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# Exploration of fungal biodiversity for the deconstruction of lignocellulosic biomass and the implementation of new biosynthetic pathways for green chemistry

Emmanuel Bertrand<sup>\*1</sup>, Jean-Guy Berrin<sup>1</sup>, Christophe Boyer<sup>1</sup>, Marianne Daou<sup>1</sup>, Michel Delattre<sup>1</sup>, Elodie Drula<sup>1</sup>, Anne Favel<sup>1</sup>, Sacha Grisel<sup>1</sup>, Mireille Haon<sup>1</sup>, Isabelle Herpoël-Gimbert<sup>1</sup>, Laurence Lesage-Meesen<sup>1</sup>, Anne Lomascolo<sup>1</sup>, David Navarro<sup>1</sup>, Sana Raouche<sup>1</sup>, Eric Record<sup>1</sup>, Marie-Noëlle Rosso<sup>1</sup>, Jean-Claude Sigoillot<sup>1</sup>, Sabine Taussac<sup>1</sup>, Annick Turbe Doan<sup>1</sup> and Craig B. Faulds<sup>\*1</sup>

<sup>1</sup>Aix-Marseille Université, INRA, UMR 1163, Biodiversité et Biotechnologie Fongiques, 163 avenue de Luminy Bat Polytech' entrée B - case 925 - 13009 Marseille, France

[\\*Emmanuel.bertrand@univ-amu.fr](mailto:Emmanuel.bertrand@univ-amu.fr)

[Craig.faulds@univ-amu.fr](mailto:Craig.faulds@univ-amu.fr)

## Collection and Conservation of the fungal biodiversity

The filamentous fungi of the Phyla Ascomycota and Basidiomycota provide a large repertoire of genes encoding a diverse combination of enzymatic mechanisms involved in the degradation of lignocellulosic biomasses. The collection of the CIRM-CF (International Centre for Microbial Resources – Filamentous Fungi, Aix-Marseille-University, Inra) hosted by the Joint Research Unit Biodiversity and Biotechnology of Fungi (UMR 1163 BBF) is responsible for the conservation and valorisation of fungal strains exhibiting selective or simultaneous degradation activities towards lignin and polysaccharides. During the last five years, several field-collecting campaigns have contributed to the development of the collection. Four were carried out over-seas and 5 on mainland France with an interest in tropical forests areas: French Guyana, French West Indies, French New Caledonia (Lechat et al 2013, 2015; Welti et al 2012). In total, 620 specimens were collected, identified by morphological criteria, cultured and added to the collection. In addition, the collection has been enriched by the deposit of strains by French or foreign mycologists. Thus, over the last five years, the collection has increased by 535 wild strains (including 38 new species that were not represented in the collection). To allow phenotype characterization and to facilitate genomic sequencing and assembly, 250 monokaryotic strains have been generated from

different wild strains. This genetically stable material is used in several genome sequencing projects such as the Community Sequencing Programs at the Joint Genome Institute and were also deposited in the collection. Finally, the integration of other collections such as 377 *Fusarium* strains also ensure the growth of the collection. Therefore, the CIRM-CF currently includes approximately 2200 strains that have been characterized based on morphological and molecular criteria. All the strains of the collection have been authenticated by molecular methods such as ITS sequencing and phenotypic characterization is currently ongoing through research projects. All the public information and data related to the strains are available through the BRFM-DB database [1]. The collected biological material is maintained under high quality standards and has achieved ISO 9001 certification for the acquisition, authentication and distribution of fungal material since 2006. It is intended to the dissemination to the scientific community through academic and industrial projects. In 2016, within the scope of the ISO 9001:2008 certification, the CIRM-CF mission was also extended to the screening of fungal strains for targeted biological functions and white biotechnological applications.

## **Exploration of the fungal adaptation and their responses to lignocellulosic biomasses**

The CIRM-CF collection is the starting point of comparative studies at the whole genomic level focused on the diversity of mechanisms used by the filamentous fungi to breakdown plant cell walls. One of the objective is to compare how the enzymatic systems acquired by the different types of filamentous fungi are used to cleave lignocellulosic polymers into available carbon sources. In fact, filamentous fungi can colonize plant tissues using different strategies: pathogenic, saprotrophic or symbiotic. The enzymatic systems acquired to decompose plant polymers into available molecules used as carbon sources for the fungus are compared by genomic and transcriptomic approaches. The response of the fungus to different substrates representative of model biomasses: grasses, gymnosperm, softwood and hardwood, is also evaluated by comparative transcriptomic and secretomic analyses (Couturier et al., 2015).

In particular, *Polyporales*, an order of wood-decaying fungi with applications in white and green biotechnologies and in medicinal chemistry, has been extensively studied. An open-access, user friendly, bioinformatics tool named FunGene-BD (<http://www.fungene-db.org>) was developed to facilitate the molecular authentication of *Polyporales* strains, otherwise subjected to morphological studies. This tool includes a curated database that contains Internal Transcribed Spacer (ITS) genes screened through a semi-automated pipeline from the International Nucleotide

Sequence Database and the similarity search BLASTn program. (Navarro et al, 2012). The restriction of the database to one reference sequence by species allowed most of the time an unequivocal analysis. It is a promising tool for molecular authentication of the Polyporales and is useful for scientists that are not experts in mycology but need to check the identity of strains for applied microbiology.

Among the *Polyporales*, the most representative group of saprophytic homobasidiomycetes causing wood decay is the genus *Pycnoporus*. It is regarded as of scientific interest through the production of a full arsenal of oxidoreductases. Historically four species were defined in this genus based on their morphological features and their distribution areas, *P. cinnabarinus*, *P. sanguineus*, *P.coccineus* and *P.puniceus*. As part of the European project BIORENEW, the phylogeny of the laccase gene lac3-1 from 36 *Pycnoporus* strains within the CIRM-CF originating from different geographical areas (Central/South America, Europe, Africa, Asia and Oceania), highlighted that enzyme sequence diversity was associated with biogeographical origin. Special attention was given to the closely related species *P. sanguineus* and *P. coccineus*, which display very similar characters but are geographically discontinuous populations, indicating that biogeography has played a strong role in determining evolutionary history of species in the genus *Pycnoporus* (Lesage-Meessen et al, 2012).

In the post-genomic era, specialized databases are very useful tools to identify the full enzymatic repertoire available to a fungus necessary for it to deconstruct a plant cell wall. In a recent work in collaboration with the research unit CNRS-AFMB ([www.afmb.univ-mrs.fr](http://www.afmb.univ-mrs.fr)), the Carbohydrate-Active enZymes database was implemented to achieve the simultaneous identification of CAZymes and oxidoreductases, especially those associated with lignin degradation (Auxiliary Activities) within a fungal genome (Levasseur et al, 2013). Forty-one available fungal genomes were initially included to study the occurrence of auxiliary activities enzymes in well-known white rot fungi (WRF), brown rot fungi (BRF) and plant pathogens from the Ascomycota (13 species) and Basidiomycota (28 species) divisions. A total of 1576 auxiliary activities were identified. All BRF strains to date lack ligninolytic AA2 enzymes whereas a single WRF strain can contain up to 26 AA2 proteins. This high prevalence of AA2 enzymes in WRF re-enforced the central role of ligninolytic class II peroxidases in wood degradation, and argues for the use of AA2 as a family “marker” for predicting the wood-decaying activities and nutritional mode of different fungal groups (White rot fungi vs Brown rot fungi).

To study the full lignocellulosic enzymatic arsenal of *P. cinnabarinus* CIRM-BRFM137, its genome was sequenced within the framework of the European project BIORENEW (Levasseur et al, 2014). CAZy analysis revealed that this genome contains all the enzymes families involved in cellulose degradation even if it presents the smallest number of glycoside hydrolases among sequenced White Rot Fungi. The number of

xylan and pectin-degrading enzymes, important in the breakdown of the hemicellulosic component of the plant cell wall are also restricted and mainly represented by families GH10 and GH43. Among the predicted lignin-degrading activities, laccases (AA1\_1), ligninolytic peroxidases (AA2), cellobiose dehydrogenase (AA8-AA3\_1), and other auxiliary enzymes were identified. *P. cinnabarinus* contains a complete ligninolytic enzymatic spectrum and may exploit different strategies for ligninolytic breakdown compared to other White Rot Fungi.

Due to the heterogeneity of lignocellulosic substrates, many enzymes with different characteristics are required to convert lignocellulose into fermentable sugar monomers. Natural fungal diversity in wood-decaying species within the CIRM-CF collected in temperate and tropical forests or recovered from international collections was explored for biomass deconstruction using wheat straw as a model lignocellulosic biomasses. Following the production of fungal secretomes (the proteins that are secreted by the fungus in its environment and that are found in the culture supernatant) under inductive conditions from 19 species, the capacity of these secretomes to supplement a commercial *Trichoderma reesei* cellulase cocktail for the improved release of soluble sugars from the wheat straw was assessed using the automatic platform developed in our unit (Navarro et al, 2010). This led to an improvement in biomass conversion into fermentable sugar yield of at least 23% as measured by the dinitrosalicylic colorimetric assay (Berrin et al, 2012). Among the isolates, the *Trametes gibbosa* secretome performed best, with an 60% improved conversion, a feature that was not universal to the *Trametes* and related genera. Enzymatic characterization of the *T. gibbosa* secretome revealed an unexpected high activity on crystalline cellulose, higher than that of the *T. reesei* cellulase cocktail. These investigations highlight the interest in a systematic high-throughput assessment of fungal biodiversity within collections to improve the enzymatic conversion of lignocellulosic biomass. It enabled the unbiased identification of new fungal strains issued from biodiversity with high biotechnological potential. Proteomic analysis of the best-performing secretomes (i.e. from *Fusarium verticillioides*, *Trametes gibbosa*, *Ustilago maydis*, *Podospora anserina*), performed in collaboration with the PAPPSO INRA platform in Jouy en Josas (<http://pappso.inra.fr/>), allowed the identification of a number of plant cell wall-degrading enzymes that may be potentially useful to improve the industrial lignocellulose bioconversion process (Berrin et al, 2012; Couturier et al, 2012; Ravalason et al, 2012; Poidevin et al, 2014). The most promising results obtained using fungal secretomes were patented in the framework of the Futurol and ANR E-TriCel projects (Sigoillot et al., 2013; Poidevin et al., 2013; Berrin et al., 2014). Furthermore, an analysis of the *Ascomycota* *P. anserina* secretomes produced during growth on Avicel and sugarbeet pulp (SBP) revealed a large array of CAZymes with a high number of GH6 and GH7 cellulases, CE1 esterases, GH43 arabinofuranosidases, and AA1 laccase-like multicopper oxidases. Moreover, a

preponderance of lytic polysaccharide monoxygenases (LPMOs) was exclusively produced under the SBP condition. This study brought new insights into the *P. anserina* enzymatic machinery. Furthermore, global transcriptomic and secretomic analyses revealed that within the fungal taxa the Basidiomycete *Laetisaria arvalis* produces a unique repertoire of carbohydrate-active enzymes including a complete set of enzymes acting on cellulose. Temporal analyses of its secretomes indicated that the unusual degradation efficiency of *Laetisaria arvalis* is related to its early response to the carbon source, and on the finely tuned sequential secretion of several Lytic Polysaccharides Mono-Oxygenases (LPMOs) and hydrolytic enzymes targeting cellulose specifically (Navarro et al., 2014).

The mangroves wamp fungi are important actors in organic matter decomposition but little is known on the fungal diversity and community structure in this ecosystem. Using tag-encoded 454 pyrosequencing of the ITS1, ITS2, nu-ssu-V5 and nu-ssu-V7 regions, the fungal communities found on the marine and aerial parts of mangrove trees in New Caledonia were studied and compared (Arfi et al. 2012a). There was a marked zonation in species distribution. Ascomycetes were the dominant phylum (82%), Basidiomycetes were very rare (3%), and 15% of the sequences corresponds to unknown taxa. Host specificity was shown to be a key factor in the distribution of these communities, in both the aerial and intertidal parts of the trees. Microscopic observations and a metagenomic approach were used to study further the communities in the mangrove (Arfi et al. 2012b). These approaches demonstrated that fungal growth occurred under anoxic and sulfidic conditions in the mangrove. To assess the fungal diversity, a 454 pyrosequencing of the nuclear ribosomal internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) was performed from mangrove soil (60 cm deep). Agaricomycetes dominate this ecosystem with 50% of the reads, but the most abundant operational taxonomic units (OTUs) were affiliated to Ascomycetes (around 80% of the ITS sequences). A Xylariales strain *Pestalotiopsis sp.* was isolated from the mangrove tree *Rhizophora stylosa* and the adaption of the strain to salinity was studied on its lignocellulolytic enzyme set (Arfi et al. 2013). Enzyme activities and proteomic analyses of the secretomes suggest that the presence of salt modifies the lignocellulolytic enzyme set with an increase in the secretion of xylanases and cellulases and an associated decrease in the production of oxidases. Thus, cellulose and hemicellulose hydrolysis is enhanced but lignin breakdown is reduced. Recently, the genome of a *Pestalotiopsis sp.* Isolated from the Baltic sea coast was sequenced (Kumar et al. 2015) and the related data allow a full access to new salt-tolerant enzymes as potentially more robust biocatalysts (Patel et al, 2016).

## **Degradation and valorization of complex and recalcitrant polysaccharide with fungal systems**

Plant polysaccharides are the most abundant renewable carbon source on earth and are highly relevant to face tomorrow's environmental and energetic concerns. However, plant carbohydrate conversion into valuable products for energy and green chemistry that can then be transformed in the emerging second and third generation biorefineries face several challenges due to the recalcitrance of these polysaccharides to the commercially available enzymatic cocktails used for their deconstruction. Indeed, the recalcitrance of biomass to enzymatic attacks limits industrial performance. It might be interpreted as a lack of knowledge in the way we understand the relationship between the structure of biomass and the catalytic mechanisms that constitute the performance of current enzyme cocktails.

However, previous research made it possible to identify fungal oxidases and hydrolases dedicated to the degradation of polysaccharides that are currently considered as recalcitrant. The objective is to characterize and utilize these novel enzymes to unlock the degradation of these recalcitrant polysaccharides from the laboratory up to the industrial scale. For instance, the screening of fungal biodiversity allowed the identification of fungi capable of effectively supplementing the cocktail produced by *Trichoderma reesei* traditionally used for deconstruction on an industrial scale. The correlations between omic data and markers of recalcitrance allow selection of the most promising hydrolases and oxidases. Our research has demonstrated the efficiency of fungal secretomes to improve lignocellulosic biomass conversion (Couturier et al., 2015). After screening hundreds of fungal strains obtained from the CIRM-CF collection, strains whose secreted enzymes improved significantly the glucose yield during the conversion of pretreated lignocellulosic biomass as compared to the *Trichoderma reesei* reference enzymatic cocktails used in industry were identified. This work highlighted the cooperation between fungal enzymes for enhanced degradation of complex lignocellulosic substrates. Several of the secreted enzymes were characterized, and enzymes of particular interest to biotransformation of plant biomasses have been produced on a large scale, allowing saccharification assays under industrial conditions.

More than fifteen oxidoreductases were successfully produced from 50 mg L<sup>-1</sup> to 1 g L<sup>-1</sup> at lab scale and at larger scale in collaboration with partners all over Europa using the host *Aspergillus*. Through these collaborations, a laccase from *Phanerochaete lavidobalba* (Benghazi et al. 2013), two cellobiose dehydrogenase (CDH), a new laccase from Ascomycete origin, and the laccase of *Trichoderma reesei* were cloned and heterologously expressed (Levasseur et al. 2010). Also, more than 50 fungal CAZymes (mainly glycoside hydrolases (GH), carbohydrate esterases (CE) and auxiliary activity enzymes (AA)) have been heterologously produced in the yeast expression system *Pichia pastoris*. Due to the success of fungal enzymes being produced to high yields, the system was improved to streamline the methodology. Protocols of cloning, transformation, screening of transformants, cultures, purification of recombinant

proteins were adapted in a high-throughput way (Haon et al 2015). We have used in-house genes encoding CAZymes (successfully expressed in *P. pastoris*) to establish simple standard protocols. Liquid transformation has been adapted to *P. pastoris* to avoid laborious plating of transformants. Liquid cultures have been miniaturized to 96-well plate format (deep well) and his-tagged recombinant enzymes are now purified using 96-well plates and visualized using the Caliper LabChip system (Perkin Elmer). Some of these steps (cultures, purification, etc) have been fully automated using an in-house robotic Tecan platform that will be adapted for this purpose. (<http://www.platform3pe.com/>). This allowed their biochemical and enzymatic characterization with respect to their substrate specificity and optimal conditions of utilization (Couturier et al., 2011a; 2011b; 2013a; Lafond et al., 2012; Bey et al., 2013; Sigquier et al., 2014; Poidevin et al., 2014; Katsimpouras et al., 2014; Puchart et al., 2015).

Fungal secretomes provide an ideal case study to identify the enzymatic and metabolic partners of LPMOs (Garajova et al, 2016) and the unit actively participates to this emerging field of research. Effort will also be focused on secreted proteins of as yet unknown function that are associated with biomass degradation markers and are candidates for the creation of new CAZyme families. Special attention is paid to new families of CAZymes (Lombard, et al., 2013), proteins of unknown function and LPMOs (Lytic Polysaccharide Monooxygenases). The works undertaken find their applications for biofuels, the production of precursor synthons of bioplastics, as well as the functionalization of oligo- and polysaccharides. Exploring fungal biodiversity has allowed the isolation and characterization of new enzymes, including oxidoreductases and carbohydrate hydrolases. A glucose dehydrogenase from the poorly characterized glucose-methanol-choline oxidoreductase family (GMCs : AA3 family) showed an ability to reduce oxidized quinones or radical intermediates, raising prospects for applying this enzyme to detoxify compounds formed during the degradation of lignins. A protein domain of unknown function identified in the coprophilic fungus *Podospora anserina* displayed yet unobserved bifunctional exo- $\beta$ -(1,3)/(1,6) and endo- $\beta$ -(1,4) activities toward beta-glucans. This fungal cellulase provided the basis for the definition of a new family of glycoside hydrolases, the GH131 family (Lafond et al., 2012). The structure-function analysis of hemicellulases brought to light the molecular mechanisms of substrate specificity and catalytic reactions, providing guidance for directed evolution strategies aimed at improving these biocatalysts.

CDH are extracellular glycosylated haemoflavoenzymes produced by many different wood-degrading and phytopathogenic fungi. They are involved in both the degradation of polysaccharides and lignins generating hydroxyl radicals by the Fenton reaction. The genomes from the basidiomycete *Coprinopsis cinerea* and *P. cinnabarinus*, and the ascomycete *Podospora anserina* were screened for candidate CDH genes which were successfully expressed in *P. pastoris* and *A. niger* (Bey et al., 2011; Turbe-Doan et



al. 2012). CDHs kinetic constants were determined for several carbohydrates including  $\beta$ -1,4-linked di- and oligosaccharides. The effect of CDHs on saccharification of micronized wheat straw by an industrial secretome was studied. X-ray crystallography has been used to solve the structures of the most promising enzymes and unveil functional residues involved in the catalytic mechanism/molecular interaction. X-ray diffraction data sets have been collected at the ESRF (Grenoble) and SOLEIL (Paris) synchrotrons. Over the past four years, we have been very successful in getting the crystal structures of several fungal CAZymes expressed in *P. pastoris* (Couturier et al., 2013; Lafond et al., 2015; Siguier et al., 2014). The use of ionic chromatography and mass spectrometry allowed us to gain complementary insights into the specificities and the mode of action of these enzymes.

In the framework of the Futurol project, we have focused on two fungal endomannanases from the coprophilous ascomycete *Podospora anserina*, which belong to the families GH5 and GH26. These enzymes improved significantly the conversion of softwood for the second-generation bioethanol production (Couturier et al., 2011b). These mannanases were further subjected to detailed comparative analysis of their substrate specificities and active site organization. Although the GH5 enzyme displays a classical mode of action, the GH26 enzyme revealed an atypical hydrolysis pattern resulting from a predominant binding mode involving the -4 subsite. The crystal structures of the two enzymes were solved at 1.4 and 2.85 Å resolution, respectively. Analysis of the GH26 enzyme crystal structure supported strong interaction with substrate at the -4 subsite mediated by two aromatic residues (Couturier et al., 2013a). More recently, we provided deep insights into their binding mode with manno-oligosaccharides at the molecular level using NMR spectroscopic techniques (Marchetti et al., 2015). A strategy of molecular engineering in the yeast *Yarrowia lipolytica* was further implemented in collaboration with the ICEO platform in Toulouse (bioinfo.genotoul.fr) to improve the specific activity of the GH5 and GH26 (Couturier et al., 2013b). Following random mutagenesis and two steps of high-throughput enzymatic screening, we identified several mutants that displayed improved kinetic constants for the hydrolysis of galactomannan. Examination of their three-dimensional structures revealed which of the mutated residues were potentially important for enzyme function. Among them, a single mutation displayed an impressive 8.2-fold increase in the catalytic efficiency due to a significant improvement of the affinity for the substrate.

Although plant biomass degradation has been studied extensively, our knowledge of the enzyme systems used to degrade cellulose has changed dramatically in the last five years. Indeed, a new class of secreted enzymes known as LPMOs has revolutionized our view on how cellulose is degraded. In contrast to typical cellulases, which are hydrolytic enzymes, LPMOs degrade cellulose by a mechanism involving molecular oxygen and redox-active molecules acting as electron donors (ascorbate or cellobiose

dehydrogenase). LPMO-catalysed cleavage leads to oxidation of one of the carbons in the scissile  $\beta$ -1,4-glycosidic bonds leading to keto and carboxylic acid functions at the surface of cellulose. These oxidases are considered as a breakthrough in the enzymatic degradation of biomass because they cleave glycosidic linkages. These LPMOs are considered as a major advance in the understanding of biomass degradation mechanisms because their oxidative cleavages of the glycosidic bonds allow a facilitated hydrolysis of the recalcitrant regions by the cellulases. The use of complementary approaches (biochemistry, biophysics, crystallography and inorganic biochemistry) makes it possible to untangle their functions and mechanisms of action (Bennati-Granier et al., 2015). LPMOs are unique since their mechanism of action requires electrons that can be provided by co-secreted enzymes i.e., sugar oxidoreductases and ligninases and small reductant molecules originating from fungal and or plant metabolites (Garajova et al., 2016). The AA9 family of LPMOs is widely distributed in the genomes of most ascomycetes and basidiomycetes. A high number of AA9 LPMOs are frequently encountered in fungal secretomes under lignocellulolytic conditions but the functional relevance of this redundancy is not fully understood. Enzyme activity of recombinant AA9 LPMOs were assayed on cellulose in synergy with the cellobiose dehydrogenases (Bey et al., 2013; Bennati-Granier et al., 2015). We showed that the total release of oxidized oligosaccharides from cellulose was higher for LPMOs that harbored a carbohydrate-binding module from the family CBM1. Investigation of their regioselective mode of action revealed that some oxidatively cleaved at both C1 and C4 positions while others released only C1-oxidized products. Rapid cleavage of cellulose was observed using the LPMO-H that was the most versatile in terms of substrate specificity as it also displayed activity on cello-oligosaccharides and  $\beta$ -(1,4)-linked hemicellulose polysaccharides (e.g., xyloglucan, glucomannan). These studies provide insights into the mode of cleavage and substrate specificities of fungal AA9 LPMOs that will facilitate their application for the development of biorefineries.

## **Degradation, modification and valorisation of aromatic compounds by fungal systems**

Due to their aromatic and complex structures, lignins resist most of the hydrolytic treatments and contribute to the recalcitrance of lignocellulosic biomass. Specialized fungi, such as saprophytes, are able to destructure the complex lignin matrix through a combination of highly efficient enzymes, natural mediators, organic acids and auxiliary proteins. These fungi are also well known in transforming these aromatic products into secondary metabolites of industrial importance, such as vanillin, the premier food flavouring compound used in the world. Our objective is to identify and

design novel enzymes for aromatic compound valorisation. In fact, Industrial lignins and other aromatic compounds present a sustainable source of a wide range of polymeric and/or monomeric molecules for manufacturing, building and green chemistry, with specific properties related to the aromatic nature of the polymers. The identification and characterization of key enzymes of saprophytic fungi capable of degrading this matrix, such as laccases, peroxidases, hydrogen peroxide-producing enzymes and novel dehydrogenase enzymes derived from the decryption of newly sequenced genomes and other enzymes involved in the specific pathways of biotransformation of aromatic compounds open up a wide range of monomers and polymers for construction and green chemistry. The functionalization of lignins and woody fibers allows the design of bio-adhesives in the design of agglomerated wood panels. The design of specific lignin-acting enzymatic cocktails will also be explored for the fragmentation of lignins leading to the release of platform molecules from lignocellulosic biomass, e.g. waste effluent or biomass treatment.

Another aspect of the research is to identify and characterize the key enzymes involved in biotransformation pathways specific to aromatics using comparative post-genomics approaches. The chemical metabolic pathway for the conversion of ferulic acid to vanillin by *P. cinnabarinus* has been previously elucidated although the enzymes involved were not identified. The bioconversion of aromatic monomers such as ferulic acid or sinapic acid allows applications in the sector of flavors, antioxidants and bioremediation. Although transformation of individual enzymes of interest is currently done in the Unit on model Ascomycetes such as *A. niger*, attempts to obtain consistent and stable transformants using the WRF *P. cinnabarinus* have failed so far. Efforts will be dedicated over the coming years with the development of new genetic tools such as the CRISPR-Cas9 system.

Expert knowledge on enzymes active on lignin has been developed that allows semi-automatic annotation of enzymes involved in lignin degradation. Integration of this knowledge to the CAZy classification in cooperation with the CNRS-AFMB allowed comparative genomics of carbohydrate- and lignin-active enzymes. This allowed the first pan-genome characterization of ligno-cellulolytic enzymes from *Pycnoporus cinnabarinus*, a white-rot fungus maintained at CIRM-CF.

From the construction of the Auxiliary Activity database within CAZy, several new enzymes were discovered and characterized. The genome analysis of *P. cinnabarinus* gives one putative glucose oxidase (GOx)-encoding gene and the corresponding protein was further studied. This enzyme was the first GOx studied in a Basidiomycete fungus. The cDNA was cloned in *A. niger* for production to achieve a yield of 640 mg L<sup>-1</sup> (Piumi et al., 2014). The recombinant enzyme was purified and biochemically characterized for comparison to the ascomycete homologs. Analysis of the enzyme properties showed that it is a glucose dehydrogenase acting with alternative electron

acceptor, not oxygen and that glucose is by far the preferred substrate, which constitutes an advantage over other sugar oxidases in the case of blood glucose monitoring. The substrate-binding domain of PcGDH turned out to be conserved in other glucose-methanol-choline (GMC) oxidoreductases. In addition, the ability of PcGDH to reduce oxidized quinones or radical intermediates was clearly demonstrated, which raised prospects for applying this enzyme to detoxify compounds formed during the degradation of lignin. Recently, three new dehydrogenases within the AA3 family of *P. cinnabarinus* were heterologously produced in *A. niger* and were fully characterized and showed to act synergistically with laccases (Mathieu et al. 2016).

Fungal pretreatment could be a low cost, environment-friendly alternative to chemical and physicochemical pretreatments commonly used in producing renewable energy (Rouches et al, 2016). In this context, a total of 176 strains from the CIRM-CF collection have been screened for their ability to grow on wheat straw and miscanthus with a low consumption of sugars for their own growth and to enhance saccharification. For this purpose, a new medium throughput multiwell plate Solid State Fermentation (SSF) method was used to select relevant fungi (Zhou et al, 2015). The six most efficient strains then underwent scaled-up SSF to perform finer investigations to select the best-adapted strain. The most effective fungus for enhancing enzymatic hydrolysis performance was *Polyporus brumalis* BRFM 985. From this work, a patent claiming this *P. brumalis* strain and the implementation process was filed (Gimbert et al, 2014). SSF operating parameters i.e. metals addition, time and temperature of culture and initial water content were optimized using response surface methodology, D-optimal design, combined with multicriteria optimization. To identify the enzymatic systems involved on the selective degradation of lignin during SSF, the genome of *P. brumalis* was sequenced and a time course analysis of the transcriptome and secretome during wheat straw pretreatment is currently underway

The influence of the structure of the high energy biomass, Elephant grass, and commercial sugar cane stems on the effectiveness of enzymatic deconstruction, was examined in collaboration with the Spanish laboratories of A Martinez (Madrid) and JC del Rio (Seville), and with the University of Sao Paulo (Brazil), VTT (Finland) and the University of Helsinki (Finland), and illustrated that in solid stem plant stalks, glycoside hydrolases preferentially degraded the more porous inner pith compared to the more lignified outer cortex (Perez-Boada et al, 2014; Varnai et al, 2014). The addition of a feruloyl esterase in both studies moderately improved the rate of hydrolysis, while the addition of an organic co-solvent such as DMSO to selectively remove xylan, while extensively increasing the rate of de-esterification (Faulds et al, 2011), reduced the overall enzymatic solubility of the biomass (Perez-Boada et al, 2014). These results show that the molecular rearrangement of the biomass and the

extent of processing will have major influences on the overall efficiency of enzymatic deconstruction of biomass for bioenergy production.

Three high redox potential laccases from *P. coccineus* and *P. sanguineus* were tested and compared, with the commercial Suberase® as reference, for their ability to synthesize natural active oligomers from rutin (quercetin-3-rutinoside), one of the best-known naturally-occurring flavonoid glycosides. Considering that *Pycnoporus* fungi are listed as generally-recognized-as-safe (GRAS) microorganisms, a process in collaboration with Yves Rocher was developed with technical parameters (solvent, temperature, reaction time and raw materials) easy to scale up for industrial production and compatible with cosmetic and pharmaceutical formulation guidelines. The aqueous mixture of glycerol/ethanol/buffer met this requirement and allowed the solubilization of rutin and its oxidative bioconversion into oligomers. Four flavonoid oligomer mixtures were synthesized using laccases and identified as dimers and trimers of rutin, different from those produced from Suberase®. These oligorutins displayed significantly improved anti-oxidant, anti-inflammatory and anti-ageing activities on specific enzymatic targets such as cyclooxygenase (COX-2) and human matrix metalloproteinase 3 (Uzan et al, 2011).

A wide range of applications in the pulp and paper industry exist for the application of fungal enzymes. Paper production requires fibres to be refined, meaning mechanically treated to present sufficient bonding potential. As it is a highly energy consuming stage, cellulase addition as a pre-treatment step was investigated to reduce the energy consumption and improve paper properties. The enzyme was added during pulp slushing and conditions of treatment were chosen to be compatible with an industrial application. Results obtained after low consistency disc refining of a softwood bleached kraft pulp were compared at a given drainage index. Enzymatically-treated samples showed a better development of fibrillation leading to a stronger paper. Moreover, fibre swelling was significantly improved. Moreover, by treating pulp with cellulase, it became possible to reduce refining intensity by 33% (Lecourt et al., 2010a). Two cellulase treatments during disc refining were applied under similar conditions as usual in paper mills leading to a 20% energy saving. Water retention value, which characterizes the water absorption inside the fibre wall, was increased by the cellulase treatment. However, tear index losses were observed upon enzyme treatment due to endoglucanase activity, but was balanced by the breaking length enhancement. It can be concluded that cellulase treatment could save 20% of electrical energy needed for refining, i.e., 50 kWh t<sup>-1</sup>, giving a high paper quality with a high tensile resistance and an acceptable lowering of tear strength (Lecourt et al., 2010 b).

Low molecular weight organic acids production by filamentous fungi (e.g. citric, gluconic, malic, itaconic lactic and fumaric acids) have attracted considerable attention

for their role in natural ecology and their potential industrial applications. To take advantage of the ability of filamentous fungi to degrade lignocellulosic biomass, 40 strains of Ascomycota and 26 strains of Basidiomycota from the CIRM-CF collection were screened in order to evaluate their potential for organic acid and ethanol production. Considering the pattern and the level of organic acid production, in a basal glucose liquid medium, this work illustrated the versatility in metabolites production among the fungal kingdom (Liaud et al., 2014). Furthermore, it enabled us to select *Aspergillus brasiliensis* BRFM103 to address some industrial issues regarding lignocellulosic biomass bioconversion into lactic acid. BRFM103 was successfully genetically modified by multicopy integration of a heterologous NAD-dependent lactate dehydrogenase gene to deviate the metabolic route toward lactic acid instead of ethanol (Liaud et al., 2015). Preliminary assays on complex substrates highlighted that the transformant was promising for lignocellulose bioconversion.

Laccase/mediator systems (LMS) were shown to be particularly efficient for pulp delignification. LMS have the disadvantage that the mediator is expensive and potentially toxic. N-hydroxyacetanilide in combination with laccase was shown very efficient to bleach Eucalyptus pulp and found to be a very promising alternative to 1-hydroxybenzotriazole (HBT) which is generally used (Valls et al, 2010). Furthermore, a chimeric ligninolytic enzyme was designed by fusing laccase lac1 of *P. cinnabarinus* to the carbohydrate binding module CBM1 of *A. niger* cellobiohydrolase B and produced in *A. niger*. By conferring to the chimeric protein the ability to bind to complex lignocellulosic substrates such as paper pulps, CBM addition was shown to greatly improve the delignification capabilities of the laccase in the presence of HBT (Ravalason et al. 2012). The presence of CBM could enhance the laccase biobleaching potential of softwood kraft pulp since a decrease in the enzymatic charge and chlorine dioxide consumption, as well as an increase in pulp brightness were observed. The bleaching of softwood could also be improved by the use of mannanases to remove hemicelluloses, mainly constituted of gallactomannan in softwood.

## **Valorisation of agro-industrial residues using fungi**

The residues of agro-industrial processes, such as vegetable oil extraction, brewing, fruit and vegetable juice production, etc., produce a low-value residue which is commonly used as an animal feed or composted. However, the potential value of some components of this material is sufficiently higher than the cost of animal feed or even for its conversion to bioenergy. The Unit has been looking at the exploitation of two high production agri-food residues, oil presscake and brewer's spent grain, and of a regionally-important crop residue (lavender), through the treatment with fungal cultures or fungal enzymes.

The *Lavandula* genus, which includes lavender (*Lavandula angustifolia*) and lavandin (*L. angustifolia* × *Lavandula latifolia*), is cultivated worldwide for its essential oils, which find applications in perfumes, cosmetics, food processing and, more recently, in aromatherapy products. Lavender and lavandin distilled straws (LLDS), traditionally regarded as waste after essential oil extraction, are mineral- and carbon-rich plant residues, the carbohydrates originating especially from cellulose, hemicelluloses and lignins. They contain terpenes, lactones and phenolic compounds of industrial interest (Lesage-Meessen et al 2015). Although they remain very limited (i.e. 32 000 tons/year for PACA region), preliminary work at BBF demonstrated the potential of filamentous fungi such as *T. reesei*, *P. cinnabarinus* and *P. sanguineus* for the saccharification of LLDS, the production of various cellulases, hemicellulases and oxidoreductases was demonstrated. The structure and composition of LLDS clearly opens new challenging perspectives for the use of LLDS in biotechnological processes,

Brewer's spent grain (BSG) is the most abundant side stream of the brewing industry, with between 15-20 kg being produced per hectolitre of beer. In 2010, 1.69 billion hL of beer was brewed worldwide (FAO-STAT, 2010). BSG is rich in polysaccharides (39.6% of Dry Matter), protein (18-27%) and lignin (14-26%) and we in collaboration with colleagues in VTT (Finland) have demonstrated that a combined mechanical pretreatment followed by sequential enzymatic extraction with glycoside hydrolase-rich cocktails (Forssell et al, 2011; Niemi et al, 2012a) can lead to the recovery of xylo-oligosaccharides (Forssell et al, 2011), which have a high prebiotic index, lignans (Niemi et al, 2012b), soluble protein (Niemi et al, 2013) and soluble and insoluble lignin-rich fractions which can be degraded in a human colonic model to stimulate the production of short-chain fatty acids (SCFA) and bioactive phenolic compounds which stimulate the growth of probiotic human intestinal bacteria (Niemi et al, 2013; Aura et al, 2013).

The continuous expansion of our knowledge on filamentous fungi, system biology, enzyme biochemistry and their associated metabolic pathways contributes to address societal challenges through the development of a circular bio-economy, the identification of new chains to produce value for the industry, by the valorisation of recalcitrant materials that were previously by-products or wastes from agro-industries and by the search for alternatives the processes that are not environmentally friendly with biological or bio-inspired processes.

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