to New Biotechnology)*

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Abstract

Spent mushroom substrate (SMS), the by-product of mushroom cultivation, represents a residual material whose disposal entails ecological and economic problems, but which could be a source of fermentable sugars. In this work, to improve SMS saccharification, the cloning, recombinant expression in *Escherichia coli* and characterization of two new GH5 family cellulases (Cel1 and Cel2) were performed. Both enzymes showed high thermoresistance and pH resistance, with Cel2 showing better properties than Cel1. Moreover, Cel1 and Cel2 were tested in the hydrolysis of pre-treated SMS/wheat straw (WS) (40/60), with Cel2 showing glucose and xylose yields higher than those of the Cel1 enzyme. Therefore, Cel2 was selected for the generation of 30,000 random mutants by directed evolution in order to develop improved biocatalysts. The Error-prone Polymerase Chain Reaction was used for diversity generation in the *cel2* gene and the screening for activity was carried out. A primary screening on a solid medium revealed 943 active mutants. These, were screened in a liquid medium, allowing the selection of 63 improved variants which were subjected to a scale up of production. Among these, 21 clones showed at worst a two-fold higher activity than Cel2 after the scale up of production, of which 13 had a higher thermoresistance after 72h. The performances of these 13 mutants in the hydrolysis of pre-treated SMS/WS were compared to the wild type Cel2 in conjunction with a commercial enzymatic booster mixture (MetZyme® SUNO™ BOOSTER 144). All the mutants exhibited a glucose yield two-fold or four-fold higher than that of wild-type Cel2 after 72h of incubation.

Keywords: Cellulases, *Streptomyces*, directed evolution, saccharification, spent mushroom substrate, biocatalysts

Introduction

During the last few years, with the progress of cultivation technologies, mushroom production has increased significantly, reaching 35 million tons in 2013 [1][2]. This has resulted in ecological and economic challenges, linked to the disposal of the by-product SMS, considering that the production of each kilogram of mushrooms generates 5–6 kg of residual material [3][4]. This has generated interest in finding new treatment technologies and new uses of this by-product [4], a factor of high relevance in the perspective of the circular economy [5]. In this context, it is noteworthy that the SMS is a lignocellulose material which can represent a resource for fermentable sugars production and the generation of added-value chemicals and materials [6][2][7][4][8][3][9].

However, nowadays, the production of second generation biochemicals at competitive levels is hindered by the high costs of the major enzymes needed for lignocellulolytic biomass conversion, namely cellulases and hemicellulases [5][10].

To reduce the enzymatic saccharification costs, research and development activities both in academic and industry sectors, have focused their attention on the development of more efficient biocatalysts for the lignocellulose hydrolysis, be means of the isolation of new microbes from different habitats, the discovery of new enzymes by metagenomic approaches or the improvement of existing enzymes by genetic engineering [11][12][10][13][14][15][16].

Bacteria and fungi from different habitats, such as soil [17][13][18], water [19], agricultural wastes [20][21], compost [22][23] and plants [24][25], represent a large natural reservoir of (hemi)cellulolytic enzymes due to the necessity for their production for the habitat colonization [15][26].

As shown in our previous communication [25], 24 Actinobacteria were isolated from the lignocellulose biomasses of *Arundo donax, Eucalyptus camaldulensis* and *P. nigra,* naturally subjected to biodeterioration, in order to find microorganisms able to produce biocatalysts for the hydrolysis of pre-treated lignocellulose biomasses. The screening for the cellulase activity of these strains allowed us to select *Streptomyces argenteolus* AE58P as the best cellulase producer. Moreover, the *A. donax*, *P. nigra* and *P. virgatum* pretreated biomasses were successfully hydrolysed using

the extracellular enzymatic mixture of *S. argenteolus* AE58P [25][14]. Thereby, the cellulolytic enzymes of *S. argenteolus* AE58P were identified and among these proteins, two proteins (ID 503810539 and 503918330) were revealed to belong to the GH5 family [25] and named Cel1 and Cel2. In this work these two proteins were chosen to be recombinantly expressed and characterized and to select a cellulase for directed evolution experiments. A strategy to generate and screen 30,000 directed evolved mutants of the GH5 cellulase Cel2 from *S. argenteolus* AE58P was developed and applied, in order to obtain improved biocatalysts in the hydrolysis of pre-treated SMS.

Materials and methods

Strains media and chemicals

The *Escherichia coli* Top 10 cells (Invitrogen) were grown in Luria–Bertani (LB) medium (10 g L^{-1} bactotryptone, 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract), using 50 μg ml^{-1} ampicillin.

The heterologous expression was performed using as the host the strain *E. coli* BL21 CodonPlus (DE3) RP (Novagen Ltd). The error-prone (ep) PCR (Polymerase Chain Reaction) mutagenesis was performed with the Gene Morph II Random Mutagenesis Kit (Agilent, La Jolla, California, USA). The restriction enzymes and buffers were provided by New England Biolabs (Beverly, MA, USA). 4-Nitrophenyl β-D-glucopyranoside (pNPG) was obtained from Alfa Aesar (Kandel, Germany). The saccharification experiments were performed using the enzymatic commercial mixture used in Pennacchio et al. 2018 [14] and the enzymes were provided by Genencor (Rochester, New York, USA).

The enzymatic mixture MetZyme® SUNO ^{TM} BOOSTER 144 was supplied by MetGen Oy

Gene sequencing

The new GH5 cellulase encoding genes from *S. argenteolus* AE58P, named Cel1 and Cel2, which in proteomics analyses showed peptide sequences matching with proteins with id 503810539 and 503918330, were synthesized by Polymerase chain reaction (PCR) on *S. argenteolus* AE58P genomic DNA, using degenerate oligonucleotides as primers. The primers sequences were designed on the basis of the sequences of peptides identified in our previous work [25].

The degenerate oligonucleotides are reported below. 1 FW/1 REV for the amplification of the central region: (gtc ggc aty gtg ggc ctg ggc/gcc ytc sgg rta rtc ytc gaa aaa and gcg acc acc tty tgy ctg gay gay gtg/ rta sga ctg gtt cag sgg gtt for Cel1 and Cel2, respectively); 2 FW/2 REV for the amplification of the 5' region (atg aay gay gay gtn ccn ggn ggt ctg gac/ ccc cac agy gar tat ccs gay ggc and gct ngc gca sgc scg gcc gtt cag/ cac rtc rtc cag rca raa ggt ggt cgc for Cel1 and Cel2, respectively); and 3 FW/3 REV for the amplification of the 3' terminal region (ccc cac agy gar tat ccs gay ggc/ sgc ytt scg cca ytc rcc ccg ggt and aac ccs ctg aac cag tcs tay/ gct ngc gca sgc scg gcc gtt cag for Cel1 and Cel2 respectively).

The sequencing of the DNA fragments was performed by Eurofins Genomics Service (Milan, Italy).
The sequences of the genes coding for the new GH5 cellulases Cel1 and Cel2 from *S. argenteolus* AE58P, are available in the EMBL Data Library (MN845760 and MN845759 respectively).

Cloning and recombinant expression
The Cel1 and Cel2 encoding genes were cloned into pBAD and pET22b (Novagen, Inc.) expression vectors, obtaining Cel1- pBAD, Cel2- pBAD, Cel1- pET22b and Cel2- pET22b and which were expressed in *E. coli* BL21- (DE3) RP (Novargen Ltd).
The cells were grown at 37°C in 0.05 L of LB (50 μg mL^{-1} ampicillin), until an optical density (O.D.) of 0.7. Different concentrations of Isopropil-β-D-1-tiogalattopiranoside, IPTG (0.01, 0.1 and 0.5 mM) and arabinose (0.002% and 0.02%), incubation times (3 hours and overnight) and temperatures (20°C and 37°C) were tested for the proteins expression.
The mutant cells were cultured at 37°C in 0.05 L of LB medium with 50 μg mL^{-1} ampicillin until an optical density (O.D.) of 0.7 and 0.02% arabinose was used as an inducer of the protein expression overnight at 20°C.
The cells were removed by centrifugation of the liquid cultures for 20 minutes at 4°C (6,000 rpm). The supernatants of the samples were used to measure the cellulase activity towards pNPG.
The results correspond to the average of three experiments, each performed in triplicate.

Determination of protein concentration
The protein concentration of the enzymatic extracts was determined as in Amore et al. 2012 [27]

Determination of optimal temperature and pH
The optimal temperature of the recombinant enzymes was evaluated using pNPG as substrate, dissolved in 0.05 M Na- citrate buffer at pH 5, with the incubation performed for 10 minutes at 37°C, 45°C, 50°C, 55°C and 60°C.
The determination of the optimal pH for the cellulase activity was performed using the substrate pNPG, dissolved in 0.05 M citrate phosphate buffers, with pH values between 4.0 and 6.0 with the incubation performed for 10 minutes at 45°C and 55°C for Cel1 and Cel2, respectively.

To determine the optimal temperature and optimal pH for cellulase activity for the best mutants, pNPG was dissolved in 0.05 M phosphate buffer at different pH (4-6), performing the incubation for 10 minutes at 37°C, 45°C, 50, 55°C and 60°C.
The results correspond to the average of three experiments, each performed in triplicate.

Effect of temperature and pH on the enzyme activity
To determine the thermoresistance of the recombinant enzymes, pNPG was dissolved in 0.1 M Na- acetate buffer at pH 5, with the incubation performed at 37°C, 45°C, 50°C, 55°C and 60°C until 72 hours.
The determination of the pH resistance of the cellulase activity was performed using the substrate pNPG, dissolved in 50 mM citrate phosphate buffers, with pH values between 3.0 and 7.0 at 45°C and 55°C for Cel1 and Cel2, respectively. The course of the enzymatic activity was followed for 72 hours.
To determine the thermoresistance of the best clones, pNPG was dissolved in 0.1 M Na- acetate buffer at the optimal pH of wild type Cel2 (pH 5), and at the optimal pH of each clone. The incubation was performed at the optimal temperature of each clone and of wild type Cel2 (55°C), for 72 hours, collecting the samples at different times.
The results correspond to the average of three experiments, each performed in triplicate.

Evaluation of substrate specificity
The substrate specificity of Cel1 and Cel2 was measured towards four substrates (pNPG, azo-CMC, azo-Avicel and filter paper). The substrate pNPG (Sigma Aldrich, Milan, Italy) was dissolved in 0.1 M sodium acetate buffer at pH 5 in a final concentration of 20 mM and was incubated, with the culture supernatants of *E. coli* recombinantly expressing Cel1 and Cel2, at 45°C and 55°C, respectively, for 10 minutes. After adding 0.5 M Na_2CO_3, the absorbance was read at 405 nm. One unit of glycoside hydrolase activity was the measure of enzyme needed to release 1 µmol min^{-1} of p-nitrophenol.
The substrate AZO-CMC (Megazyme, Ireland) was dissolved in 0.05 M Na- citrate buffer pH 5 to obtain a concentration of 2% w/v and the incubation (50°C) was performed with the culture supernatants of *E. coli,* expressing Cel1 and Cel2, for 5 minutes.

After adding the precipitating agent and centrifuging at 12,000 rpm for 10 minutes, the absorbance was read at 590 nm for each sample.
A slurry of the substrate Azo-Avicel in 0.1 M Na- acetate buffer (pH 4.5) was incubated for one hour in a final concentration of 2% w/v, with the culture supernatants of *E. coli,* expressing Cel1 and Cel2 at 40°C. The reaction was stopped by 2% trizma base solution (pH ~ 8.5). The absorbance was measured at 590 nm, after the centrifugation of the samples for 10 minutes (3,000 rpm).
The activity assay towards Filter Paper (no. 1), purchased from Whatman (Chiltern, UK) was performed as in [14].
The results correspond to the average of three experiments, each performed in triplicate.

Determination of V_{max} and K_M
The kinetics experiments for Cel1 and Cel2 were performed using the substrate pNPG (1 mM, 10 mM, 20 mM, 30 mM and 50 mM), dissolved in 0.1 M Na- acetate buffer (pH 5). The culture supernatants of *E. coli,* expressing Cel1 and Cel2, were incubated with the substrate at their optimal temperature (45° C and 55° C, respectively), for 15 minutes and the absorbance values were measured by taking the readings at 405 nm every minute. From these measurements, it was possible to calculate the kinetic constants.
The values of the Michaelis–Menten constants (K_M and V_{max}) of Cel1 and Cel2 were identified using the GraphPad software.
The results correspond to the average of three experiments, each performed in triplicate.

Feedstock and feedstock combinations
The biomass substrates used as raw materials were SMS and WS, were the same materials used in Beckers et al. (2019) [28].
For the development of the assays, feedstock combinations were prepared consisting of a mixture of SMS and WS in a wet weight ratio of 40% and 60% (20% and 80% in dry basis), respectively.

Thermochemical Pretreatment
The biomasses were treated at the CENER facilities of Biorefinery and Bioenergy Centre (BIO2C), located in Aoiz (Spain). The thermochemical pre-treatment assays were carried out in the reactor provided by Advancebio Systems LLC, USA as cited by

Beckers et al. (2019) [28]. The samples used for this particular study were treated either at 170ºC or 183ºC with a fixed residence time (10 minutes). The SMS and WS feedstocks were mixed based on dry matter content and adjusting the moisture up to 50% by adding the catalyst (NaOH 50%) using 2% of dry weight on the total solid content. This was followed by a manual mixing to reach homogenisation and, before feeding the reactor, the mixture was left overnight at room temperature. Indeed, these conditions were part of a broader experimental design for thermochemical fractionation using the feedstock selected (data not shown).
The pre-treated samples, generated under the conditions tested, were taken and, after collecting a sample for compositional analysis, were subjected to a manual filter press for solid and liquid fraction separation. The solid fraction was stored at 4ºC in zipped plastic bags until use for the enzymatic hydrolysis.

Compositional analysis
The moisture content in the SMS was calculated as in Beckers et al. (2019) [28], following the CEN/TS 14774-3:2010 procedure. The ash content was determined as mentioned in Beckers et al. (2019) [28] following UNE-EN ISO18122:2015. The elemental analysis of nitrogen determination was carried out following UNE-EN ISO 16948:2015 and using a conversion factor into protein of 5.25.
The pre-treated materials (slurries) were separated into insoluble and soluble fractions, and the cellulose, hemicellulose and lignin contents were quantified from the insoluble fraction by the NREL procedures nº 42618[1] and nº 42627[2] respectively. The oligomeric sugars were hydrolysed by adding 72% H_2SO_4 (w/w), followed by autoclaving at 121ºC for 1 hour. The samples were stored at -20ºC until analysis.
The soluble sugar analysis was performed by high-performance liquid chromatography (HPLC), quantifying the content of monosaccharides (D-glucose, D-xylose, D-fructose, D-galactose, L-arabinose), disaccharides (cellobiose, maltose) and trisaccharides (maltotriose) as reported in Beckers et al. (2019) [28].

[1] A. Sluiter, Laboratory Analytical Procedures, (2011). www.nrel.gov/biomass/pdfs/42618.pdf
[2] A. Sluiter, Laboratory Analytical Procedures, (2008). www.nrel.gov/biomass/pdfs/42627.pdf

Enzymatic hydrolysis

The saccharification experiments were performed using pre-treated SMS supplemented with WS on a rotary shaker (ThermoMixer C, Eppendorf, Milan, Italy).

The culture supernatants of *E. coli* recombinantly expressing Cel1 and Cel2 and the culture supernatants of *S. argenteolus* AE58P [25] were incubated with 10% (w/v) of pre-treated biomass in 2.5 mL of 0.05 M Na- citrate buffer (pH 5). The enzymatic commercial cocktail used as the benchmark was composed of Accelerase1500, Accelerase BG, and Accelerase XY prepared as reported in Pennacchio et al. 2018 [14].

The saccharification yields of Cel1 and Cel2 were tested, replacing Accelerase 1500 with the same amount, in terms of activity, of culture supernatant of *E. coli* expressing Cel1 and Cel2 and culture supernatant of *S. argenteolus* AE58P. The incubation was performed at 50°C (600 rpm). The samples were collected daily until 72 hours and centrifuged at 4°C for 30 minutes (6,000 rpm). The amounts of glucose and xylose released were quantified, analysing the supernatants by high-performance liquid chromatography (HPLC; Dionex, Sunnyvale, CA, USA) as in Ventorino et al. (2016) [25]. The saccharification experiments with SMS/WS (40:60) were also carried out using the culture supernatants of *E. coli* recombinantly expressing the Cel2 and epCel2 mutants added to the enzymatic mixture MetZyme® SUNO ™ BOOSTER 144 supplied by MetGen Oy. MetZyme® SUNO ™ BOOSTER 144 is a high performance booster cocktail custom designed to significantly enhance the cellulose hydrolysis efficiency of cellulases. Three different conditions were tested for two clones and the wild type enzyme, to select the best condition and apply it to the other mutants, as reported below:

•Condition 1- The enzymatic mixture SUNO ™ BOOSTER 144 and the cellulase enzyme (Cel2 wild type or epCel2 mutants) were added to the biomass in the same volume/volume ratio (50:50 V/V- in terms of unit of enzymes, 1.5 U and 0.35 U were added, respectively).

• Condition 2- 0.54 U of the SUNO ™ BOOSTER 144 and 0.54 U of the cellulase enzyme (Cel2 wild type or epCel2 mutants) were added to the biomass.

• Condition 3- 1.08 U of the SUNO ™ BOOSTER 144 and 1.08 U of cellulase enzyme (Cel2 wild type or epCel2 mutants) were added to the biomass.

The experiments for the hydrolysis of pre-treated SMS/WS were performed by incubating the enzymatic cocktails with 10% (w/v) of the biomass in 2.5 mL of 0.5 M succinic acid, at the optimal pH and temperature and of each enzyme on a rotary shaker (500 rpm). The samples were collected daily and until 72 hours and centrifuged at 4°C for 30 minutes (6,000 rpm). The monosaccharides obtained were quantified by analysing the supernatants, by HPLC [25] and the yields of the hydrolysed fractions were expressed in g L^{-1}.
The results correspond to the average of three experiments, each performed in triplicate.

Construction of the Cel2 error-prone PCR library
The pBAD-Cel2 construct was used as the target sequence for epPCR and the random mutations were introduced with the Gene Morph II Random Mutagenesis Kit. The PCR reactions were performed using the mix and program reported in Cecchini et al. (2018) [29]. About 20 epPCR were performed to obtain 48 µg total of Cel2 cDNA, for the construction of the 30,000 mutants library. The DNA regions obtained were purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), the digestion with the same restrictions enzymes used for Cel2 was performed and the cloning into a pBAD vector was carried out. The mixture obtained after the ligation was employed for the *E. coli* TOP10 cells transformation, plated into a LB agar medium with ampicillin (50 µg mL^{-1}) and grown at 37°C overnight. Liquid medium LB with 50 µg mL^{-1} ampicillin was added to the plate surface in order to collect the colonies with a scraper. After the cells centrifugation for 10 minutes at 4°C (6,000 rpm), the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) was used for the plasmid DNA preparation to transform the *E. coli* BL21 (DE3) RP cells for the mutants expression.
The cells were incubated overnight at 37°C on LB agar (50 µg mL^{-1} ampicillin), to select the error-prone Cel2 (epCel2) mutants.

Screening of the epCel2 mutants library
The Cel2 mutants were inoculated in LB medium (200 µL in 96-well plates) by the robot QPIX 450 (Molecular Devices, LLC, California, USA). The mutants were incubated at 37°C overnight and screened on solid medium in order to discriminate transformants expressing extracellular cellulase activity from those that do not

express it. Therefore, each clone of the library was transferred onto 22 × 22 cm Q-Tray bioassay plates containing LB Agar, ampicillin (50 µg mL^{-1}), the substrate 1% w/v carboxymethylcellulose (CMC) and the inducer 0.002% w/v arabinose, using a QPix colony picker with a 96-well gridding head (Molecular Devices, Sunnyvale, California).

Several trials of the solid medium assay were performed, incubating the plates at 37°C for 2, 4, 6 and 8 hours and overnight. In order to identify the transformants able to degrade the CMC. The plates were dyed using a Congo red (0.1% w/v) solution for 15 minutes, followed by three washings with 3 M sodium chloride (NaCl). The trials of the solid medium assay were performed three times.

The mutants, forming a clear halo around the colony, were selected as positive and were screened in liquid medium, in order to detect the clones more active than Cel2 towards pNPG.

In order to define the best conditions for the enzymes production, trials of recombinant expression of Cel2 were performed in microscale, using different concentrations of the inducer arabinose (0.002%, 0.02%, 0.2%) and two different times for the addition to the growth medium (at the beginning of the growth or at 0.7 OD). The experiments were repeated three times and the data were reported as means ± standard deviations.

After the mutants incubation at 37°C overnight, 200 µL of the cultures were diluted with LB medium (800 µL in 96 deep-well microplates) and ampicillin (50 µg mL^{-1}), using the robot BioMek NXP (Beckman Coulter, California, USA). The incubation was performed at 37°C. When the cultures reached 0.7 OD, arabinose (0.002% w/v) was used to induce the protein production at 37°C, overnight.

After centrifugation at 3,000 rpm for 30 minutes at 4°C, the activity of the clones was analysed towards the chromogenic substrate pNPG, dissolved in 0.1 M Na- acetate buffer at pH 5. The enzymes were incubated with the substrate at 55°C (Cel2 optimal temperature) for 10 minutes and the absorbance was measured at 405 nm with the Multi Detection SystemGloMax® Discover System (Promega, Wisconsin, USA).

Results and discussion

Cloning and recombinant expression of Cel1 and Cel2

Two new GH5 family cellulases, named Cel1 and Cel2, were identified among several cellulolytic proteins from *S. argenteolus* AE58P isolated from *A. donax, E. camaldulensis* and *P. nigra*, in our previous work [25].

The Cel1 and Cel2 encoding genes were synthesized by PCR on *S. argenteolus* AE58P genomic DNA. For both cellulase encoding genes, DNA fragments of the expected length were obtained and sequenced using specific oligonucleotide primers. The *cel1* and *cel2* genes were cloned into pBAD and pET22b vectors and were expressed in *E. coli*. Trials of the recombinant expression of both enzymes were performed to define the best conditions for the enzymes production, using different concentrations of IPTG and arabinose, incubation times and temperatures, and using pNPG as the substrate to measure the enzymatic activity (Table 1).

Table 1: Trials of recombinant expression. The values represent the means ± SD of three replicates

vector	inducer	incubation temperature °C	incubation time	Average U/ml Cel1	Average U/ml Cel2
pBAD	0.002% arabinose	20°C	3 hours	0.205±0.023	0.216±0.012
			overnight	0.320±0.019	0.249±0.024
		37°C	3 hours	0.134±0.011	0.163±0.029
			overnight	0.132±0.009	0.183±0.026
	0.02% arabinose	20°C	3 hours	0.170±0.013	0.203±0.033
			overnight	0.263±0.017	0.282±0.042
		37°C	3 hours	0.115±0.025	0.148±0.051
			overnight	0.059±0.019	0.114±0.027
pET22b	0.01 mM IPTG	20°C	3 hours	0.055±0.031	0.079±0.031
			overnight	0.071±0.027	0.097±0.034
		37°C	3 hours	0.082±0.015	0.101±0.027
			overnight	0.101±0.014	0.128±0.033
	0.1 mM IPTG	20°C	3 hours	0.110±0.026	0.148±0.032
			overnight	0.126±0.025	0.148±0.041
		37°C	3 hours	0.086±0.012	0.172±0.042
			overnight	0.137±0.035	0.159±0.009
	0.5 mM IPTG	20°C	3 hours	0.102±0.024	0.125±0.010
			overnight	0.141±0.027	0.187±0.025
		37°C	3 hours	0.126±0.009	0.148±0.039
			overnight	0.159±0.008	0.187±0.023

The best condition for the production of both enzymes was obtained when the genes were cloned into the pBAD vector and the protein expression was induced by 0.002% arabinose for Cel1 and 0.02% arabinose for Cel2 at 20°C, overnight. The culture supernatants of *E. coli* recombinantly expressing Cel1 and Cel2, obtained in these conditions, were used for the characterization of the recombinant enzymatic activity.

Characterization of the Cel1 and Cel2 recombinant enzymes
Evaluation of the substrate specificity

Substrate specificity of the recombinant cellulases, Cel1 and Cel2, was analyzed towards pNPG, azo-CMC, azo-Avicel and Whatman Filter Paper. The ability of Cel1 and Cel2 to hydrolyze both crystalline and amorphous regions of cellulose was measured towards the substrate Whatman filter paper. The amount of enzyme that releases 1 μmol $mL^{-1}min^{-1}$ of glucose was 26.48±0.03 and 33.64±0.04, respectively (Table 2). These can be considered significant levels compared to the literature. As a matter of fact, the FPCase (Filter Paper Cellulase) activity measured in this work for both the recombinant enzymes was three-fold (Cel1) and seven-fold (Cel2) higher compared to the endoglucanase JqCel5A (4.8±0.21 U mg^{-1}), which was cloned from the Actinobateria *Jonesia quinghaiensis* and functionally expressed in *Escherichia coli* Rosetta (DE3) [30]. Moreover, the FPCase activity was 40-fold higher compared to the most active strain (among the nine actinomycetes investigated) tested by Mohanta (2014) [31].

The Azo CMCase activity for Cel1 and Cel2 was about three times lower compared to the CelStrep enzyme [27], but was in the range of the activity exhibited by *S. argenteolus* AE58P after 6 days of fermentation [25].

The activity of Cel1 and Cel2, using pNPG as a substrate, was about 6 (Cel1) and 10 (Cel2) fold higher compared to the Azo-CMCase activity despite the fact that Cel5A -a cellulase belonging to the same family, GH5, as Cel1 and Cel2- was not able to hydrolyze pNPG [32].

The activity of Cel1 and Cel2 towards azo-Avicel was very low. It is worth noting that Cel1 and Cel2 showed higher activity towards the synthetic substrate pNPG than that towards azo-Avicel, an insoluble substrate with an high crystallinity. This result could underline the necessity to improve the ability of Cel1 and Cel2 to hydrolyze more recalcitrant crystalline substrates. Moreover this

aspect will be confirmed with the test of Cel1 and Cel2 on the real biomass SMS supplemented with WS.
Based on these results, pNPG was selected as the substrate for the next steps of the characterization of Cel1 and Cel2.

Table 2: Substrate specificity of the recombinant cellulases Cel1 and Cel2 measured towards pNPG , azo-CMC, azo-Avicel and Whatman Filter Paper

	pNPG (20 mM)	**Azo-CMC (2% w/v)**	**azo-Avicel (2% w/v)**	**Whatman Filter Paper (50 mg)**
	U/ml			**FPU/mL**
Cel1	2.44±0.06	0.38±0.04	<0.01	26.48±0.03
Cel2	3.11±0.16	0.29±0.05	<0.01	33.64±0.04

Determination of V_{max} and K_M
K_M and V_{max} values for Cel1 and Cel2 were calculated measuring the activity towards different concentrations of pNPG and plotting the data according to the Michaelis–Menten equations. The K_M value for Cel1 was 6.50±0.31mM and the V_{max} 0.035±0.002 mM min^{-1} while for Cel2 the K_M was 5.61± 0.20 mM and the V_{max} was 0.033±0.01 mM min^{-1}. Both enzymes exhibited a higher affinity for the pNPG substrate, compared to the β-glucosidase from *Streptomyces griseus* with K_M and V_{max} values of 8.6 ± 0.5 mM and 217 ± 5.0 $\mu moles^{-1}$ min^{-1}mg, respectively [33], which could highlight a β-glucosidase activity for these enzymes. Moreover, the kinetic parameters suggested that Cel2 showed a higher affinity for pNPG than Cel1.

Determination of the optimal temperature and pH
The optimal pH and temperature for the Cel1 and Cel2 activity were determined towards pNPG. The activity values, reported as U ml^{-1}, are shown in Figures 1a and 1b. The results show that the optimal temperatures for Cel1 and Cel2 were 45°C and 55°C, respectively. The behaviour of our enzymes conforms to those of

other bacterial cellulases, most of which showed a maximum of activity from 40°C to 55°C [34] [35] [36].
Moreover, the optimal temperatures for Cel1 and Cel2 were close to the temperature for the higher cellulase activity of *S. argenteolus* AE58P reported in our previous work [25].
In particular, the optimal temperature for Cel1 (45°C) was close to the optimal temperature of the endoglucanase from *Streptomyces sp.* B-PNG23 characterized by Azzeddine et al. (2013) [37]. On the other hand, Cel2 showed the maximum activity at the same temperature (55°C) as the cellulase Cel5A, belonging to the GH5 family and characterized by Klippel et al. (2019) [32].

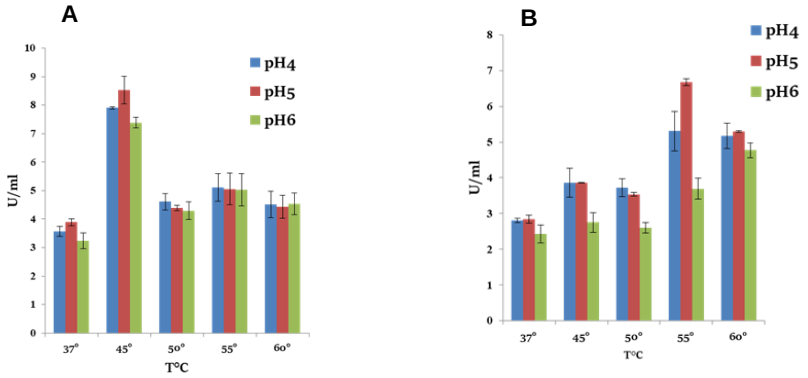

Figure 1: A Optimal temperature and pH of activity towards pNPG for Cel1; **B** Optimal temperature and pH of activity towards pNPG for Cel2

An optimal pH of 5.0 was identified for both Cel1 and Cel2. This is comparable with the cellulase Cel5A [32] and it is close to the optimal pH value reported by Oliveira et al. (2016) [38]. Moreover, in Ventorino et al. (2016) [25] we demonstrated that the optimal pH for cellulase activities produced by *S. argenteolus* AE58P is 5.

Effect of temperature and pH on enzyme activity
Considering that lignocellulosic substrate bioconversions generally take 72 hours [25][14][39][40][41], it is essential that the adopted enzymes maintain their activity to obtain a high conversion yield of the substrates. Therefore, the thermoresistance and pH resistance of the Cel1 and Cel2 enzymes were determined and compared.

As shown in Figures 2A and 2B, when we performed a one hour incubation the recombinant enzyme Cel1 maintained about 60%-70% of activity for all the tested temperatures and Cel2 retained about 80% of activity at 37°C, 45°C, 50°C. In particular, after 72 hours, both Cel1 and Cel2 retained 60% of activity at 37°C and about 40% and 50% respectively at 50°C (Figures 2A and 2B). These values indicate a better performance compared to *S. griseus* β-glucosidase which lost activity after three hours of incubation [33].

The determination of the pH resistance of cellulase activity was performed at pH values from 3.0 to 7.0 with an incubation time of 10 minutes at 45°C and 55°C for Cel1 and Cel2, respectively (Figures 2C and 2D). As shown in Figures 2C and 2D, Cel1 maintained about 70-80% of its initial activity in the pH range from 4 to 7 after 1 hour of incubation, and kept 60% of its initial activity after 72 hours of incubation at pH 5. It lost activity at the other pH values tested. The Cel1 residual activity at pH 5 after 24 hours of incubation was 80%.

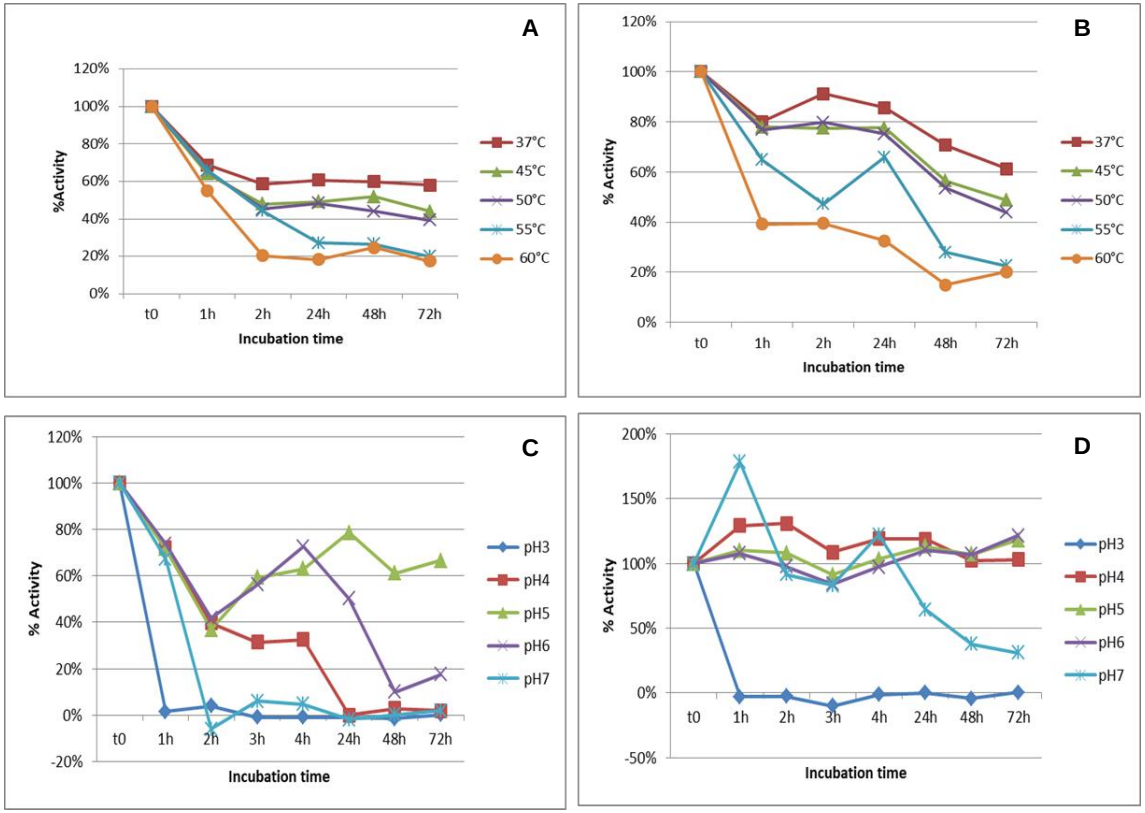

Figure 2: A Thermoresistance of activity towards pNPG for Cel1; **B** Thermoresistance of activity towards pNPG for Cel2; **C** pH resistance of activity towards pNPG for Cel1; **D** pH resistance of activity towards pNPG for Cel2

This value is comparable to the residual activity of the endoglucanase secreted by *Streptomyces capoamusin,* evaluated in the pH range from 3 to 9 at 25°C for 24 hours [38]. On the contrary, Cel2 retained 100 % of its initial activity after 72 hours of incubation at pH 4, 5 and 6, outperforming the endoglucanase secreted by *S. capoamusin* [38].

Spent mushroom substrate pretreatment

Feedstock compositional analysis and their combination

The compositional analysis of SMS revealed that its carbohydrate content is relatively low (12% and 6 % for glucan and xylan, respectively) as reported in Table 3.

Therefore, in order to increase the carbohydrate content, SMS was combined with another underutilized feedstock, WS [28]. The carbohydrate content of WS was three-fold higher than SMS: the glucan content and the xylan content in WS was 3.5-fold higher than SMS, as reported in Table 3.

Table 3: Compositional analysis of SMS and WS. WB: wet basis; DM: dry matter

	SMS		WS
	Avg.		Avg.
Moisture (%WB)	76.7±0.1	**Extractives (%DM)**	14.8±2.6
Ashes (%DM)	28.9±0.4	**Glucan (%DM)**	34.1±1.4
Protein (%DM)	11.0±0.3	**Xylan (%DM)**	20.8±1.1
Glucan (%DM)	11.8±1.5	**Arabinan (%DM)**	2.1±0.3
Xylan (%DM)	5.9±0.7	**Insoluble Acid Lignin (%DM)**	14.3±0.8
Arabinan (%DM)	0.5±0.0	**Acid Soluble Lignin (%DM)**	0.7±0.0
Lignin (%DM)	34.0±0.4	**Insoluble acid Ashes (%DM)**	0.7±0.3
Soluble sugar (%DM)s	6.1±0.1		
Organic acids (%DM)	2.8±0.0		

Table 4: Compositional analysis of the binary mixtures SMS and WS (60% and 40% in wet basis respectively) WB: wet basis; DM: dry matter

	SMS:WS **170ºC, 2% NaOH, 10 minutes**	**SMS:WS** **183ºC, 2% NaOH, 10 minutes**
% W/W	**Average**	**Average**
Moisture (WB)	55.2±0.2	61.4±0.9

INSOLUBLES (DM)		
Glucan (DM)	32.8±0.6	31.7±0.4
Xylan (DM)	18.9±0.5	14.4±0.3
Arabinan (DM)	1.7±0.0	0.9±0.0
Acid insoluble Lignin (DM)	20.4±0.4	19.3±0.2
Acid soluble Lignin (DM)	1.0±0.0	0.8±0.0
Acid soluble Ashes (DM)	2.1±0.1	1.9±0.1
Acetyl (DM)	4.9±0.0	3.1±0.0
SOLUBLES (DM)		
Glucose	0.7±0.0	0.9±0.0
Xylose	1.7±0.1	3.5±0.1
Arabinose	0.5±0.0	0.6±0.0

Thermochemical pretreatment

Several thermochemical (TC) pretreatment experiments were performed on the SMS/WS mixtures with different ratios, considering the recalcitrance of this biomass to fractionate the biomass into different components in order to obtain valuable bio-based products.

Due to the stickiness in the behaviour of SMS it was not possible to feed the horizontal continuous reactor automatically. However, it worked successfully when the SMS was mixed with WS in a 40 to 60 ratio on wet basis. Another important factor with regard to the SMS assessment through TC pretreatment is the water content which can be as high as 70%. Considering that for an optimum performance of the TC pretreatment reactor an initial moisture content of 50% in the feedstock is required, some of this required water can be supplied through the addition of the SMS, reducing the need for additional water supply.

Next, the use of SMS in combination with other lignocellulose feedstock can positively help to adjust the moisture content in the feedstock and also increase the quantity of sugars available at the end of the process.

Pretreated samples, generated under the conditions tested, were subjected to a manual filter press for solid and liquid fraction separation and their compositions were analysed as reported in Table 4.

Enzymatic hydrolysis of the lignocellulose substrate using Cel1 and Cel2

In order to compare the glucose and xylose yields of the recombinant enzymes Cel1 and Cel2 in the hydrolysis of the pretreated SMS - WS (40:60) substrate, the culture supernatant of *S. argenteolus* AE58P and an enzymatic commercial cocktail (Accelerase1500, Accelerase BG and Accelerase XY) were used as the benchmark. The performances of Cel1 and Cel2 in the biomass conversion were investigated by replacing the Accelerase 1500 with the culture supernatants of *E. coli* expressing Cel1 or Cel2 and comparing the yields with those obtained using culture supernatant of *S. argenteolus* AE58P, instead of Accelerase 1500. The reactions were incubated at 50°C (600 rpm), which is the condition most commonly used for the saccharification of cellulolytic biomasses and, as reported in our previous work [25], it

is the temperature required to obtain the higher cellulase activity of *S. argenteolus* AE58P. The samples were collected at 0, 24, 48 and 72 hours and the monosaccharide yields were determined by HPLC.

The saccharification yields after 72 hours of incubation of the culture supernatants of *E. coli* recombinantly expressing Cel1 and Cel2, with SMS-WS, were three times higher than those obtained with the commercial mixture (Table 5). Moreover, the amounts of glucose and xylose obtained with the culture supernatants of *E. coli* recombinantly expressing Cel2 (1.83±0.06 g L^{-1} of glucose and 0.62±0.04 g L^{-1} of xylose) were higher than those obtained using the culture supernatants of *E. coli* recombinantly expressing Cel1 (1.5±0.04 g L^{-1} of glucose and 0.23±0.01 g L^{-1} of xylose).

Table 5: Saccharification experiments on SMS-WS using culture supernatants of *E. coli* expressing Cel1 or Cel2, a commercial enzyme cocktail (Accelerase1500, Accelerase BG and Accelerase XY) and a culture supernatant of *S. argenteolus* AE58P.

	SMS-WS (40:60)			
	Glucose yield (g L^{-1})		**Xylose yield (g L^{-1})**	
	48 h	**72 h**	**48 h**	**72 h**
Enzymatic commercial cocktail	≤0.1	0.52±0.03	<0.1	0.51±0.03
***S. argenteolus* AE58P**	≤0.1	1.10±0.02	<0.1	0.28±0.01
Cel1	≤0.1	1.50±0.04	<0.1	0.23±0.01
Cel2	≤.0.1	1.83±0.06	<0.1	0.62±0.04

Construction and screening of a rCel2 error-prone PCR library

Cel2 was chosen as the template for the directed evolution experiments aimed at developing improved biocatalysts for the

SMS/WS substrate conversion, considering its higher thermoresistance and saccharification yields compared to Cel1. The first step of the directed evolution strategy was the introduction of random mutations in the Cel2 wild type gene sequence, using error-prone PCR (epPCR). Several PCR reactions were performed to obtain the *cel2* DNA for the generation of a library of 30,000 mutants. The second step included the cloning of the randomly mutated forms of the *cel2* gene into the expression vector pBAD and the *Escherichia coli* strain BL21(DE3) transformation. The third step was the high-throughput screening on solid medium carboxymethyl cellulose (CMC) to discriminate the transformants expressing extracellular cellulase activity from those that do not. To determine the conditions of the solid medium assay, different trials were performed and the best condition selected, namely4hours of incubation at 37°C. Among the investigated 30,000 clones investigated, 943 mutants showed a clear halo around the colonies and were selected for the screening in liquid medium in microscale. To define the best conditions for the library screening through the analysis of the enzyme activity production in liquid medium towards pNPG, different trials of recombinant expressions of Cel2 were performed in microscale.

Table 6: Trials of recombinant expressions in microscale. The values represent the means ± SD of three replicates

Inducer at beginning	**Average U/ml Cel2**	**Inducer At 0.7 OD**	**Average U/ml Cel2**
0.002% arabinose	1.70±0.23	**0.002% arabinose**	2.13±0.14
0.02% arabinose	1.12±0.29	**0.02% arabinose**	1.14±0.17
0.2% arabinose	1.48±0.22	**0.2% arabinose**	1.18±0.19

The best condition was 0.002% of arabinose inducer at 0.7 OD (Table 6). After the screening in liquid medium, 63 clones showed a higher activity than wild type Cel2 and were selected for the scale up of production in 0.05 L flasks.

Recombinant expression epCel2 mutants in flasks

After the scale up of production in 50 mL flasks, from the group of 63 mutants which were selected by means of the screening in liquid medium, 36 clones showed a higher activity compared to the wild type Cel2. Mutations of these 36 clones were identified. The ratio of the cellulolytic activity of the mutants and the wild type cellulase, including the results of the mutations are reported in Table 7.

Table 7: Ratio of activity of the variants to that of the wild-type enzyme and the mutations.

Name	**Mutation**	**Ratio of activity of the variant to that of the wild-type enzyme**
Cel2.1	A5S	1.4
Cel2.2	R152S	1.7
Cel2.3	D255H	2.3
Cel2.4	L399V	1.7
Cel2.5	V610M	2.5
Cel2.6	M396R	1.4
Cel2.7	-	-
Cel2.8	A59V	7.3
Cel2.9	S89T	2.2
Cel2.10	G186D	3
Cel2.11	V128M	2.5
Cel2.12	P427A	1.2
Cel2.13	P62A A246E	1.1
Cel2.14	A336P	1.3
Cel2.15	A580P	2
Cel2.16	Y461D	1.1
Cel2.17	S89R	3.4
Cel2.18	V61M	2.7
Cel2.19	D248E Y609N	2.5
Cel2.20	G337V	1.1
Cel2.21	P427R	3.4
Cel2.22	-	-
Cel2.23	R273S	2

Cel2.24	V128I	2.3
Cel2.25	T521S F853L	2.6
Cel2.26	V61L R152H	1.4
Cel2.27	R130H A276S	3.4
Cel2.28	R377C Q456R	6
Cel2.29	S91T G340C	3.6
Cel2.30	I72M	5
Cel2.31	-	-
Cel2.32	I30M	3
Cel2.33	D275I	1.3
Cel2.34	K429E	1.4
Cel2.35	P62A	6
Cel2.36	D275Y	3

Among these 36 mutants, three candidates showed a silent mutation (Cel2.7, Cel2.22, Cel2.31) with the activity similar to that of the WT and 21 clones showed at least a two-fold higher activity than the wild type Cel2.

Enzymatic properties of the rCel2 mutants: optimal pH and temperature and thermostability

21 mutants showed a two-fold higher activity compared to the wild type Cel2 after the scale up of production. The temperature and pH for cellulase activities as well as the thermoresistance were analysed (Figure 3).

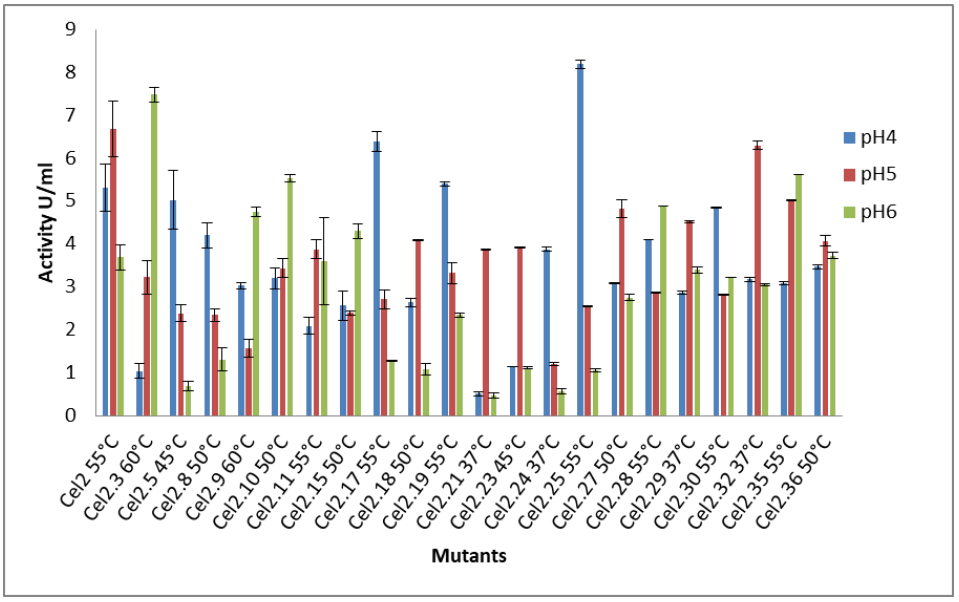

Figure 3: Optimal pH of activity towards pNPG for the Cel2 mutants measured at the optimal temperature.

Among these 21 characterized mutants, 13 clones retained their activity after 72 hours of incubation at their optimal temperature (Figure 4).
The mutants Cel2.18, Cel2.19, Cel2.28 and Cel2.29 retained about 80-90% of activity after 72 hours of incubation. Only four mutants lost 70% of activity after 72 hours of incubation and the other enzymes retained at least 40% of activity after 72 hours of incubation.

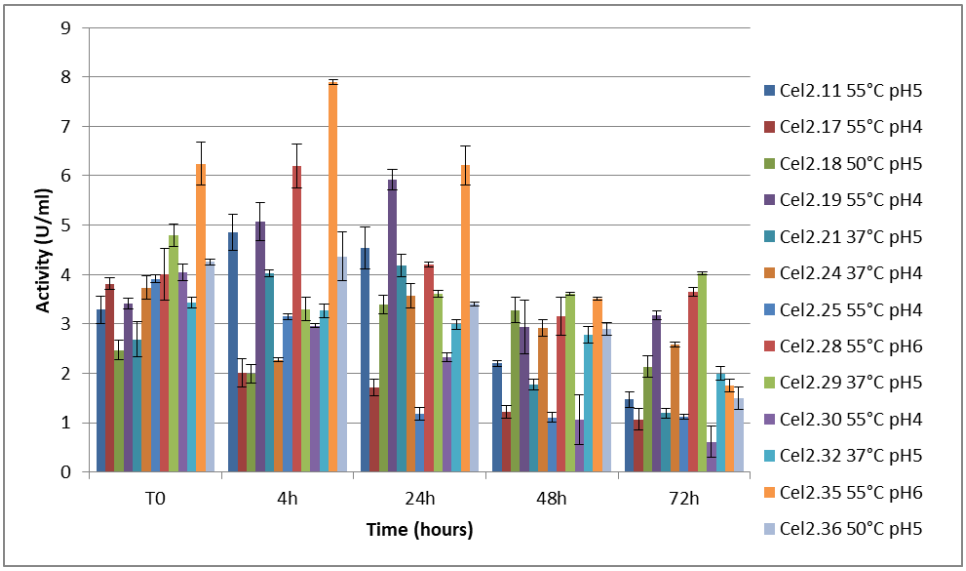

Figure 4: Thermoresistance of activity towards pNPG for the best 13 clones which maintained their activity after 72 hours of incubation at their optimal temperature and optimal pH

Saccharification of a pretreated biomass of SMS-WS by the rCel2 mutants and wild-type enzyme

To evaluate the performances of the 13 best Cel2 mutants in the bioconversion of the pretreated biomass, culture supernatants of *E. coli* expressing these mutants were added to MetZyme® SUNO™ BOOSTER 144, which is a high performance booster enzyme cocktail custom designed to significantly improve the saccharification capabilities of the cellulases. These were adopted in the hydrolysis of the pretreated biomass SMS/WS biomass (40:60) and the sugar yields were compared to those obtained with the enzymatic extract of *E. coli* expressing Cel2 together with the MetZyme® SUNO™ BOOSTER 144. In order to define the saccharification conditions, different experiments were performed for Cel2 and the two mutants, changing the amount of the enzymatic extract added to the biomass. In particular, in the third tested condition, 1.08 U of the culture supernatants of *E. coli* expressing wild type Cel2 or the epCel2 mutants and 1.08 U of the SUNO ™ BOOSTER 144, were added to the biomass. The Cel2 glucose yields, obtained in this condition, were about 1.4-fold higher than the former and about 2.1- fold higher than the latter, as

shown in Table 8. The Cel2 xylose yields, obtained using the third condition were at least 4-fold higher than the other conditions. Therefore, condition 3, was selected as the best and was applied for all the mutants.

Table 8: Glucose and xylose yields after 72 hours of SMS/WS (40:60) saccharification experiments obtained with the three conditions tested, using Cel2 and the two mutants added to the SUNO BOOSTER 144

	Condition 1		**Condition 2**		**Condition 3**	
Enzyme	**Glucose yield ($g\ L^{-1}$)**	**Xylose yield ($g\ L^{-1}$)**	**Glucose yield ($g\ L^{-1}$)**	**Xylose yield ($g\ L^{-1}$)**	**Glucose yield ($g\ L^{-1}$)**	**Xylose yield ($g\ L^{-1}$)**
Cel2	3.12±0.07	0.33±0.01	2.11±0.01	0.22±0.02	4.51±0.3	1.29±0.13
Cel2.19	5.82±0.02	0.41±0.01	5.67±0.04	1.13±0.02	9.53±0.38	2.60±0.32
Cel2.28	5.20±0.05	1.55±0.05	12.10±0.10	2.04±0.03	13.36±0.12	2.66±0.10

The monosaccharide yields of the enzymatic saccharification were estimated on samples collected after 72hours of incubation and expressed in g L^{-1} (Figure 5). It was observed that, when using Cel2 with SUNO^{TM} BOOSTER 144, the glucose and xylose yields were 4.51±0.3 g L^{-1} and 1.29±0.13 g L^{-1}, respectively, while for all the mutants the addition showed an effect on the glucose yield at least two-fold higher than the wild type Cel2. In particular, Cel2.25 and Cel2.29 exhibited a glucose yield four-fold higher compared to Cel2.

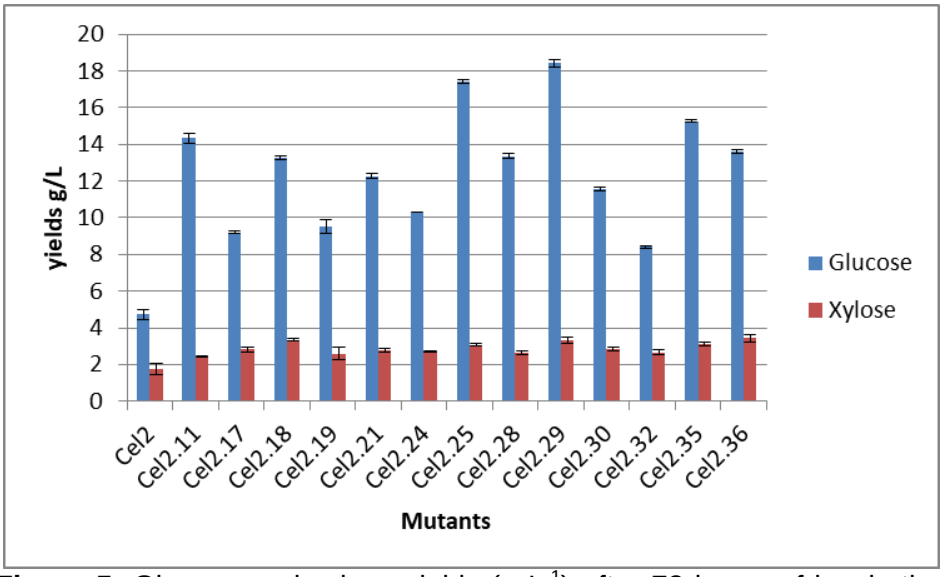

Figure 5: Glucose and xylose yields (g L^{-1}) after 72 hours of incubation of the 13 best mutants and Cel2 added to SUNO BOOSTER 144 with SMS/WS (40:60).

It is noteworthy that the substrate mixture of SMS/WS (40:60)- and the pretreatment conditions adopted in this work were specifically set up for the first time. Since different pretreatments can greatly affect the saccharification yields, it is not possible therefore to carry out a comparison of these conversion performances with those previously reported in literature. However, if we analyse the order of magnitude of the glucose (34.8 g L^{-1}) and xylose (1.03 g L^{-1}) yields obtained from the SMS saccharification reported in Zhu et al. (2016) [39] and the order of magnitude of the glucose and xylose yields obtained from the saccharification of WS, reported in Toquero et al. (2014) (i.e. unwashed WS: about 7 g L^{-1} of glucose and about 3 g L^{-1} of xylose) [41] and Sun et al. (2018) (after hydrothermal pretreatment glucose and xylose were 11.2 g $L^{-1}$and 1.7 g L^{-1}, respectively) [40], our enzymatic mixtures showed similar performances.

Conclusions

Streptomyces argenteolus AE58P had previously been isolated from lignocellulose biomasses, in order to discover new microorganisms producing biocatalysts for the hydrolysis of pretreated lignocellulosic biomasses [25]. Two new cellulases of the GH5 family (Cel1 and Cel2), responsible for the cellulase activity of the strain AE58P, were recombinantly expressed in *E. coli* and were characterized and tested in the enzymatic hydrolysis of pretreated SMS (40%)/ WS (60%). Cel2 was selected for the development of improved biocatalysts by directed evolution, considering thermoresistance and hydrolysis yields higher than Cel1. The strategy specifically set up for the generation of a library of 30,000 random Cel2 mutants and its automated screening for the identification of the most active variants allowed the selection of 63 improved variants. Among these, 21 mutants showing at least a 2-fold higher activity than the wild type Cel2 after the scale up of production were characterized. The 13 mutants exhibiting a higher thermoresistance after 72hours were selected and tested in the bioconversion of pretreated SMS/WS compared to the performance of wild type Cel2 when added to the commercial mixture SUNO ^{TM} BOOSTER 144.

All the mutants exhibited a glucose yield two-fold or four-fold higher than the wild-type Cel2, when added to the commercial mixture SUNO ^{TM} BOOSTER 144. Moreover, the magnitude order of the saccharification yields of the enzymatic mixtures tested in this work was similar to those reported in literature. However, a more specific comparison it was not possible considering that the SMS/WS mixture and the pretreatment were set up in this work.

Acknowledgements

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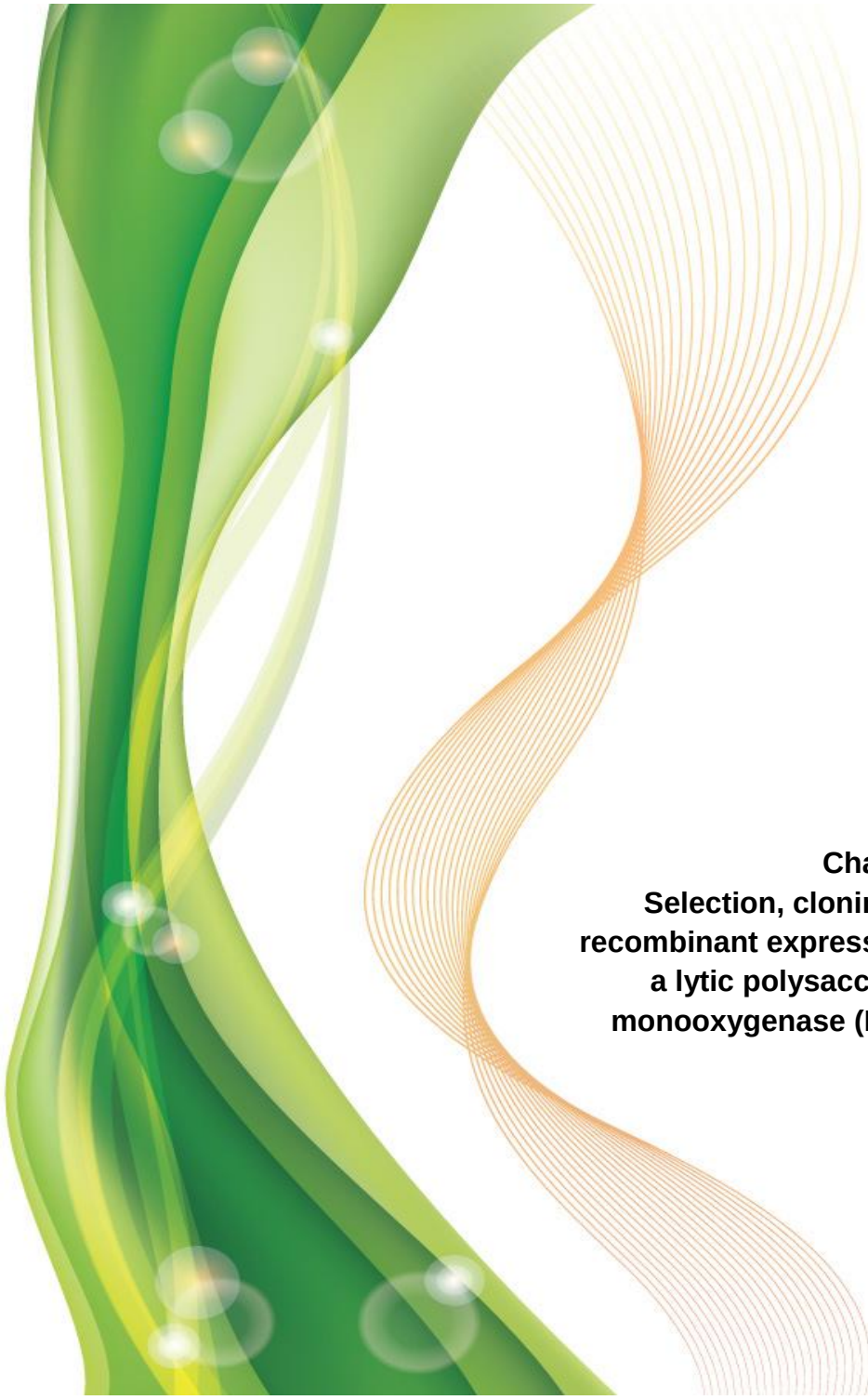


Chapter 3
Selection, cloning and recombinant expression of a lytic polysaccharide monooxygenase (LPMO)

Chapter 3: Selection, cloning and recombinant expression of a lytic polysaccharide monooxygenase (LPMO)

3.1 Introduction

LPMOs are auxiliary enzymes with a high capability of enhancing the efficiency of cellulose conversion.

The industrial importance of LPMOs for rapid and cost-efficient lignocellulose saccharification is evident from the number of patents registered that describe the technical use of LPMOs (Johansen 2016). Indeed, based on the enhanced efficiency of lignocellulose degradation and the regioselectivity of LPMOs, the introduction of these enzymes into cellulolytic enzyme cocktails could mean a significant reduction in the cost of the enzymatic process (Johansen 2016).

A recent work, performed in the same laboratory where the PhD thesis was carried out, allowed the discovery of genes coding for carbohydrate-active enzymes by the metagenomic analysis of lignocellulosic biomasses (Montella et al. 2017) and a database of sequences coding for around 5,000 enzymes of different classes was developed. Bioinformatics analyses of this database aimed at identifying sequences coding for LPMOs were performed by Bernard Henrissat from the Centre National de la Recherche Scientifique (CNRS) of Marseilles and revealed the presence of nine bacterial LPMOs from the T3PSB sample (biomass sample from *P. nigra* after 135 days of natural biodegradation in the underwood used for the metagenomic analysis performed by UNINA) (Montella et al. 2017).

Sections 3.2.1 and 3.2.2 describe, respectively, the selection and the set-up of a recombinant expression system of a LPMO, usable for directed evolution experiments or in an enzymatic cocktail in order to improve the hydrolysis of cellulose.

3.2 Results

3.2.1 Selection of a lytic polysaccharide monooxygenase

The sequences of the LPMOs, previously identified by the metagenomics study were analysed, as summarized in Tables 3 and 4.

Table 3: Bacterial LPMOs belonging to the T3PSB metagenome

Gene	Family	Subject ID	Enzyme	Microorganism
gene_T3PSB_4631	AA10	YP_001828367.1; ZP_08240587.1; BAG23684.1; EGE46501.1	chitin-binding protein	*Streptomyces griseus* subsp. griseus NBRC 13350
gene_T3PSB_5894	AA10	YP_004800577.1; AEN08037.1	chitin-binding domain 3 protein	*Streptomyces* sp. *SirexAA-E*
gene_T3PSB_1185	AA10	YP_001827285.1; BAG22602.1	cellulose-binding protein	*Streptomyces griseus* subsp. griseus NBRC 13350
gene_T3PSB_6954	AA10	-	-	-
gene_T3PSB_8	AA10	YP_004926016.1;	LPXTG-motif cell wall	*Streptomyces pratensis* ATCC

886		ADW06499.1	anchor domain protein	33331
gene_T3PSB_13034	AA10	YP_004921233.1; ADW01716.1	chitin-binding domain 3 protein	*Streptomyces pratensis* ATCC 33331
gene_T3PSB_29955	AA10	-	-	-
gene_T3PSB_42511	AA10	YP_001824468.1; ZP_08236609.1; BAG19785.1; EGE42523.1;	chitin-binding domain 3 protein	*Streptomyces griseus* XylebKG-1
gene_T3PSB_53014	AA10	NP_625478.1; CAB61600.1	cellulose binding protein	*Streptomyces coelicolor* A3(2)

The selection of the LPMO was based on an alignment with LPMO sequences and literature studies on bacterial monooxygenases and their producer microorganisms, as described below.
The sequences of two of the LPMOs from the T3PSB metagenome were shown to be fragmentary (LPMOs coded by the genes 6954 and 29955) and therefore they were not considered in the following steps.
Members of the *Streptomyces* genus are known for their ability to degrade a variety of complex and recalcitrant polysaccharides, a property reflected in the abundance of carbohydrate active enzymes encoded in their genomes (Nakagawa et al.2015; Takasuka et al. 2013).

Streptomyces spp. genomes are an exception compared to most genomes of biomass degrading bacteria that have one or two LPMOs encoding genes on account of the fact that *Streptomyces* has up to seven (*S. coelicolor*) genes coding for LPMOs. Until now, cellulose and chitin targeting LPMOs from *Streptomyces* spp. have been characterized (Nakagawa et al.2015).

Based on literature, LPMOs produced by *Streptomyces griseus*, *Streptomyces* sp. *SirexAA-E*, *Streptomyces coelicolor* A3(2) for cellulose and chitin degradation were investigated and are described below. On the other hand, studies on the LPMOs produced by *Streptomyces pratensis* ATCC 33331 for cellulose degradation have not yet been reported.

Streptomyces griseus was isolated from leaf litter and enhanced the mild alkaline solubilisation of lignins, also producing high levels of the cellulase complex when growing on wood substrates (Saritha et al. 2012). The *Streptomyces griseus* genome contains six LPMO-encoding genes (Table 4) that phylogenetically cluster with cellulose or chitin targeting LPMO sequences. Among the six LPMOs, the enzymatic properties of one small LPMO (15kDa) from *Streptomyces griseus* have been described. The enzyme was active towards both α- and β-chitin and showed a stronger binding and a greater release of soluble oxidized products for the latter allomorph. In chitinase synergy assays, however, the LPMO worked better for α-chitin, increasing chitin solubilisation yields by up to 30-fold and 20-fold for α- and β-chitin, respectively (Nakagawa et al. 2015).

Streptomyces sp. SirexAA-E (ActE) has been identified as a highly cellulolytic actinobacterium capable of deconstructing lignocellulose biomasses. This actinobacterium secretes a large number of enzymes targeting carbohydrates, including abundantly expressed LPMOs (Takasuka et al. 2013; Bianchetti et al.2013). Two of the six LPMOs are upregulated and secreted during growth on pure cellulosic substrates or plant biomasses, and three other LPMOs are upregulated when chitin serves as the substrate (Forsberg et al. 2014a). However, none of the six LPMOs has been characterized yet (Table 4) (Nakagawa et al. 2015). Furthermore *Streptomyces sp. SirexAA-E* (ActE) most frequently contains a carbohydrate-binding module from family 2a (CBM2a) (Lim et al. 2014).

Homologues of the putative LPMOs secreted by *Streptomyces* sp. SirexAA-E (ActE) also exist among the seven LPMOs encoded in the *Streptomyces coelicolor* A3 genome (Table 4). Some studies have indicated that one of these LPMOs is coexpressed with a cellulase and is a C1-oxidizing LPMO acting in synergy with cellulases (Forsberg et al. 2011; Forsberg et al. 2014a). Two LPMOs encoded in the *Streptomyces coelicolor* A3 genome have been structurally and functionally characterized (Forsberg et al. 2014b; Sprenger et al. 2016; Tanghe et al. 2017). The two enzymes display synergy when acting on cellulose, providing a possible explanation for the occurrence of a multitude of LPMOs in biomass-degrading microorganisms (Forsberg et al. 2014a).

Among the seven investigated LPMOs, three have a C-terminal extension. The cellulose binding protein [*Streptomyces coelicolor* A3(2)] coded by the gene_T3PSB_53014 has a CBM2, cellulose binding domain; (two tryptophan residues involved in cellulose binding). Members of the CBM2a subfamily are known to bind cellulose (Courtade et al. 2018). Kruer-Zerhusen analysed the significance of the domains of a LPMO from *T. fusca*. Variants with the CBM2 domain removed displayed a 20% residual binding and 60% residual activity compared to that of wild type proving that CBM2 is crucial for substrate binding (Meier et al. 2018; Kruer-Zerhusen et al. 2017). The cellulose-binding protein [*Streptomyces griseus* subsp. griseus NBRC 13350] coded by the gene_T3PSB_1185 has a C-terminal transmembrane domain. The LPXTG-motif cell wall anchor domain protein [*Streptomyces*

pratensis ATCC 33331] coded by the gene_T3PSB_8886 has a C-terminal extension with a probable Sortase domain.

Based on the literature studies, *Streptomyces pratensis* ATCC 33331 has not yet been studied for cellulose degradation. This microorganism produces two LPMOs: a LPXTG-motif cell wall anchor domain protein with a probable Sortase domain, of interest to researchers mainly on account of its immobilization to accumulate cells in bioreactors or for biofilms; a chitin-binding domain 3 protein, a protein with a greater affinity for chitin rather than cellulose.

The sequences of the LPMOs found in literature were aligned with the sequences of the LPMOs identified by the metagenomic analysis in order to verify the sequence identity, as reported in Table 4.

Table 4: LPMOs of the family 10 (LPMO10s) coded by the *Streptomyces griseus*, *Streptomyces* sp. *SirexAA-E*, *Streptomyces coelicolor* and *Streptomyces pratensis* genomes

Microorganism	**Number of genes coding for LPMOs**	**Corresponding LPMO sequences in the metagenome**			**Characterized LPMO10s**	**C-terminal extension**	**References**
		T3PSB LPMOs	**Literature LPMOs**	**% sequence identity**			
Streptomyces griseus	6	gene_T3PSB_4631	SgLPMO10A	-			Nakagawa et al. 2015
			SgLPMO10B	78%			
			SgLPMO10C	43%			
			SgLPMO10D	43%			
			SgLPMO10E	26%			
			SgLPMO10F	**100%**	SgLPMO10F		
		gene_T3PSB_1185	SgLPMO10A	38%			
			SgLPMO10B	28%			

			SgLPMO10C SgLPMO10D **SgLPMO10E** SgLPMO10F	35% 39% **100%** 26%			
		gene_T3PSB_42511	SgLPMO10A **SgLPMO10B** SgLPMO10C SgLPMO10D SgLPMO10E SgLPMO10F	11% **100%** 96% 97% 98% 99%			
Streptomyces pratensis	-	gene_T3PSB_8886 gene_T3PSB_13034	-	-	-	-	-
Streptomyces sp. SirexAA-	6	gene_T3PSB_5894	**SsLPMO10A**	**100%**	-		Takasuka et al. 2013

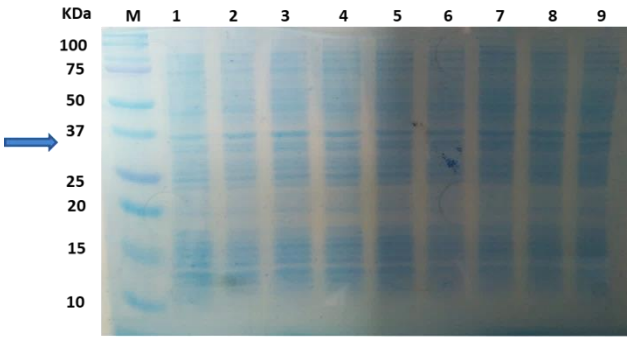
E			SsLPMO10B SsLPMO10C SsLPMO10D SsLPMO10E SsLPMO10F	28% 41% - 24% 43%		CBM2 domain (SsLPMO10D)	Nakagawa et al. 2015
Streptomyces coelicolor A3	7	gene_T3PSB_53014	ScLPMO10A ScLPMO10B **ScLPMO10C** ScLPMO10D ScLPMO10E ScLPMO10F ScLPMO10G	- 30% **100%** 42% 31% 31% 33%	ScLPMO10B ScLPMO10C	CBM2 domain (ScLPMO10C)	Forsberg et al. 2014a Forsberg et al. 2014b Tanghe et al. 2017 Sprenger et al. 2016 Courtade et al. 2017 Courtade et al. 2018

For *Streptomyces griseus* the gene_T3PSB_4631 has a 100% of identity with SgLPMO10F, the gene_T3PSB_1185 has a 100% of identity with SgLPMO10E and the gene_T3PSB_42511 has a 100% of identity with SgLPMO10B. For *Streptomyces* sp. SirexAA-E the gene_T3PSB_5894 has a 100% of identity with SsLPMO10A and for *Streptomyces coelicolor* A3 the gene_T3PSB_53014 has a 100% of identity with ScLPMO10C (Table 4).

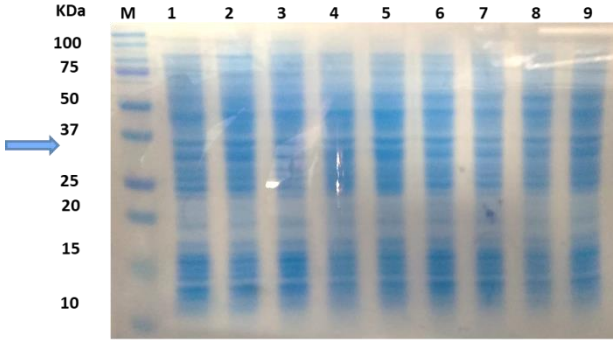
In conclusion, the protein from *Streptomyces coelicolor* A3(2) coded by the gene_T3PSB_53014 was selected for the setting up of the recombinant expression system, since its involvement in cellulose degradation has previously been investigated. Moreover, the *S. coelicolor* LPMO stands out as an interesting enzyme since it presents a cellulose binding domain, CBM2, which has been demonstrated to be crucial for substrate binding.

3.2.2 Setting up of a LPMO recombinant expression system

The LPMO from *Streptomyces coelicolor* A3(2) was selected for the setting up of a recombinant expression system usable for directed evolution, considering the importance of the LPMO class in the enhancement of the yield of cellulose conversion.
The LPMO gene from *Streptomyces coelicolor* A3 (2) was synthesized by GeneArt in pMA-RQ and cloned into the expression vector pET22b (Novagen, Inc.), using *EcoRI* and *HindIII* restriction enzymes. It was recombinantly expressed in the *E. coli* strain BL21 CodonPlus (DE3) RP (Novargen Ltd).
The cells were cultured at 37°C in 50 mL of LB medium supplemented with ampicillin (50 µg mL^{-1}), until an optical density (O.D.) of 0.7. In order to test the protein expression different inducers were used such as 5 mM lactose and IPTG at different concentrations (0.01, 0.1 and 0.5 mM), incubation times (4 hours, 3 hours and overnight) and temperatures (30°C and 20°C).
The protein expression was analysed by SDS page, as shown in Figure 4. Based on these results it was not possible to claim that the protein was expressed in all conditions. However, with regard to the qualitative analysis, only a slight difference was observed when the induction was performed using 0.5 mM IPTG for 3 hours at 30°C. In Courtade et al. 2017 it is reported that induction with IPTG results in a mixture of native and premature (non-cleaved signal peptide) LPMO and cell lysis. In any case, in order to confirm the protein production and use the LPMO in a mixture of enzymes for cellulose degradation, it would be necessary to carry out a purification step.

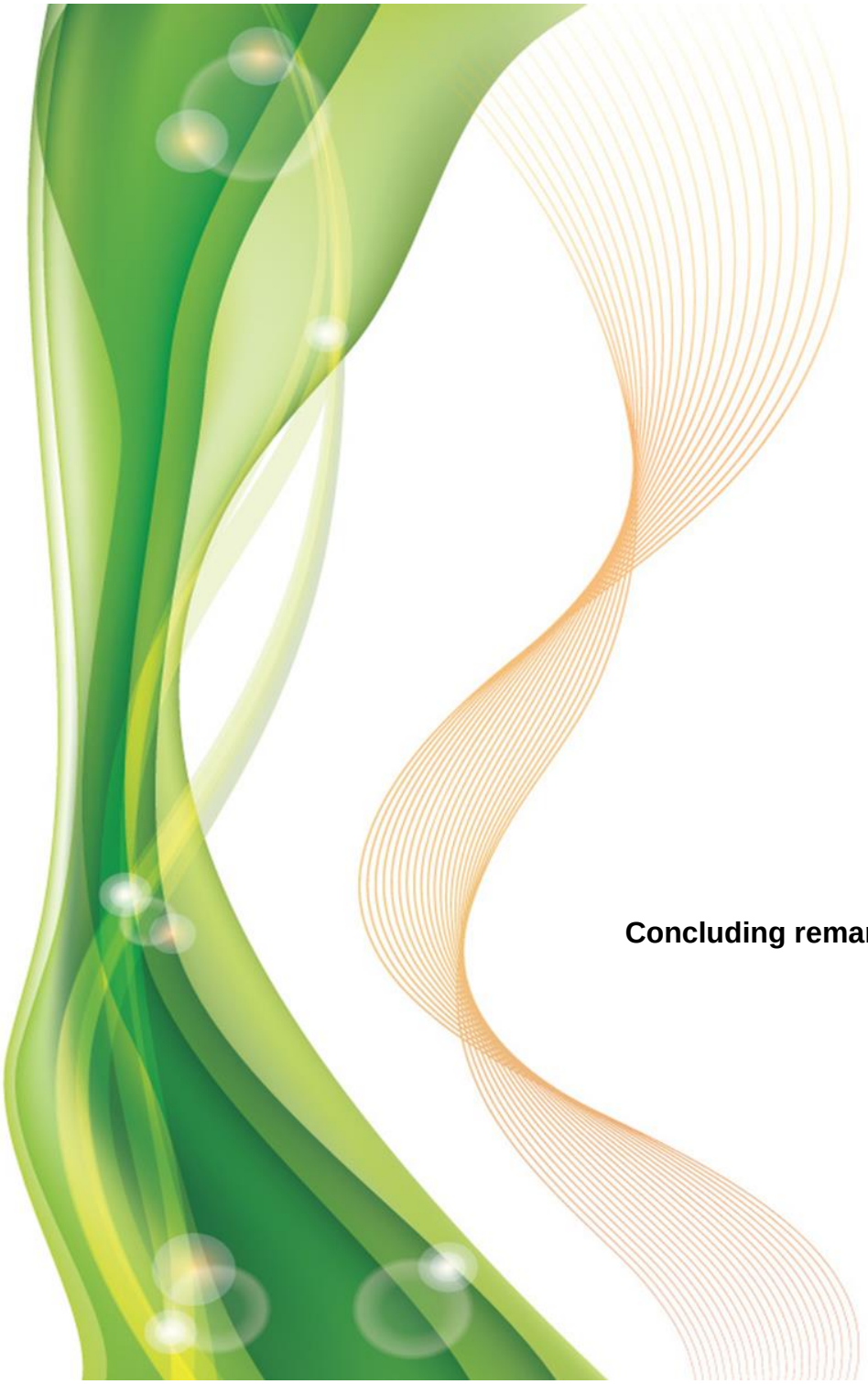


M: marker
1: not induced
2: 0.1mM IPTG induction 3h 30°C
3: 0.5 mM IPTG induction 3h 30°C
4: 0.1 mM IPTG induction 3h 20°C
5: 0.5 mM IPTG induction 3h 20°C
6: 0.1 mM IPTG induction o.n. 30°C
7: 0.5 mM IPTG induction o.n. 30°C
8: 0.1 mM IPTG induction o.n. 20°C
9: 0.5 mM IPTG induction o.n. 20°C



M: marker
1: not induced
2: 0.01mM IPTG induction 4h 30°C
3: 0.01 mM IPTG induction 4h 20°C
4: 0.01 mM IPTG induction o.n. 30°C
5: 0.01 mM IPTG induction o.n. 20°C
6: 5mM lactose induction 4h 30°C
7: 5mM lactose inducion 4h 20°C
8: 5mM lactose induction o.n. 30°C
9: 5mM lactose induction o.n. 20°C

Figure 4: Trials of the recombinant expression of a LPMO in different conditions



Concluding remarks

Concluding remarks

The overall aims of this project are the development of novel improved enzyme variants with an increased efficiency to hydrolyse the carbohydrates present in SMS, combined with WS into fermentable sugars, and the selection of a bacterial LPMO, to set up a recombinant expression system, considering the capability of this class of enzymes to enhance the yield of cellulose conversion.
Two new cellulases of the GH5 family (Cel1 and Cel2), previously identified as responsible for the cellulase activity of the strain *S. argenteolus* AE58P, were recombinantly expressed in *E. coli*, characterized and tested in the enzymatic hydrolysis of *P- nigra*, *P-virgatum* and pretreated SMS/WS (40:60%). Cel2 was selected for the development of improved biocatalysts by directed evolution, considering that it had a higher thermoresistance and produced greater hydrolysis yields than Cel1. The strategy specifically set up for the generation of a library of 30,000 random Cel2 mutants and performing its automated screening in order to identify the most active variants allowed the selection of 63 improved variants. Among these, 21 mutants showing at least a 2-fold higher activity compared to the wild type Cel2, after the scale up of production, were characterized. In addition, 13 mutants exhibiting a higher thermoresistance after 72hours were selected and tested in the enzymatic hydrolysis of pretreated SMS/WS in comparison with the wild type Cel2 when added to the commercial mixture SUNO TM BOOSTER 144. All the mutants exhibited a glucose yield 2-fold or 4-fold higher than the wild-type Cel2, when added to this commercial mixture.

With the aim of setting up a LPMO recombinant expression system, considering the importance of these enzymes in terms of their capability to enhance the yield of cellulose conversion, the protein from *Streptomyces coelicolor* A3(2) was chosen since the involvement in cellulose degradation has been previously investigated. The bacterial monooxygenase was cloned and recombinantly expressed in *E. coli.* However, the protein expression level was low and therefore, in order to claim that the protein was expressed and in order to use it in a mixture of enzymes for cellulose degradation, it would be necessary to carry out a purification step.

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Appendices

Appendices

Appendix I: List of communications

- Vincenza Faraco, Anna Pennacchio, **Valentina Mauriello.** 2018**.** Cloning, recombinant expression and characterisation of two cellulases of family GH5, Cel1 and Cel2. 4CIAB congress 4th IBEROAMERICAN CONGRESS ON BIOREFINERIES. Poster session. Jaèn, Spain
- Organizing committee of BioID&A- Biotechnology Identity and Application. 2019. University of Naples Federico II.

Appendix II: List of publications

- **Valentina Mauriello**, Anna Pennacchio, Irantzu Alegria Dallo, Laura Garcia Saez, Petri Ihalainen, Antoine Mialon, Valeria Ventorino and Vincenza Faraco*. **Development of improved cellulase variants for spent mushroom substrate conversion by directed evolution.** (submitted to New Biotechnology)