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Identification of *Dictyopanus pusillus* as a promising candidate for enzymatic lignocellulose pretreatment of oil palm tree residues

Par

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ABSTRACT

Basidiomycete fungi are the most important organisms involved in wood recycling in nature trough production of ligninolytic enzymes able to degrade lignocellulose to obtain energy. Bioprospection of basidiomycete fungi and their ligninolytic enzymes is a valuable asset for the development of biotechnological delignification processes. Now, lignocellulolytic residue accumulation has been increasing over the years due to worldwide population growth and lignocellulose is one of the most promising renewable sources for bioethanol production. It encourages the use of by-products derived from the agro-industry as a practical alternative strategy for handling the accumulation of waste products with high energetic value. Bioethanol production using lignocellulose necessitates a selective pretreatment removing lignin to release cellulose and hemicellulose, which are then hydrolyzed to obtain sugars that can then be fermented to produce ethanol. Until now, the pretreatment process is the only step that has been supported by an effective biotechnological strategy, and still depends on chemicals or high energy demand strategies. Enzymatic pretreatment of lignocellulose would be a green alternative for the delignification process that occurs within cellulosic ethanol industry.

Among basidiomycete oxido-reductase enzymes, peroxidases, such as lignin peroxidases, manganese peroxidases and versatile peroxidases, and catalases as laccases are ligninolytic enzymes that oxidize lignin present in wood that could offer an environmental-friendly alternative for the exploitation of lignocellulosic by-products. Various agroindustries generate such by-products but oil palm (*Elaeis guineensis*) is the principal source of oil in the world, with Malaysia and Indonesia being the main producers followed by Nigeria, Thailand and Colombia. This specific culture generates important quantities of residues and for instance for Colombia, it means that in 2014 from 1.1 million tons of palm oil obtained, 3.3 million tons of wastes were generated.

Thus, the objective of this research project was to perform bioprospection of white-rot native Colombian basidiomycete fungi with ligninolytic activity and explore their genome to identify enzymes capable of handling the pretreatment of palm oil

tree lignocellulosic residues. Selection of basidiomycete fungi with ligninolytic activity was achieved using Solid State Fermentation and delignification capacity of enzymatic extracts was evaluated over lignocellulose from oil palm.

It arose from this study that the isolation of a Colombian native *Dictyopanus pusillus*-LMB4 basidiomycete fungus showed a relevant laccase activity (267.6 $U.L^{-1}$ after 28 days of fermentation). Interestingly, this specie had not yet been reported as an efficient ligninolytic organism. Enzymatic crude extract with laccase activity from native *D. pusillus*-LMB4 was used for a pretreatment process of empty fruit bunches lignocellulose to obtain reducing sugars. Ligninolytic protein characterization, mainly laccases, of this enzymatic crude extract showed the presence of at least one enzyme with laccase activity by zymogram analysis and mass spectrometry peptide identification. Genomic exploration of *D. pusillus*-LMB4 revealed the existence of 14 genes that have similarity with other reported laccase enzymes. To our knowledge this work presents the first genomic exploration of a *Dictyopanus* species with an emphasis on laccase enzymes that could be used for lignocellulose pretreatment during cellulosic ethanol production and bioremediation of soils and wastewaters, among others. Hence, these results report *Dictyopanus pusillus*-LMB4 as a new ligninolytic species, and its laccase enzymes as possible future green alternatives for the pretreatment of lignocellulose biomass from the agroindustry.

RESUMEN

Los hongos basidiomicetos son los organismos más importantes en el reciclaje de la madera en la naturaleza a través de la producción de enzimas ligninolíticas capaces de degradar lignocelulosa para la obtención de energía. La bioprospección de hongos basidiomicetos y sus respectivas enzimas ligninolíticas son de gran valor para el desarrollo del proceso biotecnológico de deslignifcación. Actualmente la acumulación de residuos ligninolíticos ha incrementado en los últimos años, como consecuencia del crecimiento de la población mundial, siendo la lignocelulosa una de las más prometedoras fuentes renovables para la producción de bioetanol. Lo anterior incentiva el uso de subproductos provenientes de la agroindustria como una alternativa estratégica para el manejo y la acumulación de productos de desecho de alto valor energético. Para la producción de bioetanol es necesario un pretratamiento para la remoción de la lignina y así permitir la liberación de la celulosa y hemicelulosa, que permite la hidrolisis de estos carbohidratos para la obtención de azúcares que finalmente son fermentados para la producción de etanol. Hasta el momento, el pretratamiento es la única etapa que no ha sido soportada por una estrategia biotecnológica efectiva, dependiendo de compuestos químicos o procesos que demandan alta energía. El pretratamiento enzimático de la lignocelulosa podría ser una alternativa verde para el proceso de deslignificación en la industria del bioetanol celulósico.

Entre las enzimas de los hongos basidiomicetos se encuentran peroxidasas como manganeso peroxidasas, lignina peroxidasas y versátil peroxidasas, y catalasas como las lacasas, estas enzimas ligninolíticas son capaces de oxidar la lignina presente en la madera, ofreciendo una alternativa amigable con el medio ambiente para la explotación de subproductos lignocelulósicos. Existen varias agroindustrias que generan subproductos lignocelulósicos, un ejemplo es la industria de la palma aceitera (*Elaeis guineensis*), que es la principal fuente de aceite a nivel mundial, siendo Malasia e Indonesia los mayores productores de aceite, seguidos por Nigeria, Tailandia y Colombia. El cultivo de palma genera importantes cantidades de residuos, como sucede en Colombia, donde se

produjeron 1.1 millones de toneladas de aceite de palma, generando 3.3 millones de toneladas de desechos lignocelulósicos para el año 2014.

Con base a lo anterior, el objetivo de este proyecto de investigación fue realizar la bioprospección de hongos basidiomicetos nativos colombianos con actividad ligninolítica y explorar su genoma para identificar enzimas con la capacidad de realizar el pretratamiento de residuos lignocelulósicos de la palma aceitera. Aislamiento y selección de hongos basidiomicetos con actividad ligninolítica fueron logrados por medio de fermentación en estado sólido, al igual que la capacidad ligninolítica de los extractos enzimáticos obtenidos sobre lignocelulosa de palma aceitera.

Como resultado de esta investigación se obtuvo el hongo basidiomiceto colombiano *Dictyopanus pusillus*-LMB4, el cual mostró una relevante actividad lacasa, interesantemente, esta especie no ha sido aún reportada como un eficiente organismo ligninolítico. El extracto enzimático crudo con actividad lacasa del asilamiento nativo *D. pusillus*-LMB4 fue usado para el pretratamiento de lignocelulosa de racimos vacíos de palma aceitera para la obtención de azúcares reductores; la caracterización de las proteínas de este extracto enzimático crudo mostró la presencia de al menos una enzima con actividad lacasa; la exploración genómica de *D. pusillus*-LMB4 mostró la existencia de 14 genes que poseen similitud con otras enzimas lacasas reportadas. A nuestro conocimiento esté trabajo muestra la primera exploración genética de una especie de *Dictyopanus* con énfasis en enzimas lacasas. Enzimas que podrían para el pretratamiento de lignocelulosa en la producción de etanol celulósico. Por consiguiente, estos resultados reportan a la especie *Dictyopanus pusillus* como un nuevo organismo lignolítico y sus enzimas lacasas como una posible alternativa verde para el pretratamiento de biomasa lignocelulósica de la agroindustria.

RÉSUMÉ

Les champignons basidiomycètes forment le principal groupe d'organismes vivants responsables du recyclage du bois grâce à une production d'enzymes ligninolytiques qui leur permettent d'en retirer une source d'énergie. La bioprospection de ces champignons et de leurs enzymes ligninolytiques est donc un atout pour le développement de procédés biotechnologiques de délignification. De plus, l'accumulation de résidus en lignine s'est accentuée au cours des dernières années due à la croissance mondiale de la population, mais la lignocellulose est une source renouvelable pour la production de bioéthanol. Cette pratique encourage l'utilisation des sous-produits dérivés de l'agro-industrie comme stratégie alternative pour prévenir l'accumulation de résidus à haute valeur énergétique. La production de bioéthanol nécessite un prétraitement visant à enlever la lignine pour libérer la cellulose et l'hémicellulose, qui sont ensuite hydrolysées en sucres fermentables pour la production d'éthanol. Or, le prétraitement pour enlever la lignine est la seule étape qui n'a pas encore été adaptée efficacement au sein d'un procédé biotechnologique et elle repose actuellement sur l'utilisation de méthodes chimiques ou de procédés énergivores. Un prétraitement enzymatique offrirait donc une alternative écologique pour le processus de délignification effectué par l'industrie de bioéthanol cellulosique.

Parmi les enzymes des champignons basidiomycètes, on trouve les péroxydases telles que les péroxydases de manganèse, les péroxydases de lignine et les péroxydases polyvalentes, et les catalases telles que les laccases, des enzymes ligninolytiques qui oxydent la lignine qui compose le bois et qui pourraient former une alternative écologique à l'exploitation de sous-produits de lignocellulose. Différentes agro-industries génèrent de tels sous-produits, mais le palmier à huile (*Elaeis guineensis*) est la principale source d'huile végétale dans le monde, la Malaisie et l'Indonésie étant les plus importants producteurs, suivis du Nigeria, de la Thaïlande et de la Colombie. Cette culture génère de grandes quantités de résidus puisque, par exemple pour la Colombie en 2014, 3,3 millions de tonnes de résidus ont été générés pour 1,1 millions de tonnes d'huile de palme obtenus.

Donc, l'objectif de cette étude était d'effectuer la bioprospection de champignons basidiomycètes de la Colombie ayant une activité ligninolytique et une exploration de leur génome afin d'identifier des enzymes pouvant effectuer le prétraitement de résidus ligneux provenant du palmier à huile. La sélection des espèces de basidiomycète s'est effectuée par fermentation sur phase solide et leur pouvoir de délignification a été évalué en utilisant de la lignocellulose de palmier à huile.

L'étude a démontré qu'un isolat du basidiomycète *Dictyopanus pusillus*-LMB4 possédait une activité laccase intéressante. Or, cette espèce n'avait pas à ce jour été identifiée parmi les organismes lignivores. Un extrait brut enzymatique de *D. pusillus*-LMB4 ayant une activité laccase a été utilisé pour le prétraitement de fruits vides du palmier à huile et a engendré la production de sucres réducteurs. De plus, la caractérisation de cet extrait enzymatique a mis en évidence la présence d'au moins une enzyme ayant une activité laccase. L'exploration génomique de *D. pusillus*-LMB4 a démontrée l'existence de 14 gènes possédant des similarités avec différentes laccases précédemment identifiées. Cette étude constituerait la première documentation du génome de *D. pusillus*-LMB4 mettant l'emphase sur les laccases qui pourraient être utilisées pour le processus de prétraitement menant à la production d'éthanol cellulosique.

Bref, ces résultats inscrivent *Dictyopanus pusillus* parmi les organismes lignivores ainsi que ses laccases comme une alternative pour le développement de procédées écologiques pour la délignification de la biomasse lignocellulosique provenant de l'industrie agro-alimentaire.

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LIST OF ABREVIATIONS

ABTS ……..…… 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

ADF …… Acid detergent fiber

ADL …… Acid detergent lignin

AFEX ……..…… Ammonia fiber expansion

AP-PCR …….. Arbitrary primed - polymerase chain reaction

ANOVA ……..... Analysis of variance

BMMY …… Buffered methanol-complex medium

BLAST ……. Basic local alignment search tool

BLASTn ……..... Basic local alignment search tool for nucleotides

BLASTp …… Basic local alignment search tool for proteins

BLASTx …….. Translated basic local alignment search tool

BR …… Brown-rot fungi

CAZy ……..…. Carbohydrate-active enzymes

CDSs ……..…….. Coding sequences

DNS ……...3,5-dinitrosalicylic acid to 3-amino,5-nitrosalicylic acid

E-value …….. Expect value

EBI ……..…….. European bioinformatics institute

EE-LMB4 ……. Enzymatic extract from native isolation *D. pusillus*-LMB4

EFB ……..…….. Empty fruit bunches

ExPASy ……..…….. Expert protein analysis system

FPLC …… Fast protein liquid chromatography

FPU ……. Filter paper units

GMQE ……..…… Global model quality estimation

HBT ……... 1-Hydroxybenzotriazole

ITS …… Internal transcribed spacer regions

LB …….. Lysogeny broth

LMS ……..…….. Laccase mediator system

MEA ……..... Maltose extract agar

MUSCLE ……. Multiple Sequence comparison by log-expectation

NCBI ……..... National center for biotechnology information

NDF ……...…… Neutral detergent fiber

NIC ……... Natural intermediate compounds

PCR …….. Polymerase chain reaction

PDA ……..…… Potato dextrose agar

PDB ……... Protein data bank

PSI-BLAST ……..… Position specific iterated-basic local alignment search tool

RNA-seq …… RNA sequencing

SDS-PAGE …….. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SIC …… Synthetic intermediate compounds

SMRT ……...… Single molecule real-time

SPS …….. Simultaneous pretreatment and saccharification

SSF …… Solid-state fermentation

TAIL-PCR ……..... Thermal asymmetric interlaced-polymerase chain reaction

TEMPO …… 2,2,6,6-tetramethylpiperidin-1-yloxy

tBLASTx …… Translated nucleotide basic local alignment search tool

VA …… 3,4-dimethoxybenzyl alcohol

WBEA ……..…….. Wheat bran extract agar

WR …….. White-rot fungi

YDPS …….. Yeast extract peptone dextrose agar

Chapter 1: Introduction

Enzymes are the catalysts of all biological processes with various outcomes including energy production, biomolecule synthesis or bioremediation. Enzymes implementation in biotechnology applications began many centuries ago with the use of whole cells capable of specific biochemical transformations such as alcoholic fermentation. With a better understanding of biological processes, enzymatic extracts from cells with specific properties were then prepared to perform specific tasks such as protein and starch hydrolysis (Underkofler *et al.*, 1958). The use of enzymes in an industrial context rapidly highlighted the need to increase their production to reduce costs and to favor the transition from chemical industrial procedures to enzymatic processes (Abbas *et al.*, 2005; Pellis *et al.*, 2018). Paper, textile, leather, detergent, food, beverage, pharmaceutical and fine chemistry are industry examples that have incorporated enzymes into their industrial-scale processes, decreasing the negative environmental impact of massive industrial production (Jegannathan & Nielsen, 2013). To this end, huge scale fermentation for production of exo-enzymes and heterologous expression systems were explored. Moreover, protein engineering studies were conducted to improve the catalytic activity of selected enzymes (Porter *et al.*, 2016).

Regardless of the enzyme, the process to bring biocatalysts from microorganism to an industrial application and commercialization is similar: isolation and screening of microorganisms for a specific activity, production, purification, and characterization of the desired enzyme, overexpression in heterologous systems and improvement of its catalytic characteristics (Sharma *et al.*, 2018). Also, Fungi, bacteria, plants, and animals are enzyme sources for industrial processes, although, fungi have been the main source of industrial enzymes (Chambergo & Valencia, 2016). An industrial point of view, the fundamental characteristic of enzymes, that is to perform a chemical reaction with less energy and time, is the main appeal. Besides, as biocatalysts, enzymes reduce the use of hazardous compounds and, the release of pollutants by the industry while avoiding harsh operational conditions. As such, enzymes have improved traditional industrial processes such as production

of alcohols, sugars, polymers or organic molecules among others, making these macromolecules very useful biotechnology tools for modern industry (Sheldon, 2017).

Energy from liquid fuels has been supported by enzymes with the production of sugars from polysaccharides to be fermented to generate fuel alcohols, as well as starch and cellulose hydrolysis. This is especially true during years when prices for traditional sources of liquid fuels were high, and such enzymatic strategies were used by Brazil between 1970 to 2000 (Amorim *et al.*, 2011). Liquid fuels are the main source of energy for transport, and petroleum-based liquid fuels the principal product used. However, liquid fuels are among the main sources of greenhouse gases besides being a non-renewable source of energy, leading a search for new alternatives to fulfill the demand. In opposition, biofuels are a renewable source of energy, bioethanol and biodiesel being the most common biofuels used for transport.

Bioethanol is produced by fermentation of sugars from food and crops, mainly. It is categorized based on the sugar source for its production, namely first-, second- and third-generation biofuels (Ramos *et al.*, 2016). First generation biofuels are obtained from edible crops, using sugars as sucrose from sugar cane or fermentable syrups from starch hydrolysis. The process relies on the fermentation of sugars to ethanol which is directly possible with fermentable syrups but necessitates a prior hydrolysis step with more complex molecules such as starch. Production of first-generation biofuels has started many years ago with Brazil and USA being the most important producers. The main drawback of this bioethanol production is that it diverts food to the ethanol fermentation process, reaching 15.3% and 18.4% of the corn and sugar cane production, respectively, for 2013 (Koizumi, 2015).

Second-generation biofuels are obtained from biomass with lignocellulose being the principal raw material component. An advantage of these biofuels is the use of residual biomass without compromising food security as it can occur with first generation biofuels. However, the actual production of lignocellulosic ethanol is low, due to higher production costs. In fact, lignocellulose pretreatment, the first step in this second-generation ethanol production, involves more production costs than saccharification or fermentation, as well generating inhibitors of cellulose hydrolysis

and sugar fermentation (Gomez *et al.*, 2008). Thus, an effective and low-cost pretreatment in this second-generation ethanol production has yet to come but could lead to massive industrial development in this field, due to the extensive availability of cheap sources of lignocellulosic biomass residues. (Gerbrandt *et al.*, 2016; Khoo, 2015; Kumar *et al.*, 2016).

Third-generation biofuels are lipids obtained from algae, and production focuses on biodiesel applications. A main inconvenient of this technology is the use of huge bioreactors since the leading source of energy for their production comes from the sun. Moreover, difficult extraction of oil from the algae due to their strong cellular walls and the formation of residual biomass before oil extraction are other drawbacks of this approach. Furthermore, the majority of transport vehicles are still not adapted to biodiesel for the combustion (Alaswad *et al.*, 2015).

Independently of biofuel type, biotechnology is playing an important role in the development of alternative energy fuel production. Indeed, enzyme incorporation into the hydrolysis step for bioethanol production has been possible through cost reduction of cellulolytic enzyme production, enzymatic bioprospection and boosting of hydrolytic enzymes by protein engineering (Ellilä *et al.*, 2017). These improvements in polysaccharide hydrolysis have increased the sugar release while replacing the acid or alkali solutions previously used for starch and cellulose hydrolysis (Viikari *et al.*, 2012). Hence, hydrolytic enzymes could offer now a good cost:benefit ratio for the cellulose hydrolysis process with more efficient cellulases for sugar release and their integration into ethanol fermentation (Kuhad *et al.*, 2016; Liu *et al.*, 2016). However, the use of enzymes in the lignocellulose pretreatment process has not yet been successful since lignin is a more complex biopolymer than cellulose or starch, increasing the challenges to incorporate enzymes into the delignification process (Plácido & Capareda, 2015). Nevertheless, considerable efforts are still put towards bioprospection of various species in order to find an appropriate enzyme to tackle this issue.

Fungi-wood and fungi-fungi interactions have been described in wood colonization, in particular basidiomycete species, showing their relevance in this process (Hiscox *et al.*, 2018). Basidiomycete fungi are the main microorganisms

capable of wood decay due to a production of ligninolytic enzymes for carbon recycling from lignocellulose in forests. Thus, bioprospection of new basidiomycete fungi and identification of enzymes involved in wood decay can offer new biocatalysts for the delignification process, replacing traditional pretreatment methods and englobe the second-generation bioethanol industry in a real green chemistry concept (Porter *et al.*, 2016).

1. Literature review

1.1. Lignocellulose

Lignocellulose, the main component of leaf cell walls, branches, and tree trunks, is the most abundant renewable biomass material of Earth. Lignocellulose is a polymeric matrix formed by three polymers: cellulose, hemicellulose and lignin, that give plants structural, and mechanical support and protection, mainly, from environmental damages or phytopathogen microorganisms (Figure 1). Lignocellulose has been used to produce paper and energy by combustion or fermentation (Guo *et al.*, 2015). Nevertheless, a clean energy production from lignocellulose such as bioethanol involves 1) elimination of lignin to release cellulose; 2) breaking down cellulose and hemicellulose to release sugars; 3) fermentation of sugars to produce bioethanol; 4) purification of produced bioethanol (Aditiya *et al.*, 2016).

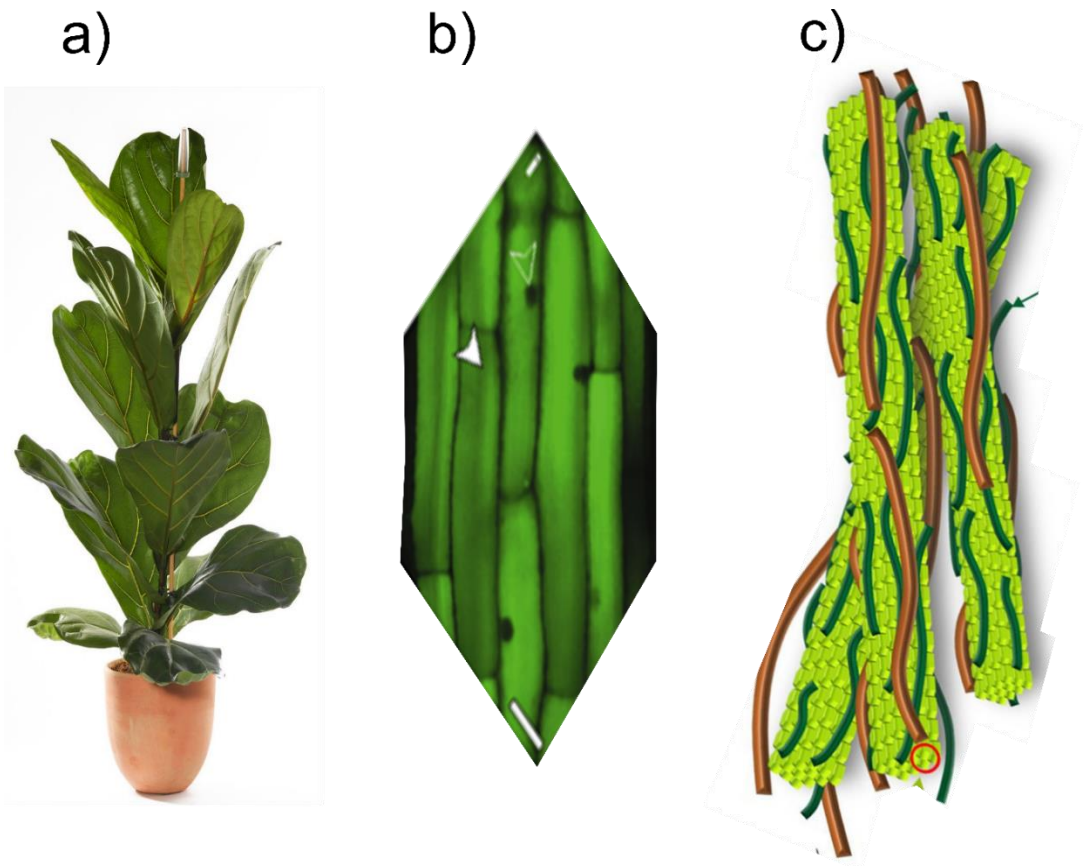


Figure 1. Lignocellulose structural organization.
a) healthy plant; b) microphotography of a plant tissue showing vegetal cells as a brick wall to give resistance and rigidity to plants; c) lignocellulose microfibrils representation with cellulose chains in bright green in the center, hemicellulose chains in dark green intertwined with cellulose chains and lignin polymers in brown covering lignocellulose microfibrils. Figure adapted from (Jensen *et al.*, 2017) (Brandizzi *et al.*, 2002).

Sources of lignocellulosic biomass to produce bioethanol include mainly residues from crops, followed by forestry wastes, energy crops along with municipal and industrial wastes. Sugar cane bagasse (Cardona *et al.*, 2010), corn straw, rice straw (Binod *et al.*, 2010) and wheat straw (Talebnia *et al.*, 2010), are the major crops for lignocellulosic biomass production. Estimation of total bioethanol that could be produced from crop residues arises to 491 billion liters per year in the world (Saini *et al.*, 2015), but the low development stage of lignocellulosic ethanol technology and the industry inclination to obtain first-generation bioethanol have not favored the growth of industrial production of lignocellulosic ethanol (Ullah *et al.*, 2015).

1.1.1. Cellulose

Cellulose is an unbranched homopolysaccharide composed of glucose monomers bonded by β-1,4-glycoside linkages forming a chain called microfibril. Microfibrils are packaged and stabilized by hydrogen bonds forming cellulose fibers. Cellulose can be crystalline or amorphous depending on the packing degree, higher packing being associated to cellulose crystallinity. High degree of packing hinders hydrolysis, due to the fact that it limits access to enzymes or acids during cellulose hydrolysis processes (Brown, 2004) (Figure 2).

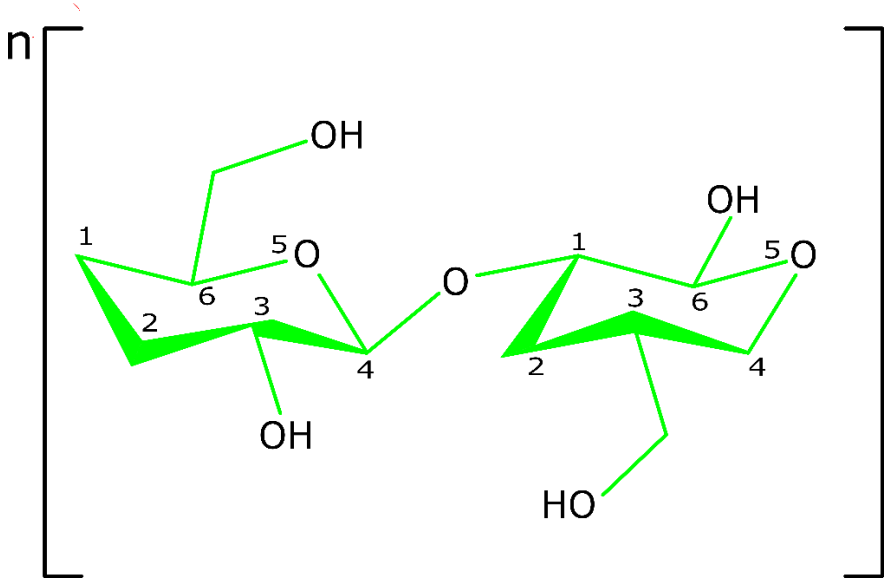


Figure 2. Structure of cellulose chains.
Glucose molecules are oriented with a 180° rotation to form the β-1,4-glycoside link between the carbon 1 of the first glucose and the carbon 4 of the second glucose. The left and right ends are called non-reducing and reducing end, respectively.

Cellulose is a commercially valuable polymer because it is used for paper and bioethanol production, mainly. In fact, cellulose paste, from trees or recycled paper, is the only raw material used to produce paper although agricultural wastes are a promising source for cellulose since it would not use additional land, water or technology to obtain the desired raw material (Gupta & Verma, 2015; Sarkar *et al.*, 2012). Production of bioethanol from cellulose begins with cleavage of β-1,4-glycoside linkages forming cellulose microfibrils by a cellulolytic pool of hydrolytic

enzymes called cellulases. The three main groups of cellulolytic enzymes are endo-1,4-β-glucanases (EC 3.2.1.4), exo-1,4-β-glucanases (E.C 3.2.1.74, 3.2.1.91) and β-glucanases (EC 3.2.1.21) (Eriksson, 1978). Those enzymes work in a synergic way to obtain glucose syrups, that can be used in the fermentation process for bioethanol production. More specifically, endo-1,4-β-glucanases randomly break down internal links of cellulose chains and generate cellulose oligosaccharides. Exo-1,4-β-glucanases progressively cleave non-reducing ends of cellulose chains, releasing glucose or cellobiose. Finally, β-glucanases break down reducing ends from oligosaccharides and cellobiose produced by the two other enzymes to generate glucose (Juturu & Wu, 2014).

1.1.2. Hemicellulose

Hemicellulose is a branched heteropolysaccharide chain principally composed of glucose, mannose or xylose mainly bond by β-1,4-glycoside linkages. Hemicellulose is attached to cellulose and lignin by hydrogen bonds to form a lignocellulose structure. Hemicellulose chains are named according to the principal monosaccharide present in the backbone of the chain. The most abundant hemicelluloses are mannan, xylan and xyloglucan (Scheller & Ulvskov, 2010). Hemicellulose content in lignocellulose varies between crops and also according to plant components. Moreover, linkage diversity, polymerization degree, and backbone structure make the classification and hydrolysis of hemicellulose more complex compared with cellulose (Juturu & Wu, 2014) (Figure 3).

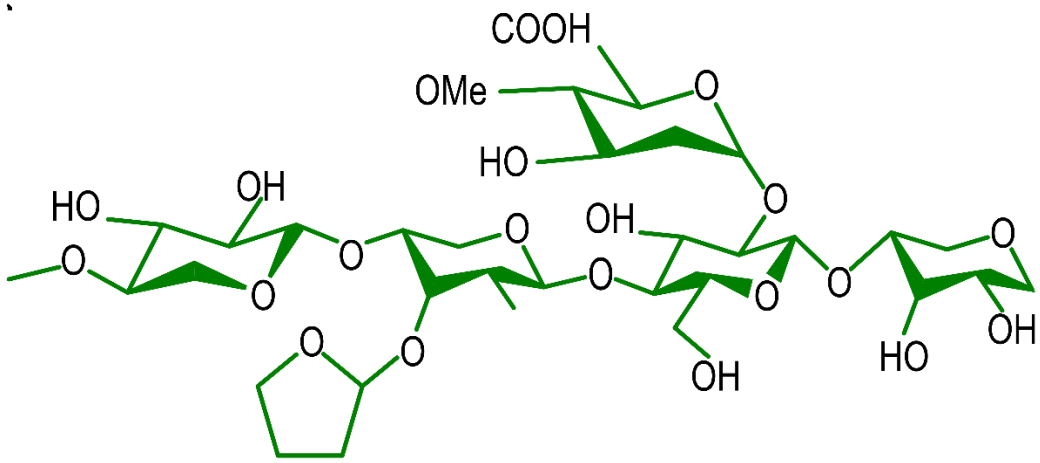


Figure 3.Structure of hemicellulose chains.
In a similar fashion as cellulose, glucose molecules are the main component of the chain but pentoses and hexoses are grafted to this structure to form different kinds of hemicellulose.

Hemicellulose as raw material for bioethanol production has been explored since fermentable sugars can be obtained from its hydrolysis (Collins *et al.*, 2005). In particular, the exploration of xylanase enzymes for the hydrolysis of various hemicelluloses has promoted the interest for this polysaccharide in bioethanol production (Uday *et al.*, 2016). Nevertheless, the use of hemicellulose for bioethanol production is mainly hindered by the formation of fermentation inhibitors as 2-furaldehyde (furfural) from hemicellulose due to high temperature and pressure conditions used during the lignocellulose pretreatment, along with inefficient use of pentoses by yeasts during the fermentation process (Sànchez Nogué & Karhumaa, 2015). As alternatives to integrating hemicellulose into bioethanol production, biotechnological strategies have been developed, such as detoxification by ligninolytic enzymes to reduce furfural content, enhancing the bioethanol fermentation (De La Torre *et al.*, 2017; Kudahettige Nilsson *et al.*, 2016). Moreover, isolation and modification of new yeast strains along with fermentation process improvements have allowed the use of carbohydrates of five carbons such as xylose to obtain bioethanol from sugar cane bagasse with high efficiency and a good cost-benefit ratio (Antunes *et al.*, 2016; Losordo *et al.*, 2016).

Aside from bioethanol production, hemicellulose has been incorporated into biotechnological processes, to take advantage of the various carbohydrate compounds from lignocellulose, and recent publications have shown promising results for the production of biomaterials and other biofuels including butanol or hydrogen (Peng & She, 2014). Interestingly, hemicellulose possesses hydrophobic and thermoplastic characteristics that can be chemically modified or incorporated into new polymers for specific applications. For instance, hemicellulose modified with chitosan has been produced to generate biofilms that can be used in food packaging (Chen *et al.*, 2016).

1.1.3. Lignin

Lignin is an amorphous polymer of lignocellulose composed mainly of three phenolic compounds, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, called monolignols. Monolignols are linked together by C-C and C-O-C bonds between carbons from two benzene rings or from a phenolic radical and a benzene ring (Boerjan *et al.*, 2003). The diversity of monolignols, their random linkages and their low polarity are responsible for the difficulties associated with lignin degradation (Cyril Heitner, 2010). Lignin provides a protective barrier for cellulose and gives rigidity to plants. Its content in plants varies between 15-36% and plant rigidity is directly proportional to its quantity and type giving rise to a plant organization according to lignin. More precisely, the main monolignol component of the lignin structure delineates these plant categories: gymnosperms are composed of 90% guaiacyl, known as softwood plants, angiosperms having the same proportion of guaiacyl and syringyl known as hardwood plants, and gramineae with guaiacyl and p-hydroxyphenyl in the same proportion, known as grass (Higuchi, 2006) (Figure 4).

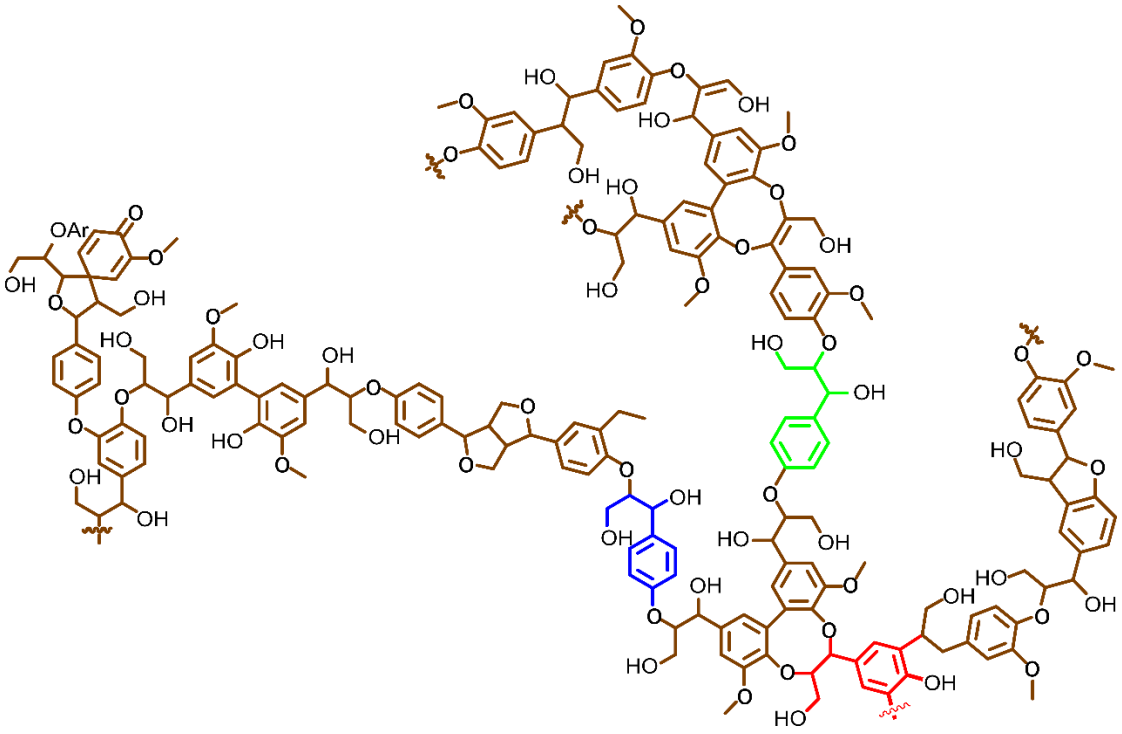


Figure 4. Lignin structure.
Several chains in a random sequence compose lignin backbone. Monolignols, coniferyl alcohol (blue), sinapyl alcohol (red) and *p*-coumaryl alcohol (blue), are its main components and linkage diversity make lignin a hard molecule to degrade either chemically or biologically. Modified from (Zakzeski *et al.*, 2010).

Lignin appears to be the main barrier to overcome in a second-generation bioethanol production due to the difficulty to extract cellulose and hemicellulose from this structure. In the past, lignin was oxidized, and undesired lignin-related compounds were discarded with the acid or basic solutions used. Nowadays, lignin catalytic valorization along with development of biorefinery skills are taking advantage of this natural polymer, closing the cycle of an integral management of industrial crop residues (Zakzeski *et al.*, 2010). Also, it is important to highlight the fact that lignin is the only source of renewable aromatic compounds, giving another value aside from second-generation ethanol industry substrate (Beckham *et al.*, 2016; Liang & Wan, 2017; Ragauskas *et al.*, 2014; Sun *et al.*, 2018). For instance, lignin isolated compounds have been used to produce jet fuel (Bi *et al.*, 2015); vanillin (Fache *et al.*, 2016); and antioxidants (Kurakake *et al.*, 2015) through

fractionation using chemical catalysis; biological approaches are still mainly unexplored for these applications.

1.2. Lignocellulose pretreatment

Pretreatment is the fractionation of lignocellulose into cellulose, hemicellulose, and lignin, and it is the first step in bioethanol production. A successful pretreatment will allow an optimal recovery of cellulose that will be directly proportional to the amount of ethanol produced (Kumar *et al.*, 2009). Pretreatment relies on breaking lignin bonds to release cellulose and hemicellulose to render these complexes accessible to hydrolytic enzymes.

Ideal lignocellulose pretreatment for a successful second-generation biofuel production would incorporate the following characteristics: low cost, simple and adaptable process for bioethanol production, ability to boost hydrolysis and fermentation steps, integral use of all lignocellulose polymers for the production of high-value products, and reduced emission of hazardous compounds as acid and alkali solutions through the use of biotechnological tools (Chen *et al.*, 2017). Lignocellulose pretreatment can be accomplished by physical, chemical or biological processes. Physical and chemical methods are mainly used by the industry to obtain cellulose due to their ease of execution and low cost, respectively. However, both approaches generate hazardous compounds and fermentation inhibitors as drawbacks. Biological pretreatments are not currently used industrially, but research in this field is increasing since it is the only green chemistry strategy for lignocellulose pretreatment (Sindhu *et al.*, 2016). Even though lignocellulose biomass pretreatment has been focused on bioethanol production, it would be desirable to open this process to other biorefinery approaches (Galkin & Samec, 2016; Isikgor & Becer, 2015). Development of new valorization strategies using lignocellulose biomass obtained from enzymatic pretreatment is an interesting challenge to obtain various compounds that petrochemistry or organic synthesis has developed over the years (Figure 5).

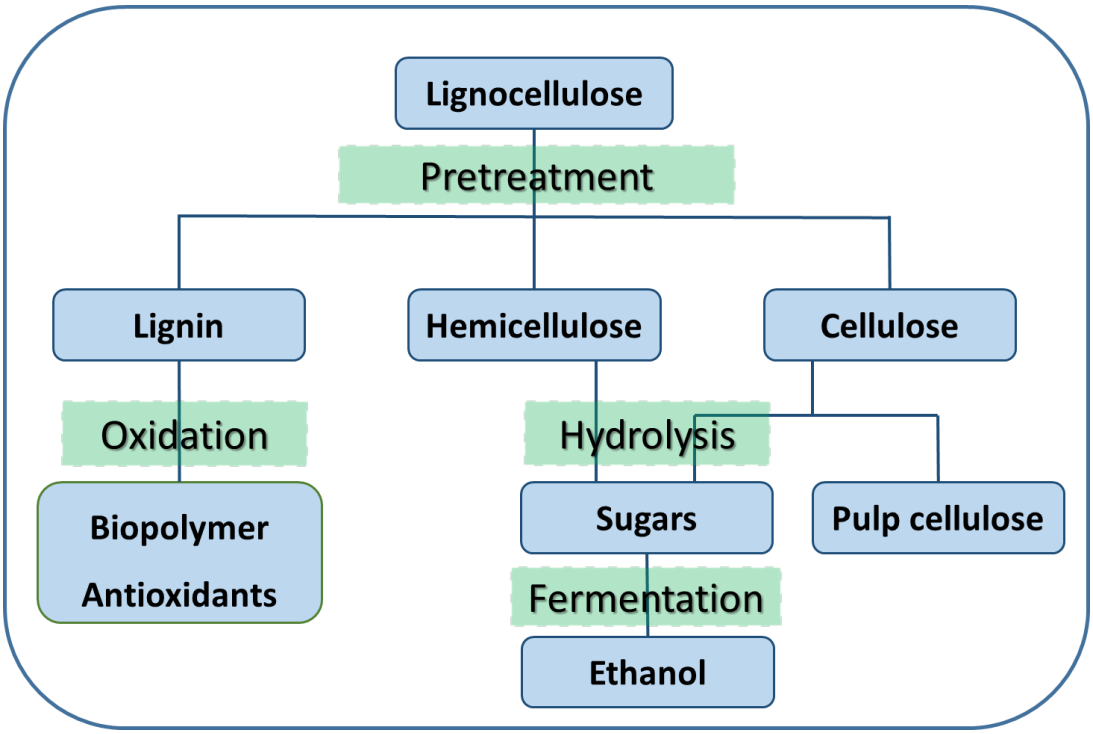


Figure 5. Lignocellulose handling flow chart.

The flow chart depicts steps in green and products in blue during lignocellulose handling. The first step is the pretreatment, where the three principal components of lignocellulose are separated. To obtain ethanol by fermentation, hemicellulose and cellulose must be hydrolyzed by cellulolytic and hemicellulolytic enzymes to release the sugars. The fermentation process is carried by bacteria or yeasts leading to ethanol production through catabolic pathways.

1.2.1. Physical pretreatment

Physical pretreatments are focused on particle size reduction or decrease of lignocellulose polymerization degree. Mechanical pretreatment is based on crushing, grinding or compression to reduce the particle size, and is performed before any chemical or biological pretreatment. Particle size reduction is not enough for an efficient lignocellulose pretreatment but necessary for the success of the up-coming steps (Dionisi, 2013). High cost of physical pretreatments, despite the fact that it relies on simple technologies constitute its main disadvantage (Cadoche & López, 1989).

Microwaves, ultrasounds pyrolysis and syngas are the typical physical pretreatments. However, a successful physical pretreatment often uses acid or alkali solutions. Microwaves can accelerate the process and are especially effective when

combined with alkali solutions (Binod *et al.*, 2012; Zhu *et al.*, 2016). Ultrasounds can also reduce pretreatment time but necessitate temperatures of 75°C and NaOH concentration of 3.25% (Velmurugan & Muthukumar, 2012). Pyrolysis only needs a fraction of the time for the process, but it requires temperatures between 300 and 500 °C for lignin depolymerization (Liaw *et al.*, 2013).

1.2.2. Chemical pretreatment

Chemical pretreatments are the most common for cellulose recovery due to their low cost and high efficiency to remove lignin from the lignocellulose (Behera *et al.*, 2014). Indeed, phosphoric acid (Siripong *et al.*, 2016), sulfuric acid (Li *et al.*, 2016), and alkali solutions have been used successfully for the pretreatment of lignocellulose to increase the production of reducing sugars in bioethanol production.

Chemical pretreatment, mainly the use of acid solutions, has the disadvantage of decreasing effectiveness of hydrolysis and fermentation processes in bioethanol production due to generation of inhibitors (Toquero & Bolado, 2014). Inhibitors such as acetic acid, furfural, formic acid and hydro methyl furfural from chemical pretreatment have been reported and their effective elimination is generally associated with a high production of reducing sugars and ethanol (Gupta *et al.*, 2016; Jönsson & Martín, 2016).

1.2.3. Physicochemical pretreatment

Ammonia Fiber Expansion (AFEX) and steam explosion are the most common physicochemical pretreatment. Both processes allow depolymerization of lignin and cellulose microfibrils to improve saccharification. Moreover, solutions used for AFEX and steam explosion can be recirculated and recycled, respectively, for a better energy efficiency of the process (Abdul *et al.*, 2016; Pielhop *et al.*, 2016). Lignin depolymerization by a physiochemical pretreatment releases organic compounds to favor the recovery of molecules with added value without the production of cellulose hydrolysis and sugar fermentation inhibitors. However, the use of physicochemical pretreatments is an expensive technology to be adapted in an industrial environment, due to high temperatures required (around 200°C) and time needed

for boilers pre-heating (about 1h), necessary to reach ideal conditions for lignocellulose pretreatment (Zhu *et al.*, 2015).

1.2.4. Biological pretreatment

Due to lignin complexity, a biological pretreatment requires a consortium of enzymes for lignin breakdown. Fungi (Salvachúa et al., 2011) and bacteria (de Gonzalo, Colpa, Habib, & Fraaije, 2016) constitute the organisms with most lignocellulose degradation activity reported, having enzymatic machineries able to breakdown lignin through oxido-reduction mechanisms. In the forests, ligninolytic microorganisms are responsible for lignocellulosic biomass recycling from wood decay and constitute a great source to find perfect enzyme candidates for lignocellulose pretreatment, especially considering the fact that they express ligninolytic and cellulolytic enzymes (Rytioja *et al.*, 2014).

The use of whole microorganisms for lignocellulose pretreatment is not favored by the industry since it would result in an accumulation of biomass from the fungi or bacteria used over the ligninolytic substrates. Also, these microorganisms thrive for cellulose that will not be available anymore to produce ethanol. Thus, efforts are focused on research to obtain enzymes by fermentation for lignin degradation and cellulose breakdown. However, ligninolytic microorganism consortia are essential to fully understand the complexity and requirements of lignocellulose biological pretreatment (Cragg *et al.*, 2015).

Bacteria are certainly a source of ligninolytic enzymes but this enzymes seem to be better adapted to organic molecule transformation such as dyes and pharmaceutical derivatives (Brown & Chang, 2014; Brown *et al.*, 2011). This restricts the search of ligninolytic enzymatic complexes for delignification processes among bacterial isolates. Moreover, reports of lignin degradation by bacteria are scarce due to the fact that the relation between bacteria and lignocellulose is associated to cellulose hydrolysis (Montella *et al.*, 2017).

Fungi are the main organisms associated to wood decay colonization (Smith *et al.*, 2017), due to their ability to secrete oxido-reductase enzymes and their affinity for phenolic polymers such as lignin. In fungus, there are many factors that induce

the production and secretion of ligninolytic enzymes, varying with fungal species and environmental conditions. Depletion of nutrients (carbon, nitrogen or sulfur), presence of compounds acting as enzyme expression inductors such as saccharides (glucose, xylose, cellobiose), metal ions (copper, manganese, cadmium and silver) or organic compounds (phenols, diphenols) all contribute to modulate production and secretion of ligninolytic enzymes. More specifically, ligninolytic enzyme genes are regulated by specific transcription factors and promoter sequences (Furukawa *et al.*, 2014; Penttilä *et al.*, 2005). During wood decay, saccharides are the main trigger of ligninolytic enzyme expression. Sugars released by decomposing leaves and wood, are recognized by membrane proteins of white or brown rot fungi cells which initiates, and regulates, secretion of lignolytic enzyme complexes. (figure 6) (Fritsche & Hofrichter, 2005; Shary *et al.*, 2008).

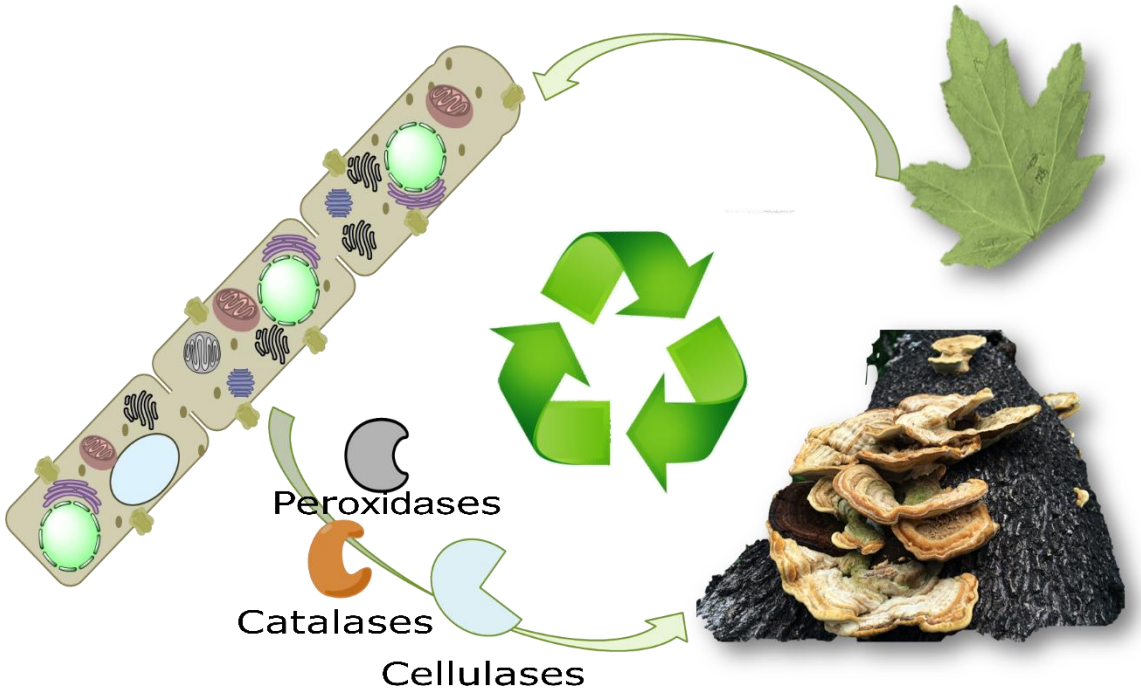


Figure 6. Lignocellulolytic enzymes expression.

Ligninolytic enzymes production from fungal cells representation. Saccharides and organic compounds derived from lignin, hemicelluloses and cellulose serve as the trigger for the production in the fungal cells. Peroxidases, catalases and hydrolases enzymes secreted are involved in delignification and hydrolysis processes of lignocellulose.

Studies on fungi related to lignocellulose decomposition have demonstrated that species involved in wood decay produce a pool of many enzymes that act against the three lignocellulose components (Abbas *et al.*, 2005; Martínez *et al.*, 2009). Furthermore, a recent study has shown the importance of fungi stage colonization during wood decomposition in regards to enzyme production, the youngest and oldest stages being associated with the expression of several enzymes with ligninolytic and cellulolytic activities, respectively (Presley & Schilling, 2017; Zhang *et al.*, 2016).

Delignification process in nature has been well described and enzymes responsible for lignin depolymerization have been characterized. However, the *in vitro* delignification process using enzymatic extracts with ligninolytic activity is still not well understood (Chen & Wan, 2017). In fact, ligninolytic enzymatic mechanisms have been studied with simple substrates, such as phenolic compounds, but the precise mechanism that laccases use on complex molecules as lignin is still not properly defined. Moreover, laccases not only depolymerize lignin, but can also induce polymerization (Munk *et al.*, 2015). Hence, understanding ligninolytic enzyme complexes and their synchrony in order to circumvent actual limitations and improve their use in the delignification process for biofuel and/or organic compounds production is an on-going process (Abdelaziz *et al.*, 2016).

1.2.4.1. Wood-decay fungi

As was highlighted previously, fungi are the main wood litter organism, nevertheless, the Agaricomycetes class from *Basidiomycota* division into fungi kingdom are the species that during the course of evolution, have developed an efficient enzyme system to mineralize lignin, cellulose and hemicellulose substantially (Blanchette, 1991; Bushell, 1995; Velmurugan & Muthukumar, 2012). Inside of Basidiomycota division exists two fungal groups according to the enzymatic profile and colonization ways to degrade lignocellulose, called Brown-Rot (BR) and White-Rot (WR) fungi (Goodell *et al.*, 2008; Krah *et al.*, 2018; Schwarze, 2007). BR are the lignocellulolytic organisms that have been specialized on enzymatic hydrolysis of cellulose and hemicellulose, through the expression of multiple

Carbohydrate-Active Enzymes (CAZy) with low degradation of lignin by ligninolytic enzymes. However, BR supporting the lignin oxidation into the lignocellulose colonization by the generation of Radical Oxygen Species (ROS) to favor the cellulose hydrolysis (Castaño *et al.*, 2018).

WR are the Basidiomycetes more reported as delignificant organism due to a major production of ligninolytic enzymes, compared with BR (Riley *et al.*, 2014). The major of WR reported as important delignificant organism belong to the Polypolares order, also, the recent genomes from WR reported have shown the abundance and close phylogenetic relationship between the ligninolytic enzymes into the four Polypolar clades: Polyporoid, Phlebioid, Antrodia and Residual Polyporoid (Binder *et al.*, 2013), highlighting the presence of catalases such as laccases for the delignification function (Glazunova *et al.*, 2018; Savinova *et al.*, 2019). To conclude, is relevant to highlight the exploration and identification of ligninolytic enzymes from WR, due that those kinds of enzymes are the main components of the extracellular enzymatic complex for wood decay. Further, the WR could be the clue to design biotechnological strategies for lignocellulose pretreatment in the bioethanol industry.

1.3. Enzymes involved in lignocellulose pretreatment

Enzymes involved in delignification processes are known as ligninases. Ligninases are extracellular enzymes with oxide-reducing activity capable of tackling recalcitrant compounds as lignin and lignin fractions released during wood decay (Mester & Tien, 2000). Moreover, these enzymes are able to perform dye degradation in industrial effluents (Wesenberg *et al.*, 2003) and medicinal molecule degradation (Asif *et al.*, 2017). Their versatility toward degradation of recalcitrant compounds have rendered these enzymes as true biotechnological assets in industrial and agroindustrial residue management. However, the use of ligninases for industrial processes has not been successful yet due to a poor cost-benefit ratio.

Ligninases are classified either as phenol oxidases (laccases) or heme peroxidases (lignin peroxidases, manganese peroxidases and versatile peroxidases) (Dashtban *et al.*, 2010; Wong, 2009). Laccase enzymes constitute the main ligninase group studied. Laccases can be found in trees, bacteria and fungi,

showing a diversity in structure but keeping the same oxido-reduction function. Peroxidases are more similar in structure, typically having two structurally conserved Ca^{+2} binding sites and one tetrapyrrolic ring with a ferrous nucleus responsible of the oxidoreduction process. However, peroxidases differ from each other in regards to manganese dependence for the enzyme activity (Martínez, 2002).

1.3.1. Laccases

Laccases (EC 1.10.3.2) are multicopper oxidases capable of hazardous compounds transformation, ring cleavage of aromatic moieties, synthesis of organic substances using amines or phenols such as substrates and formation of dimers or oligomers by coupling of radicals generated from the phenolic rings. (Agrawal *et al.*, 2018).

Laccases are glycosylated proteins formed by three protein domains called cupredoxins that shelter four copper atoms. Copper atoms (Cu^{+2}) within laccases are denominated T1, T2, and T3 in accordance with their position into the enzyme, each copper having a different function (figure 7). T1 copper is located in the third laccase enzyme domain and its position is coordinated by two histidines, one cysteine, and one methionine. T2 and T3 coppers form a cluster between the first and the third domain of the enzyme. Each one of the two T3 copper is coordinated by three histidine residues while two histidines share coordination bonds with the T2 copper in laccase enzymes (Zhukhlistova *et al.*, 2008). Known fungal laccase enzymes are generally characterized by a molecular weight around 66 kDa, an isoelectric point of 3.9, an optimal temperature of 55 °C and optimal pH of 3, 4, 4.5 and 6 for 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-Dimethoxyphenol, guaiacol and syringaldazine, respectively (Baldrian, 2006).

Laccase enzymatic reaction begins with oxidation of the substrate by the T1 copper (Cu^{+2} to Cu^{+}) followed by an electron transfer from the T1 copper to the T2 and T3 coppers. It is necessary to accomplish four successive oxidation states of the substrate with electron transfer to copper atoms to attain the complete reduction state of the enzyme. The electron transfer from the substrate to the T1 copper is not a rate-limiting step, but the electron transfer from T1 copper to the T2/T3 copper

cluster and the reduction of a new substrate by the T2 copper are rate limiting for the laccase activity. The enzyme reduced state has a high affinity for reducing substrates such as dioxygen that can produce two water molecules for each laccase complete cycle (Figure 7).

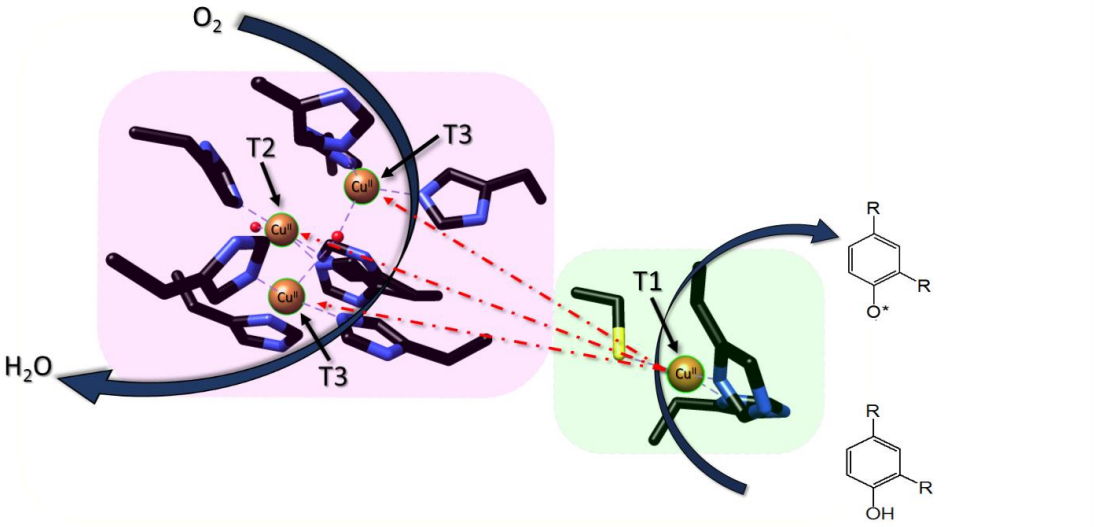


Figure 7. Structure of laccase catalytic site.

Structure of Basidiomycete *Trametes hirsuta* catalytic site (PDB accession number 5LDU) obtained using UCSF Chimera software. The four copper atoms involved in the oxido-reduction laccase activity are shown as brown spheres. Cu-T1 is attached to two histidine residues and one cysteine while Cu-T2 and Cu-T3 are bound to 2 and 3 histidines, respectively. The red dotted line represents the electron flow from T1 to T2/T3. Pink and blue shadows depict clusters involved in reduction and oxidation of substrates, respectively.

1.3.1.1. Laccase mediator systems

As it was described, laccase enzymes are mainly described for their preference to oxidizes phenolic compounds. However, these enzymes also have the capacity to oxide non-phenolic compounds through the involvement of laccase mediator systems (LMS) that can oxidize intermediate substrates by electron transfer or radical hydrogen atom transfer mechanisms (figure 8). Intermediate substrates participating in LMS are split into two groups: synthetic and natural intermediate compounds (Hilgers *et al.*, 2018).

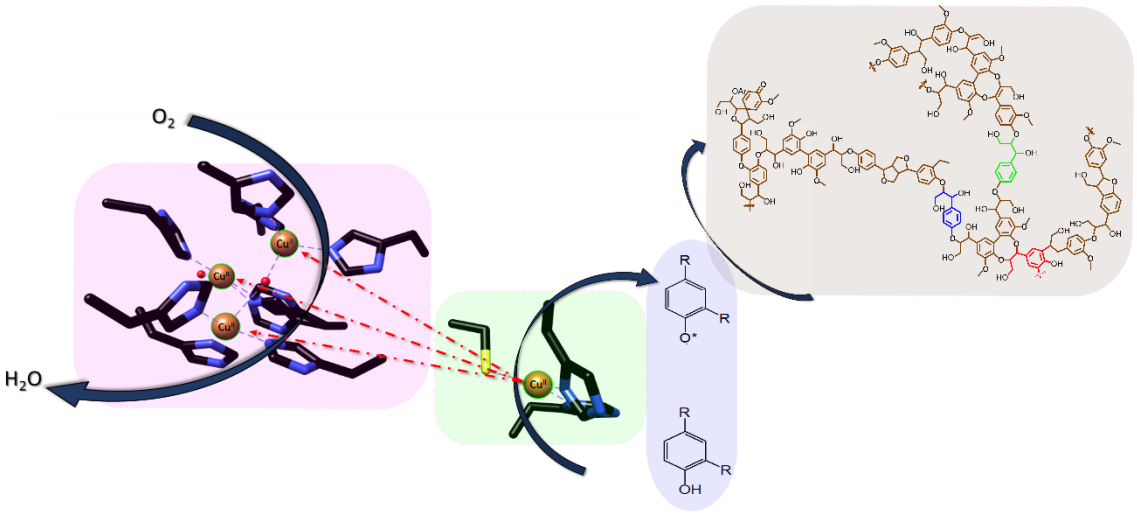


Figure 8. Laccase mediator system.

Follow the laccase catalytic site from figure 7 from Basidiomycete *Trametes hirsuta.* Pink and blue shadows depict clusters involved in reduction and oxidation of substrates, blue represent the intermediary phenolic compound and brown the oxidized substrate for the intermediary.

Synthetic intermediate compounds (SIC) characteristically comprise heterocyclic atoms along with hydroxyl and/or amine functional groups in their structure, for instance ABTS, HBT (1-Hydroxybenzotriazole), and TEMPO (2,2,6,6-tetramethylpiperidin-1-yloxy), among others. SIC have been used to boost the oxidation of non-phenolic compounds and lignin in lignocellulosic substrates. However, their usage was limited due to high costs and poor substrate oxidation. Natural intermediate compounds (NIC) are mainly phenolic compounds such as p-coumaric acid, 4-hydroxybenzylic alcohol, hydroquinone, syringaldehyde and vanillin among others that are released during lignin degradation by organisms in nature. NIC have been used to boost the biodegradation of non-phenolic compounds including dyes and detoxification of soil and industrial effluents (Christopher *et al.*, 2014).

1.3.2. Ligninolytic peroxidases

Ligninolytic peroxidases are a group of enzymes that are similar in structure and function; the structure is composed of two protein domains and one ferric heme prosthetic group responsible of the activity. All heme peroxidases depend on the

presence of hydrogen peroxide (or other organic hydroperoxides) for their catalytic oxidation. The catalytic cycle is composed of three simple reactions using hydrogen peroxide or hydrogen as an electron acceptor for oxidation of the substrates, generating as final products the oxidized molecule such as lignin or another organic compound and water. However, evolution of ancestral peroxidases has improved and amplified substrate range of ligninolytic peroxidases to include phenolic and non-phenolic compounds, to finally diverge in different specialized ligninolytic peroxidases (Ayuso-Fernández *et al.*, 2018). Ligninolytic peroxidases are produced by bacteria and fungi but basidiomycete fungi account for the largest number of enzymes reported (Plácido & Capareda, 2015).

Optimal pH, substrates and redox potential of ligninolytic peroxidases are similar aside from the fact that some enzymes use manganese as an additional enzymatic cofactor during the oxidation process. The additional Mn cofactor is a way for ligninolytic peroxidases classification in three different ligninolytic enzymes: lignin peroxidases that are not dependent of Mn, manganese peroxidases that necessitate Mn and versatile peroxidases than can use Mn as a cofactor but which is not mandatory for their enzymatic activity (Hofrichter *et al.*, 2010).

As laccases, ligninolytic peroxidases are also responsible for wood decay and can accomplish degradation of recalcitrant compounds. Thus, ligninolytic peroxidase exploration and modification by protein engineering is trending to improve its catalytic properties for the cellulosic biofuels industries (Gonzalez-Perez & Alcalde, 2018).

1.3.2.1. Lignin peroxidases

Lignin peroxidases (EC 1.11.1.14) are heme peroxidase enzymes composed of a distal and proximal domain that shelter one porphyrin heme moiety (Choinowski *et al.*, 1999). This porphyrin heme is responsible for the catalytic activity, using H_2O_2 as the final electron acceptor in the oxidation process (Falade *et al.*, 2017). Lignin peroxidase enzymes are glycoproteins with several isoenzymes reported in the same microorganism, with an average molecular weight of 40 kDa and low pH value

of 3 for the optimal enzymatic activity, using veratryl alcohol as the substrate (Furukawa *et al.*, 2014).

1.3.2.2. Manganese peroxidases

As for lignin peroxidases, manganese peroxidases have a porphyrin heme responsible for the oxidation process. But in contrast with the other peroxidases, these enzymes have a Mn^{++} ion that conform the pyrrolic structure in the catalytic pocket (Hofrichter, 2002). Manganese peroxidases are glycoproteins, with a molecular weight between 38 and 63 kDa and 43 % of identity with other sequences of ligninolytic peroxidases, which proves the close structural similarity although these enzymes have a wide activity range for phenolic compound oxidation (Sundaramoorthy *et al.*, 1994).

1.3.2.3. Versatile peroxidases

Versatile peroxidases, just like manganese peroxidases, have a manganese binding pocket but their activity does not depend on the presence of this atom (Knop *et al.*, 2016). Versatile peroxidases have a molecular weight between 38 and 45 kDa and some authors describe these enzymes as hybrids between lignin peroxidases and manganese peroxidases (Manavalan *et al.*, 2015).

1.4. Simultaneous pretreatment and saccharification (SPS)

One alternative to reduce cost in the incorporation of ligninolytic enzymes in the production of lignocellulosic ethanol is Simultaneous Pretreatment and Saccharification (SPS). Ligninolytic enzymes from basidiomycete fungi are released along with cellulases and xylanases mainly, during wood decay. Hence, SPS uses ligninolytic and cellulolytic enzymes in one step to boost saccharification and fermentation processes. This simultaneous strategy for lignocellulose pretreatment is a relatively new approach and mainly crude enzymatic extracts from basidiomycetes associated with delignification have been used along with commercial cellulolytic cocktails for the saccharification process. Such SPS has

shown the ability to increase reducing sugar production for enhanced bioethanol production (Althuri *et al.*, 2017; Kumar *et al.*, 2017).

Based on studies on the cellulosome of ascomycete fungi for the saccharification process, another alternative SPS approach has been evaluated with the construction of enzymatic immobilized modules using an inert support with immobilized proteins. The use of enzymatic immobilized units has shown better results in reducing sugar production compared with the use of free enzymes (Arfi *et al.*, 2014; Artzi *et al.*, 2016). Construction of lignocellulolytic enzymatic modules trough enzyme evolution of efficient catalytic complexes (cellulases, xylanases and ligninases) could enhance the incorporation of biocatalysts in lignocellulose pretreatment. Likewise, immobilized lignocellulolytic units led to the incorporation of laccase, xylanase and cellulase enzymes, increasing reducing sugar production, as it was done for lignocellulose pretreatment of wheat straw (Davidi *et al.*, 2016).

1.5. Bioprospection of ligninolytic organisms

Exploration of ligninolytic organisms has increased in recent years, especially supported by genomic tools helping to discover enzymes from new species and their potential industrial applications (Blackwell, 2011). As mentioned earlier, the main source of enzymes are fungi and bioprospection is the key to uncover proteins with new catalytic properties which could be incorporated into current industrial processes. Generally, industrial process enzymes require thermostability, acid tolerance, fast regeneration to the catalytic basal state and reuse the enzyme for several batches, among others. Newly discovered enzymes can be candidates for a given application, with or without the need to improve specific characteristics, but can also serve as a model to improve existing enzymes by protein engineering (Choi *et al.*, 2015; Damborsky & Brezovsky, 2014).

Comparative studies of fungal genomes from isolated ligninolytic species have supported the relevance of evolutive interaction between basidiomycete fungi and wood decay (Floudas *et al.*, 2012). Genomic information from ligninolytic fungi can serve as a guideline for enzyme identification with delignification and hydrolysis properties. Such genomic and proteomic data about ligninolytic enzymes can

certainly deepen our understanding of the delignification process and complete gathered information from *in vitro* studies (Nagy *et al.*, 2017; Nagy *et al.*, 2016). However, the production of ligninolytic enzymes from fungi is a long process that involves the isolation, screening and enzyme production by fermentation. Metagenomic studies can offer an alternative approach to explore several unknown enzymes in regard to potential enzymatic activities involved in delignification (Ferrer *et al.*, 2016; Madhavan *et al.*, 2017; Upadhyay *et al.*, 2016).

The important diversity of living organisms on earth and the wide variety of biological characteristics certainly reflects evolutive pressure towards adaption to diverse conditions. Numerous studies have identified and characterized living organisms but still it is generally accepted that many others, mostly microorganisms including fungi, have not been yet discovered. Most importantly, these unknown or uncharacterized organisms might present unexpected properties. With over 30 000 basidiomycete species but only a small number of fully characterized fungus, it appears relevant to pursue the exploration of basidiomycete fungi with ligninolytic activity, especially with the importance of ligninolytic enzymes as potential green catalysts for various applications.

1.6. Ligninolytic basidiomycete fungi genome exploration

The close relation between basidiomycete fungi, ligninolytic enzymes and wood decay has been described through this chapter. Gathered genomic information has allowed to adapt ligninolytic enzymes to *in vitro* degradation of recalcitrant compounds, but lignocellulose delignification has not yet attained good efficiency. Most genomic information about ligninolytic enzymes and isoenzymes is limited to codifying genes from fungi and bacteria, but studies on expression regulation of these enzymes during the delignification process are scarce and focused on cellulolytic enzymes (Presley & Schilling, 2017; Zhang *et al.*, 2016). In fact, genome sequencing of basidiomycete fungi is increasing, reaching around 80 complete genomes assembled, but enzyme exploration in those genomes has been focused on cellulolytic enzymes mainly (Li *et al.*, 2016; Ohm *et al.*, 2014; Riley *et al.*, 2014). However, the ligninolytic basidiomycete genomes that exist have opened the

exploration and analysis to evolutionary pathways leading a characterization of ligninolytic enzymes (Ayuso-Fernández *et al.*, 2017).

Based on our current understanding, ligninolytic enzymes expression is mainly regulated by external factors such as metal ions, xenobiotics compounds and nutrients depletion as it was described previously in section 1.2.4. However, the complete regulation mechanisms are not fully understood (Janusz *et al.*, 2013; Yang *et al.*, 2017). In fact, the ligninolytic secretome from basidiomycetes has been analyzed from fungi, but the information is not enough to comprehend and understand the delignification process *in vitro* (Alfaro *et al.*, 2014). New sequencing techniques along with fungi bioprospection can likely increase our knowledge on the enzymatic delignification process performed by fungi during lignocellulose recycling. Such knowledge can then serve as basis to develop biotechnological alternatives to handle lignocellulosic residues from agroindustry, and to lead that biomass to the production of bioethanol and/or organic compounds.

PROBLEM STATEMENT

Industrial development and the digital era are direct causes of the increasing energy demand. Consequently, reserves of traditional energy sources, such as charcoal and petroleum, have been decreasing by excessive extraction to satisfy the global energy demand. Besides, charcoal, oil and gas are the main sources of greenhouse gases. The upcoming exhaustion of non-renewable energy, has favored a switch to alternative and renewable energy sources such as wind, sun and biomass, among others (Ellabban *et al.*, 2014; Obama, 2017). Diversification of renewable energy sources in the world, based on geographical location (sun, wind, sea, and rivers) and biomass production can be a suitable strategy to supply future energy demands (Grueneich, 2015). Energy production from lignocellulosic biomass is the most promising alternative for biofuels production, taking advantage of the use of agricultural residues, besides to reduce the accumulation of biomass post-harvest (De Corato *et al.*, 2018).

Accumulation of agroindustrial lignocellulosic residues are a direct consequence of population growth forcing an increase of crops, either for nutrition or energy source purposes, and the lack of strategies for a responsible disposal of these compounds can represent an environmental problem. An example of this situation is the fast growing of palm oil extraction in the world, where the production has increased by 43% in the last 10 years, mainly for the palm oil transformation in biodiesel obtention (FAO, 2016). At the past, the most common strategy to recycle lignocellulose from palm oil crops was the combustion to obtain caloric energy for oil extraction factories. However, this methodology has low energetic performance and leads to the emission of greenhouse gases. Hence, incorporation of agroindustrial lignocellulosic residues into an efficient elimination process that could generate high-value products is desirable (Saini *et al.*, 2015).

The oil palm (*Elaeis guineensis*) is the principal source of vegetable oil in the world, accounting for above 34.5% of the global oil production, followed by soybean and canola oils with 29.1% and 15.1%, respectively (FAO, 2016). The raw oil can be either refined for human consumption or converted into biodiesel by

transesterification of the fatty acids (Hosseini & Wahid, 2012; Lim & Teong, 2010). There are numerous negative impacts of the palm oil industry, including social and economic aspects, but for the purpose of this research, the accumulation of by-products from the increase of palm oil extraction is the primarily target. Empty Fruit Bunches (EFB), leaves and stems are the most abundant lignocellulosic solids obtained from the extraction of palm oil (Hassan *et al.*, 2011; Singh *et al.*, 2013). The main components from lignocellulosic by-products of oil palm are: cellulose (53%); hemicellulose (24%); and lignin (18%) (Shinoj *et al.*, 2011). From each hectare of planted oil palm, it is possible to obtain between 50-70 tons of lignocellulosic by-products. These by-products are a waste management problem, an example of these is Malaysia, where lignocellulosic biomass from oil palm have reached 85.5% of all biomass wastes generated in the country (Shafie *et al.*, 2012; Shuit *et al.*, 2009).

Malaysia and Indonesia are the principal producers of palm oil in the world, owning 47% and 45% of the production respectively, followed by Nigeria (1.7%); Thailand (1.7%) and Colombia (1.2%). In 2015, the world's annual palm oil production was 52 million tons, contrasting with the staggering amount of 274 million tons from oil palm fruits needed for this production (FAO, 2016). In Colombia, 753000 tons of palm oil were produced in 2010 and 1.1 million tons in 2014, a 30% increase in the palm oil production (Fedepalma, 2015). This increase of the oil palm sector in Colombia is due to government incentives (Castiblanco *et al.*, 2015). Overall, the ratio of palm oil production and lignocellulosic by-products is of 1:3 (Sulaiman *et al.*, 2011), and in the case of Colombia, it means that from 1.1 million tons of palm oil obtained in 2014, 3.3 million tons of wastes were generated after the oil extraction process.

Apart from burning, there are three main strategies for the handling of lignocellulosic by-products, which aim to reduce wastes accumulation and give them an added value. The first alternative is the degradation of these "wastes" into compost, that can then be used for soil enrichment. Composting, requires space, long time and the addition of macronutrients (Tuomela *et al.*, 2000). The second strategy is the anaerobic digestion for biogas production, a process that demands

weeks to obtain a significant amount of gas without demonstrated a positive cost-benefit ratio (Gomez-Tovar *et al.*, 2012). The third alternative is the production of ethanol by fermentation of syrups obtained from the hydrolysis of cellulose and hemicellulose (Dwivedi *et al.*, 2009). The production of lignocellulosic ethanol appears the optimal choice to handle by-products originated from agroindustry, since it brings at the same time a source of renewable energy (Orts & McMahan, 2016).

Second-generation bioethanol from agroindustry wastes is a well-established alternative to reduce the dependence on fossil fuels. However, the above mentioned strategies to handle lignocellulosic residues, are not economically viable to compete with the fossil fuel industry Thus, it would be highly desirable to integrate new strategies into the delignification step of bioethanol production to reduce production costs (Chen & Fu, 2016). Lignin removal is directly linked to the costs of second-generation ethanol production, and high expenditure of ligninolytic enzyme production makes the use of these biotechnological tools almost unprofitable (Moreno *et al.*, 2012; Oliva-Taravilla *et al.*, 2016). Nevertheless, physico-chemical methods to lignin removal have many drawbacks such as pollutants generation, that an efficient biotechnological approach could overcome.

The primary sources of ligninolytic enzymes are basidiomycete fungi. However, these fungi have the disadvantage of taking between 15 to 30 days to produce ligninolytic enzymes by fermentation, with a low titer and activity. Also, lignin biodegradation usually needs more than one kind of ligninolytic enzyme. Accordingly, enzymatic extracts from basidiomycete fungi with ligninolytic activity, mainly composed of laccases, have been used for agro-industry residue delignification to produce fermentable sugars and ethanol (Avanthi & Banerjee, 2016; Chintagunta *et al.*, 2017). Those reports confirm that for a successful enzymatic pretreatment of lignocellulose by recombinant ligninolytic enzymes, it is probably advisable to construct heterologous systems allowing the production of several ligninolytic enzymes that work synchronously (Davidi *et al.*, 2016).

Bioprospection of ligninolytic basidiomycete fungi has helped increase our knowledge of the delignification process in nature; data about structure, function and activity of ligninolytic enzymes has allowed overexpression of proteins with potential

industrial applications (Martínez *et al.*, 2017). However, incorporation of ligninolytic enzymes into an industrial process as yet to come. As mentioned earlier, expression of ligninolytic enzymes in a heterologous system is probably the best way to reduce the production cost and to adoption of these enzymes in industrial process. In addition, ligninolytic enzymes could be used for multiple industries such as food, textile, cosmetics, paper, among others, increasing the industrial demand for those kind enzymes, decreasing the costs production of the ligninolytic enzymes (Alcalde, 2015; Mate & Alcalde, 2015).

For the oil palm industry, the management and exploitation of lignocellulosic residues from oil palm agro-industry is a priority to reduce the negative impact of lignocellulosic residues accumulation, product of the rising oil palm production of the last years. However, but in order to achieve this goal, it is important to find economic and competitive biotechnological alternatives with low ecological impact for the treatment of lignocellulosic products derived from palm oil extraction. For the above reason, this project proposes to find enzymatic extracts or to identify ligninolytic enzymes from basidiomycete fungi by fungal bioprospection and its genomic exploration, in order to offer a future biotechnological tool to handling this enormous underused biomass for oil palm industries.

HYPOTHESIS

Native basidiomycete fungi offer a new source of valuable ligninolytic enzymes to develop biotechnological tools to improve the delignification pretreatment step of bioethanol production from oil palm residues.

OBJECTIVES

General objective

Bioprospection of Colombian native basidiomycete fungi with ligninolytic activity to identify enzymes capable of handling the pretreatment of palm oil tree lignocellulosic residues.

Specific objectives

1) To Select Colombian native basidiomycete fungi with ligninolytic activity.

2) To evaluate delignification capacity of enzymatic extracts from Colombian native basidiomycete fungi.

3) To characterize ligninolytic enzymes in extracts from Colombian native basidiomycete fungi.

4) To explore codifying genes for ligninolytic enzymes from Colombian native basidiomycete fungi.

2. Chapter 2: Isolation and selection of native Colombian basidiomycete fungi enzymatic extracts with ligninolytic activity

2.1. Introduction

Basidiomycete fungi stand at the base of the lignocellulose recycling process occurring in forests, playing a fundamental role in the carbon cycle (Mäkelä *et al.*, 2014; Riley *et al.*, 2014). The recovery of nutrients such as carbon and nitrogen from lignocellulose through microbial decomposition maintains the equilibrium between wood formation and decay. Wood colonization by fungi begins with the release of simple sugars from decaying vegetal cell wall. The sugars released are recognized by fungal spores which triggers the fungal hyphae formation. As the hyphae grows and develops branches, the fungus penetrates and colonizes internal cell walls of the tree thanks to the production and excretion of enzymes (Schwarze, 2007). During wood fungal colonization, secretion of enzymes with different activities, including proteases, esterases, ligninases, and cellulases, is mandatory. Ligninases and cellulases form the most important enzyme groups involved in wood decay since lignin oxidation and cellulose hydrolysis are essential steps of the process. Secretion of these enzymes occurs chronologically, ligninolytic enzymes being mainly expressed during the hyphae initial state whereas cellulolytic enzymes are more present during the hyphae late state. This enzyme production time course with lignin oxidation prior to cellulose hydrolysis guarantees a proper sugar release for fungi to use as their main energy source (Presley & Schilling, 2017; Zhang *et al.*, 2016).

These enzymes are not only valuable for fungi survival but constitute the basis of various biotechnological processes. Although previous studies have focused on understanding fungal wood colonization and on uses of some enzymes for specific applications, no industrial process makes a direct use of fungal delignification enzymes. Low production costs and proper adaptation of the proteins for the specific needs of the industry are the main obstacles. Hence, with bioprospection of fungus, especially basidiomycetes, it is conceivable to find uncharacterized species expressing a ligninolytic machinery that would be suitable for the pretreatment of lignocellulose for bioethanol production from oil palm agro-industrial residues (Sánchez, 2009).

This chapter describes the isolation of basidiomycetes fungi with wood colonization capacity, recovering of enzymatic extracts with ligninolytic activity by Solid-State Fermentation (SSF) using lignocellulose from oil palm residues, and the identification of ligninolytic activities in the enzymatic extracts. Bioprospection of basidiomycete wild fungi from places with high biodiversity rate, such as humid tropical forest, remains a suitable method to uncover and isolate new species that exhibited a ligninolytic enzymatic activity. Also, those new native species would be a new source for ligninolytic enzymes with industrial requirements such as high temperature and low pH conditions. In this study, species of white rot fungi were collected from Colombian forests to isolate fungal strains based on laccase, lignin peroxidase, and manganese peroxidase activities measured in enzymatic crude extracts from solid-state fermentations using empty bunch oil palm as lignocellulose source.

2.2. Materials and methods

2.2.1. Fungi isolation and growth conditions

Fungi collection was planned under the regulations of Colombia's Environmental Ministry. The research permit in biological biodiversity was obtained from the Corporación Autónoma de Santander (file number 153-12 REB) and with the agreement of the Ministerio del Interior, certifying the absence of ethnic groups in the area of sampling (application number 1648, August 14, 2012). Fungal collection was done in Yariguíes national park vicinities in Santander state, Colombia. The collect area was localized between coordinates 6°51´48" N and 73°23´00" O. Yariguíes national park is composed of a forest area (26%), an agricultural area (35%) with coffee and cocoa crops mostly, pastures (35%) and natural intact vegetation (38%). Temperature varies between 12°C and 20°C with an average humidity of 90% with frequent ground-level clouds. (Donegan & Huertas, 2005).

Fruiting bodies from basidiomycete fungi growing on decaying wood were collected in a tropical humid forest in Colombia, following previously published parameters to favor the presence of delignification enzymes (Blanchette, 1984;

Worrall *et al.*, 1997). Macroscopic characteristics from fruiting bodies were used as taxonomic keys to identify the genera of each fungus collected (Lodge *et al.*, 2004). The main inclusion criteria were macroscopic properties belonging to the orders of *Agaricales*, *Russulales*, and *Polyporales* due to the close relationship between these fungi and ligninolytic enzymes (Floudas *et al.*, 2015; Peláez *et al.*, 1995; Stalpers, 1978). Collected fungi were kept in wax paper bags to prevent deterioration. Isolation of the collected fungi was performed in Wheat Bran Extract Agar (WBEA) composed of 18 $g.L^{-1}$ agar, 10 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ peptone, 2 $g.L^{-1}$ yeast extract, 0.1 $g.L^{-1}$ KH_2PO_4, 0.1 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.085 $g.L^{-1}$ $MnSO_4$, 1000 mL wheat bran extract (175 g of wheat bran soaked for one hour in 1 L of distilled water and then, pressed and filtered using 5 gauze layers. The filtrate is considered the wheat bran extract), 0.1 $g.L^{-1}$ chloramphenicol and 600 $U.L^{-1}$ nystatin. High antibiotic concentrations are necessary since fruiting bodies are exposed over the culture media without previous sterilization process. Pilei were adhered to the top cover of Petri dishes, allowing spores to fall and, eventually, to germinate on the culture media. Top covers were rotated every 24 h for 3 days and top covers containing the pilei were replaced for new sterilized ones (Choi *et al.*, 1999). Sub-cultures in the same media were incubated at 25°C to obtain axenic strains from these isolates. The presence of microscopic sexual basidiomycete properties was checked, including septate hyaline hyphae and clamps (Figure 9).

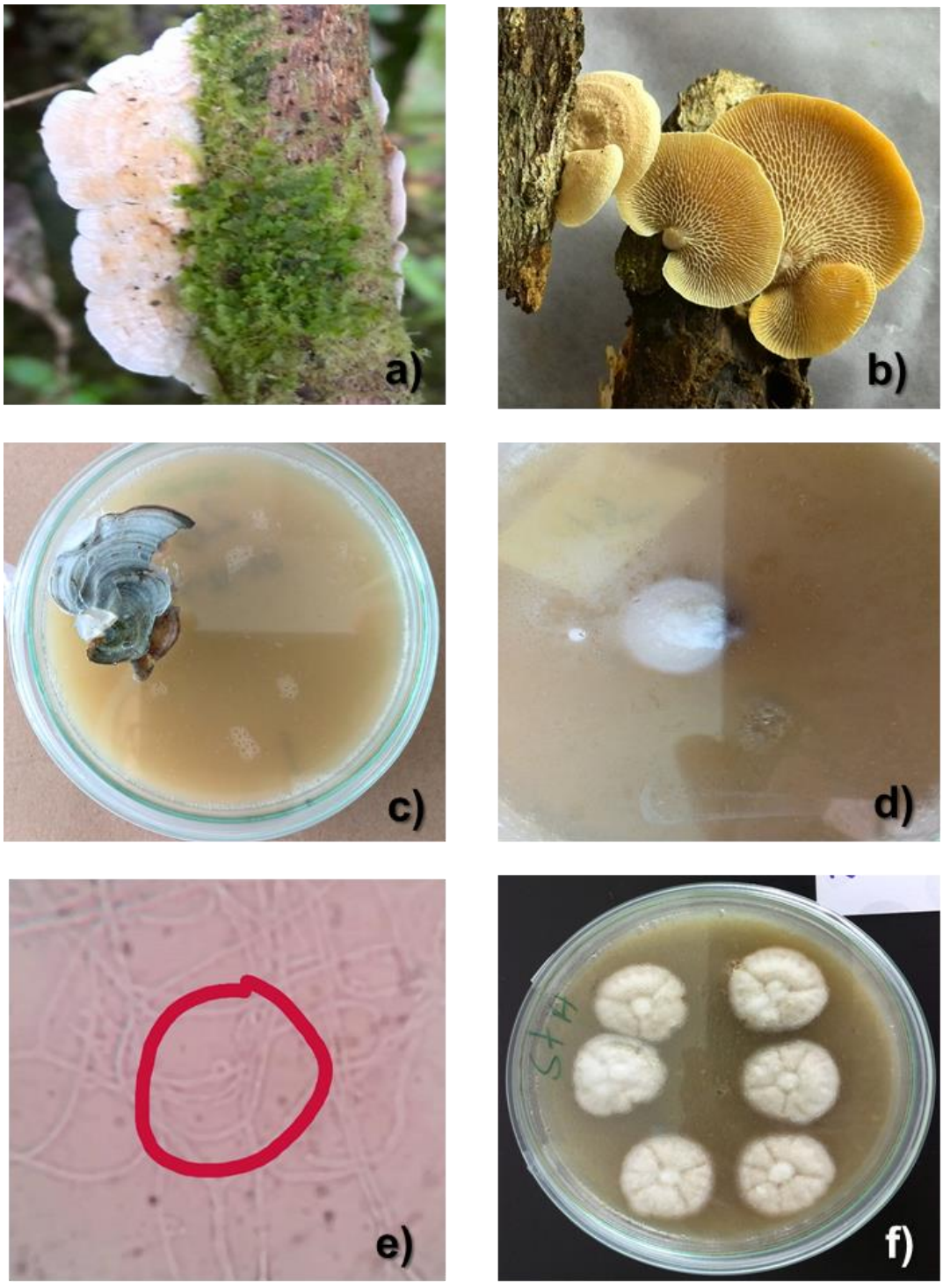


Figure 9: Isolation steps for basidyiomicete fungi isolations

a) Observation and collect of basidiomycete fungi over wood decay, b) Taxonomic identification of fruiting bodies collected, c) basidiomycete isolation by spores dropping, d) identification of colonies identified, e) microscopic identification of typical structures for basidiomycete fungi, clamp formation, red circle and f) axenic culture of basidiomycete species.

2.2.2. Conservation of fungi isolations

Fungi identified as basidiomycetes were conserved at 4°C over sterilized wood chips. To do so, wood chips of 1 cm x 1 cm were sterilized twice in different days to prevent future contamination during the storage and conservation, submerged in maltose extract broth (MEB) and sterilized one more time. 10 soaked chips with MEB were placed in Petri dishes that contained maltose extract agar (MEA) and 15 days old isolations, and incubation was prolonged at 30°C until total chip colonization was observed. The colonized wood chips were removed from the Petri dish and placed in a 50 mL tube that contained 5 mL of MEA at the bottom to keep humidity and kept closed at 4°C. Additionally, for each Petri dish, one colonized wood chip was placed in potato dextrose agar (PDA) to check growth and viability of the isolation (Nakasoke, 2004) (Figure 10). Biological Collection was called Cepario of de hongos, Laboratorio de micología, identified with the acronym Cepario UIS-F, in the Natural History Museum of Universidad Industrial de Santander, registered before at Registro Nacional de Colecciones Biologícas from Instituto Humboldt, Ministerio del medio ambiente, Colombia.

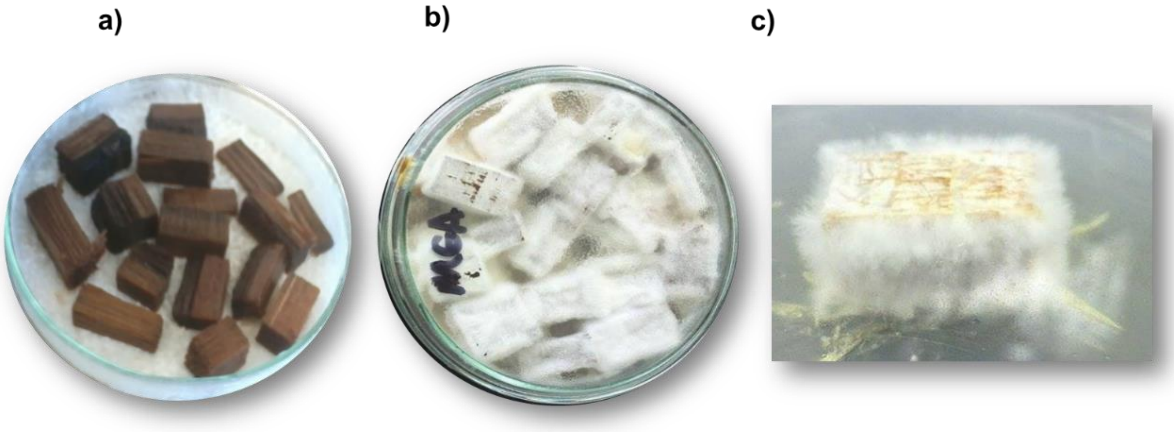


Figure 10. Conservation of fungi isolation on wood chips.
a) Sterilized wood chips over 15 days old fungi culture. c) Colonized wood chips by the fungi culture b) Fungi isolation recovery over a culture medium.

2.2.3. Fiber analysis of palm empty fruit bunches

I order to know the cellulose, hemicellulose and lignin percentages in the palm empty fruit bunches used as substrate in the SSF, Neutral Detergent Fiber (NDF),

Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL) were determined by the Van Soest method using the fibercap^{tm} system (Foss Analytical AB, Denmark) at Integrated laboratory of animal nutrition, biochemistry and pastures and fodder, Universidad de Antioquía, Medellín, Colombia. Cellulose and hemicellulose percentages were estimated as the difference between ADF and ADL percentages and NDF and ADF percentages, respectively. While lignin concentrations corresponded to ADL percentage in dry weight of oil palm by-products. Additionally, values were used to estimate the total carbon concentration in fermentations. Assays were performed in duplicate.

2.2.4. Isolation screening through solid-state fermentation (SSF)

The selection criteria of isolated wild-type fungi were quantification of ligninolytic activity by the enzymatic extracts produced through SSF. Lignocellulosic material from oil palm by-products was used as lignin source and fermentation support, taking the advantage of its low cost and suitability to favor laccase enzyme production from basidiomycete fungi (Rodríguez-Couto, 2018; Rodríguez Couto & Sanromán, 2005). SSF was performed in 250 mL flasks in sterile conditions. Each flask contained 12 mL of basal media in deionized water, comprising 0.2 $g.L^{-1}$ yeast extract, 0.76 $g.L^{-1}$ peptone, 0.3 $g.L^{-1}$ Urea, 0.25 $g.L^{-1}$ $CuSO_4 \cdot 5H_2O$, 1.4 $g.L^{-1}$ $(NH_4)_2SO_4$, 2 $g.L^{-1}$ KH_2PO_4, 0.3 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.4 $g.L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.005 $g.L^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.0016 $g.L^{-1}$ $MnSO_4$, 0.0037 $g.L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.0037 $g.L^{-1}$ $CoCl_2 \cdot 6H_2O$, and 2.5 $g.L^{-1}$ of empty fruit bunches (EFB) chopped into chunks of approximately 2 cm. Each flask was inoculated with eight agar plugs cut from actively growing fungal mycelium grown on WBEA for two weeks. Each SSF batch isolation contained thirty flasks and fermentation was held without agitation or light, in an incubator at 25˚C with an average humidity of 55% for 30 days. Every three days, three flasks were used to collect crude enzymatic extracts (Figure 11).

In order to increase the ligninolytic activity of SSF, a second screening was made with isolations that showed higher enzymatic activity. This second screening was performed with a 10-fold increase of C/N ratio, from 1,9 to 19, by addition of glucose at a concentration of 10 g/L. Also, copper concentration was increased from 1 mM

to 5 mM into the basal media. Because ligninolytic activity was substantially increased by these new carbon and copper conditions and since SSF requires long incubation time that would have handicapped the pursue of other aspects of this study, C/N ratio and copper concentration was not further optimized.

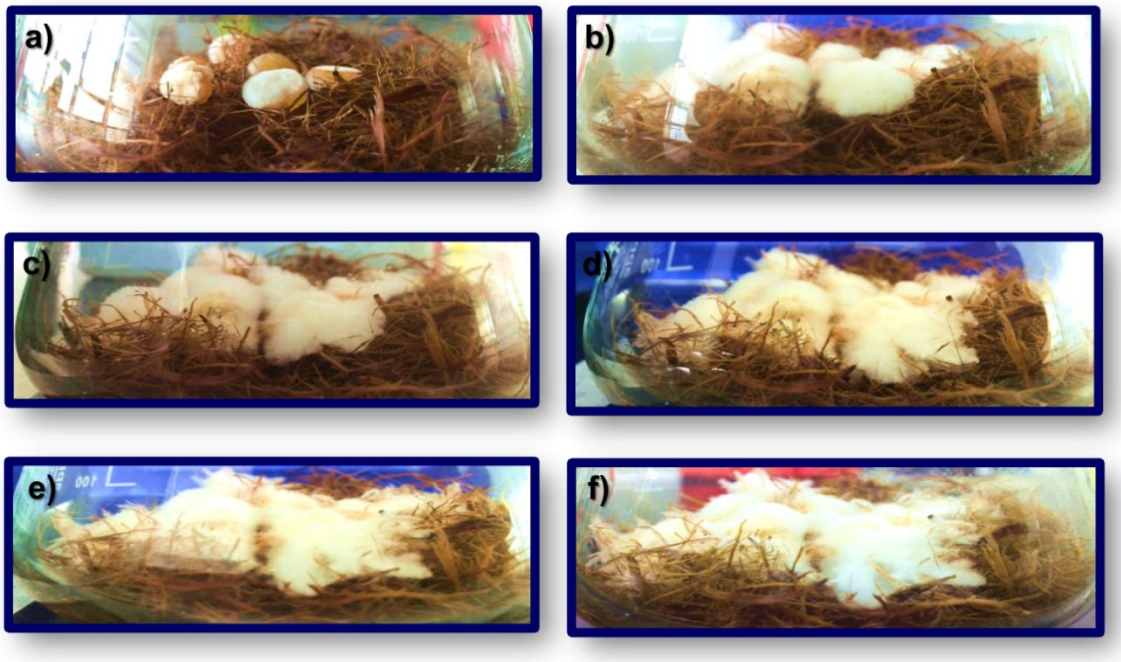


Figure 11. **SSF of *D. pusillus*-LMB4 isolation over EFB for 28 days.**
a) day 0: fungi agar plugs inoculation over sterilized EFB and basal media, b) day 4: fungi growing over agar plugs, c) day 7: beginning of EFB colonization by fungi, d) day 15: spread of fungi over EFB, e) day 20: fungi colonization of EFB surface, f) day 28. Pictures depict the same flask and flank.

2.2.5. Crude enzymatic extracts from SSF

Crude enzymatic extracts were obtained by addition of 30 mL of 60 mM sterile phosphate buffer into the fermentation flask that was shaken for 24h at 150 rpm. Whole flask contents were then collected in 50 mL tubes, vortexed for 15 minutes at 1500 rpm and centrifuged twice at 9000 rpm for 15 minutes to remove suspended solids (Lim *et al.*, 2013). Supernatants were taken as crude enzymatic extracts. Each supernatant was assayed for laccase, manganese peroxidase and lignin peroxidase activities.

2.2.5.1. Laccase activity

Laccase activity was measured by following the oxidation of ABTS to its oxidized state $ABTS^{+\bullet}$ (ε_{420} = 36.000 M^{-1} cm^{-1}) (Sigma-Aldrich, USA) (Johannes & Majcherczyk, 2000). Reactions were initiated by mixing 40 µl of crude enzymatic extract, 150 µl of 50 mM acetate buffer (pH 4.5) and 10 µl of 1.8 mM ABTS and were followed at 420 nm for one minute using a Thermo-fisher Multiskan™ GO Microplate Spectrophotometer. Enzyme activity was expressed in units per liter ($U.L^{-1}$). One unit of enzymatic activity was defined as the quantity of enzyme needed to transform 1 µmol of ABTS to $ABTS^{+\bullet}$ per minute.

2.2.5.2. Manganese peroxidase activity

Manganese peroxidase activity was measured by the formation of Mn^{+3} −malonate complex (ε_{270} = 11.590 M^{-1} cm^{-1}) at pH 4.5 in 50 mM sodium malonate buffer with 0.5 mM of MnSO4 (Järvinen *et al.*, 2012). Reactions were done by mixing 20 µL of culture supernatant, 100 µL of 20 mM citrate buffer pH 4.5, 40 µL of sodium malonate buffer and initiated with 40 µL of fresh 0.8 mM H_2O_2. Reactions were followed at 270 nm for one minute using a Thermo-fisher Multiskan™ GO Microplate Spectrophotometer. Enzyme activity was expressed in units per liter ($U.L^{-1}$). One unit of enzymatic activity was defined as the quantity of enzyme needed to oxide1 µmol of ion Mn^{+2} per minute.

2.2.5.3. Lignin peroxidase activity

Lignin peroxidase activity was measured by the transformation of 3,4-dimethoxybenzyl alcohol (VA) (Sigma-Aldrich, USA) to veratryl alcohol cation radical ($VA^{+\bullet}$), that has a yellow color (Khindaria *et al.*, 1995). Reactions were achieved by mixing 20 µL of culture supernatant, 100 µL of 20 mM citrate buffer pH 3, 40 µL of 10 mM VA and initiated with 40 µL of fresh 0.8 mM H_2O_2. Reactions were followed at 310 nm for one minute using a Thermo-fisher Multiskan™ GO Microplate Spectrophotometer. Enzyme activity was expressed in units per liter ($U.L^{-1}$). One unit of enzymatic activity was defined as the quantity of enzyme needed to transform 1 µmol of VA to $VA^{+\bullet}$ per minute.

2.2.6. Molecular identification of selected isolates

Total genomic DNA was extracted from selected isolates following a standard phenol-chloroform protocol (Płaza G. A., 2004). Briefly, fungi were grown in WBEA for 15 days and 0.5 g of mycelium was placed in a tube with a lysis solution (0.1 M NaCl2, Tris-HCl pH 8, 5% SDS) and 0.5 mm diameter glass beads, and shaken until mycelium breakdown was observed. The volume was centrifuged at 11,000 x g for 10 minutes, the supernatant was mixed in the same proportion with a phenol-chloroform-isoamyl alcohol solution (25:24:1) and centrifugated at 11.000 g for 5 minutes. The upper phase was extracted and mixed in the same proportion with a chloroform-isoamyl alcohol (24:1) and centrifugated at 14.000 g for 10 minutes. Finally, the aqueous fraction was collected, and the fungus DNA was precipitated with cold isopropanol. The DNA pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) (Plaza et al., 2004). A pair of primers within the Internal Transcribed Spacer regions ITS1-F (5´-CTTGGTCATTTAGAGGAAGTAA -3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) was used to amplify ribosomal DNA by Polymerase Chain Reaction (PCR) (Gardes & Bruns, 1993). iProof™ High-Fidelity DNA Polymerase kit (Bio-Rad, Canada) was used to amplify this DNA region with the following PCR conditions: 98°C for 5 min, followed by 35 cycles of 98°C for 1 min, 51°C for 1 min, and finally 3 min at 72°C (Izzo & Mazzola, 2009). PCR products were loaded onto 1% agarose gels, separated by electrophoresis, stained with GelRed® (Biotium, USA) and viewed with UV light. One Kb Plus DNA Ladder (Thermo-Fisher Scientific, Canada) was used to determine size of amplified products. PCR products were sequenced by the Sanger method using the same PCR primers at Génome Québec Innovation Centre (McGill University, Montreal, Canada). Sequences were aligned by Basic Local Alignment Search Tool (BLAST), using the nucleotide-BLAST, highly similar sequences (MEGA BLAST algorithm) at National Center for Biotechnology Information (NCBI) server, to confirm the identity of selected isolates.

2.3. Results and discussion

2.3.1. Fungi isolation

Twelve ligninolytic fungi belonging to genera *Aleurodiscus, Byssomerulius, Dictyopanus, Hyphodontia, Mycoacia, Phellinus, Pleurotus, Stereum, Trametes* and *Tyromyces* were axenically isolated of 43 collected fruiting bodies. Morphological characteristics from the twelve isolations are listed and showed in table 1 and figure 12, respectively. Among difficulties for axenic isolation of basidiomycete fungi collected in such natural environments, ecological and physiological factors could have hampered axenic recovery of all fruiting bodies (Hiscox *et al.*, 2018). However, in our case, biota mycoparasitism associated to basidiomycetes, mainly Trichoderma species, was the principal contaminant organism found in isolate culture media. Those fungi genera possess fungicide and antagonistic activity against basidiomycete cell walls, in addition to releasing enzymes such as chitinases and glucanases (Boddy & Hiscox, 2016; Colavolpe *et al.*, 2014; Schubert *et al.*, 2008). Moreover, basidiomycete recovery from collected samples can also suffer from competition with ascomycete fungi. Competition between these two fungi heavily relies on nutrient accessibility, a growth factor favoring ascomycetes due to their faster growing pace in complete culture media, or even in the presence of simple nutrient sources observed in advanced stages of wood decay (Boddy, 2000).

Table 1: **Macroscopic characteristics of isolated fruiting bodies.**

Isolation	Characteristics					
	Surface	**Color**	**Texture**	**Fruiting body margin**	**High cm**	**Width cm**
Hyphodontia sp.	Front	White	Pruinose	Irregular	--	--
	Back	N.D.	--			
Byssomerulius sp.	Front	Light brown	Jagged	Irregular	--	--
	Back	Light brown	Pruinose			
Mycoacia sp,	Front	Brown	Spined	Irregular	--	--
	Back	--	--			
Trametes sp..	Front	Green	Pruinose	Serrated	2.5	3.5

	Back	Light brown	Porioid			
Aleurodiscus sp.	Front	Light brown	Rugged	Eroded	--	1.7
	Back	Light brown	Spined			
Aleurodiscus sp.	Front	Light brown	Rugged	Eroded	--	1.7
	Back	Light brown	Spined			
Phellinus sp.	Front	Dark red	Porioid	Eroded	2.0	2.0
	Back	Dark red	Rugose			
Dictyopanus sp.	Front	Light brown	Anastomosed lamellas	Even	1.5	2.2
	Back	Brown	Smooth			
Pleurotus sp. 1	Front	White	Regular lamellas	Wavy	4	5.2
	Back	White	Smooth			
Stereum sp.	Front	Dark green / Light green / Light brown	Smooth	Even	3.0	5.5
	Back	Light green	Smooth			
Pleurotus sp. 2	Front	White	Regular lamellas	Wavy	4.2	5.0
	Back	White	Smooth			
Tyromyces sp.	Front	White	Porioid	Wavy	2.9	2.5
	Back	White	Rugose			

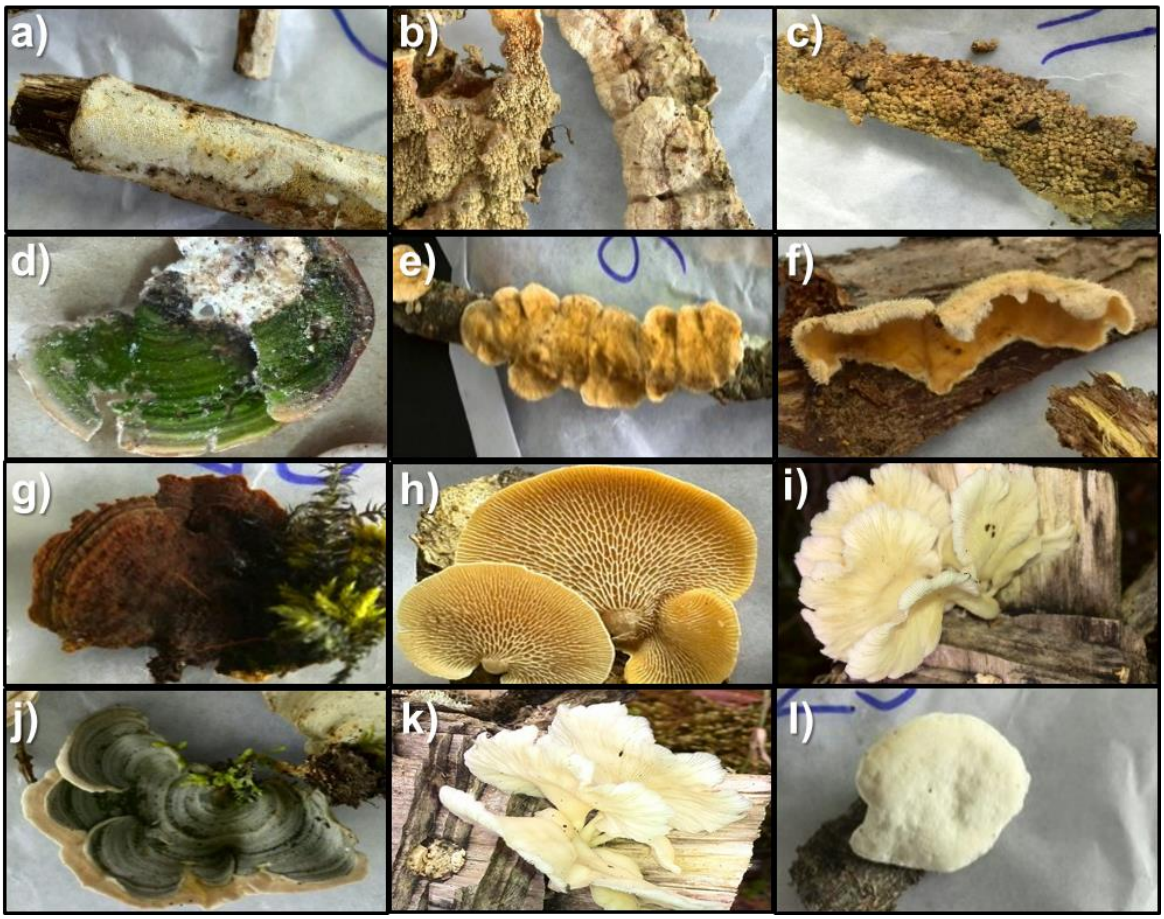


Figure 12. Basidiomycete fruit bodies collected

Genera of basidiomycete fungi selected a) *Hyphodontia sp.*, b) *Byssomerulius sp.*, c) *Mycoacia sp.*, d) *Trametes sp.,* e) *Aleurodiscus sp.,* f) *Aleurodiscus sp.,* g) *Phellinus sp.,* h) *Dictyopanus sp.,* i) *Pleurotus sp.* 1, j) *Stereum* sp., k) *Pleurotus sp.* 2, l) *Tyromyces sp*.

Based on macroscopic properties, twelve fungi isolates were identified. Isolated genera all belong to the *Hymenochaetales*, *Polyporales*, and *Agaricales* orders: *Aleurodiscus sps.* (2 isolates), *Byssomerulius sp.* (1 solate), *Dictyopanus sp*. (1 isolate), *Hyphodontia sp.* (1 isolate), *Mycoacia sp.* (1 isolates), *Phellinus sp.* (1 isolate), *Pleurotus sps.* (2 isolates), *Stereum sp.* (1 isolate), *Trametes sp.* (1 isolate), and *Tyromyces sp.* (1 isolate). The last order members are part of the Agaricomycetes class, which has been evolutionarily recognized in the production of oxidoreductases and hydrolases involved in wood decay (Eastwood, 2014; Floudas *et al.*, 2015; Kim *et al.*, 2016) (Figure 13).

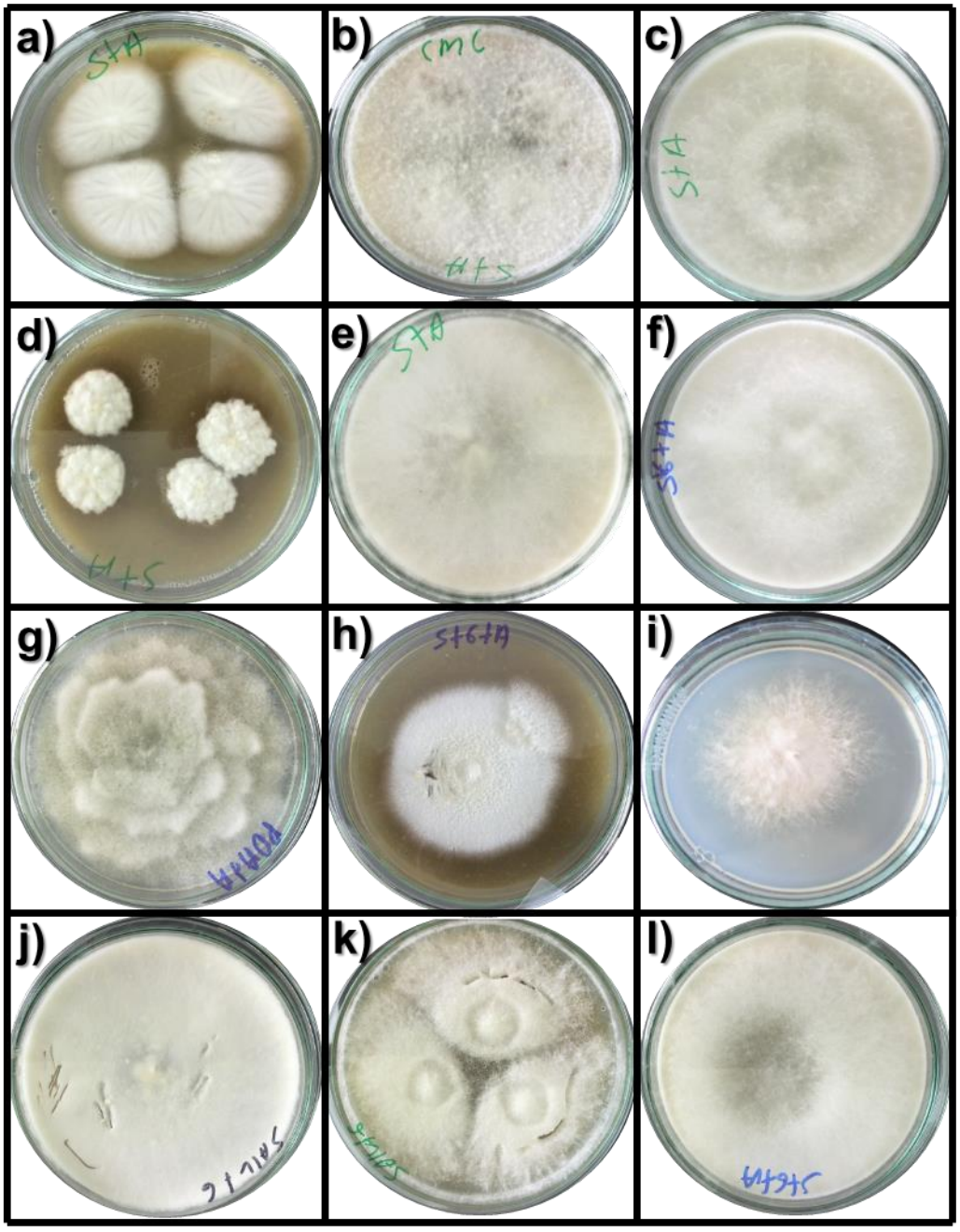


Figure 13. Fungal colonies of basidiomycete fungi isolated.

Axenic cultures in WBEA after two weeks of growth. a) *Hyphodontia sp.*, b) *Byssomerulius sp.*, c) *Mycoacia sp.*, d) *Trametes sp.*, e) *Aleurodiscus sp.* 1, f) *Aleurodiscus sp.* 2, g) *Phellinus sp.*, h) *Dictyopanus sp.*, i) *Pleurotus sp.* 1, j) *Stereum sp.*, k) *Pleurotus* sp. 2, l) *Tyromyces sp*.

2.3.2. Fiber analysis of palm empty fruit bunches

Fiber analysis of palm empty fruit bunches revealed 77.53% NDF, 58.32% ADF and 17.15% ADL. These values indicate that the EFB composition in lignocellulosic

polymer used for the SSF was 40.79% cellulose, 19.21% hemicellulose, 17.15% lignin, and 22.47% impregnated oil and ashes, those percentages were calculated following the indications at section 2.3.3. These results are in accordance with typical reported EFB composition, with cellulose being the main component, followed by hemicellulose and lignin (Chang, 2014).

2.3.3. Isolation screening through solid-state fermentation (SSF)

Enzymatic extracts were screened for laccase, manganese peroxidase and lignin peroxidase activity, enzymes known to participate in the delignification process. From the crude enzymatic extracts obtained by SSF, only five isolates showed laccase activity by ABTS oxidation in our screening assay. Surprisingly, we were unable to detect any peroxidase activity by Mn^{+3} or $VA^{+\bullet}$ formation for manganese and lignin peroxidases, respectively. We believe that peroxidase activity from the SSF supernatant enzymatic extracts was too low to be quantified by the oxidation of VA under the conditions described in section 2.3.5.3. A previous secretome profile analysis of WR from Agaricales order, species *Pleurotus ostreatus*, *Trichaptum abietinum* and *Phlebia radiata*, has shown that laccase was the prevalent ligninolytic activity during fermentation assays using lignocellulose as substrate or synthetic culture media (Fernández-Fueyo *et al.*, 2016; Mali *et al.*, 2017). Those results support our observation that peroxidases activity was undetectable in our SSF extracts. The cause of this laccase activity predominance over peroxidase activity for WR is not clear yet, but carbon or nitrogen source, lignocellulose substrate and some organic compounds can regulate the organism physiology and could act as inducers to benefit a major transcription and secretion of laccases (Elisashvili & Kachlishvili, 2009; Janusz *et al.*, 2013).

Regardless, the use of lignocellulosic by-products from oil palm as substrate to screen fungi isolates allowed the direct evaluation of laccase enzyme production, which could potentially serve as pretreatment of oil palm by-products. These isolates were identified as *Dictyopanus*-LMB4 (22.3 $U.L^{-1}$), *Pleurotus*-LMB2 (69.5 $U.L^{-1}$), and *Pleurotus*-LMB3 (57.2 $U.L^{-1}$). Laccase activity of the *Hyphodontia*-LMB8 and

Trametes-LMB15 isolates was considered too low to warrant further characterization (Table 2).

Table 2**: Laccase activity for native basidiomycete isolations from SSF over EFB**

Isolation	Laccase activity ($U.L^{-1}$)	SSF days
Pleurotus-LMB2	69.53 ± 4.4	20
Pleurotus-LMB3	57.17 ± 7.9	20
Dictyopanus-LMB4	23.31 ± 2.0	20
Byssomerulius -LMB8	4.02 ± 0.5	12
Trametes-LMB15	2.03 ± 1.0	20

For the three most active isolates, the highest laccase activity was detected after 20 days of fermentation (Figure 14). Using these three isolates, laccase activity conditions were optimized by increasing the copper concentration and carbon-to-nitrogen ratio (C/N) (Baldrian & Gabriel, 2002; Li *et al.*, 2011). As a result, the isolate exhibiting the highest laccase activity under these newly optimized conditions was *Dictyopanus*-LMB4 (267.6 $U.L^{-1}$ after 28 days of fermentation). To the best of our knowledge, this represents the first observation of a crude *Dictyopanus sp.* enzymatic extract exhibiting significant laccase activity. Furthermore, this activity is similar to a previously reported *Trametes sp*. Laccase activity evaluated under comparable fermentation conditions using lignocellulosic by-products from oil palm (218.6 $U.L^{-1}$) (Singh *et al.*, 2013). The maximal laccase activities of the *Pleurotus* isolates were significantly lower than the one observed for *Dictyopanus*-LMB4, with 98 $U.L^{-1}$ for *Pleurotus*-LMB2, and 66.9 $U.L^{-1}$ for *Pleurotus*-LMB3 (Figure 15).

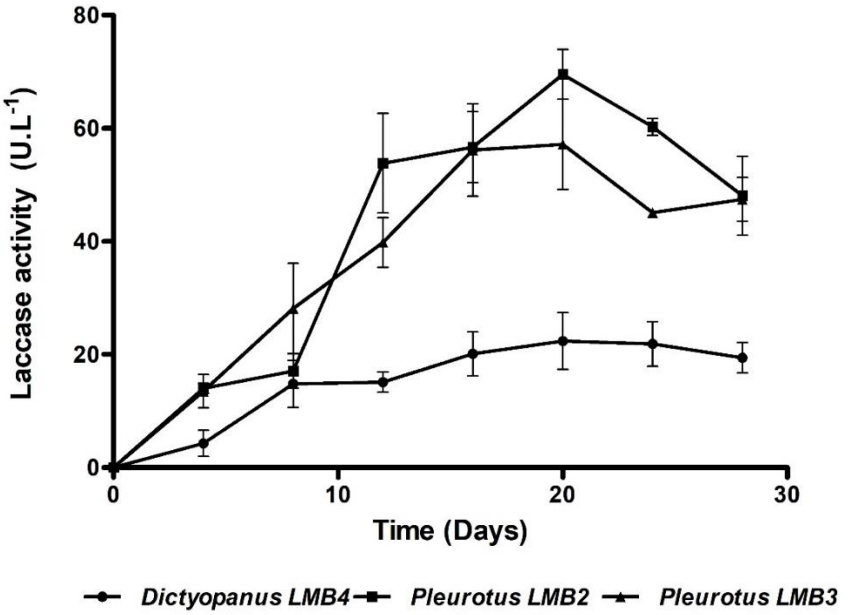


Figure 14. Laccase activity of SSF isolates.

Enzyme activity was measured from culture supernatants with a 1.9 C/N ratio without copper. Laccase activity was only detected in five isolates, with the highest activities observed for *Pleurotus* and *Dictyopanus* genera. Results represents mean ± SD of 3 replicates.

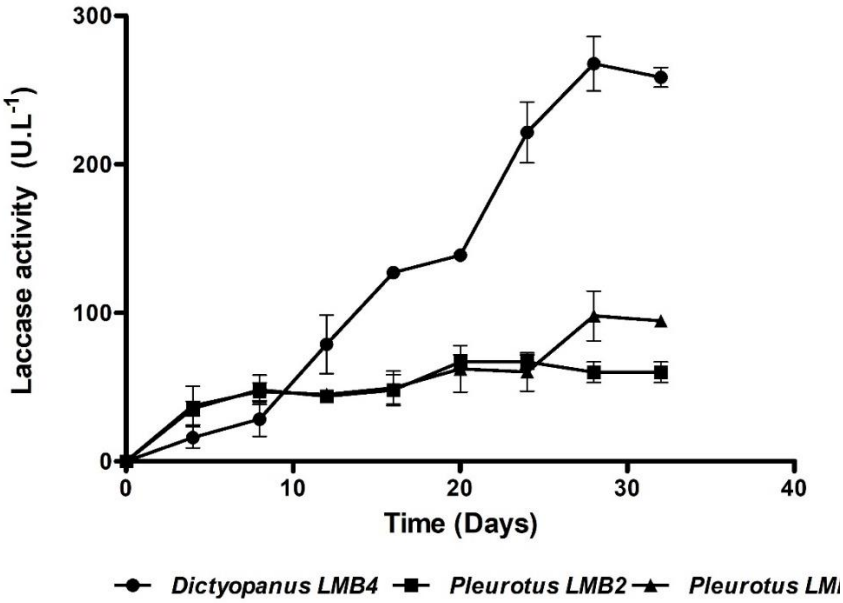


Figure 15. Laccase activity of selected isolates under optimized assay conditions.

Enzyme activity was measured from supernatants obtained through SSF with a 19 C/N ratio and 5 mM copper. Isolates from *Dictyopanus* and *Pleurotus* genera were evaluated. *Dictyopanus* LMB4 demonstrated the highest laccase activity (267 ± 18 $U.L^{-1}$), a 12-fold increase from the first screening. Results are depicted as mean ± SD of 3 replicates.

Upon optimization of the growth conditions, the crude enzymatic activity of *Dictyopanus*-LMB4 increased 6- and 12-fold after 20- and 28-day incubations, respectively, highlighting the importance of copper increase and changes in the C/N ratio by the addition of glucose for proper enzyme expression. Similar observations were reported on other laccase extracts. Indeed, a 4-fold increase in laccase activity was observed for a *Ganoderma lucidum* isolate when glucose was added to the fermentation medium (Hailei *et al.*, 2015). The authors concluded that accessibility to a ready bioavailable carbon source such as glucose improved mycelial growth, which is proportional to the amount of laccase secreted by the fungi. Similarly, laccase activity was improved when copper was added to submerged fermentations of *Colorios versicolor*, due to the fact that copper ions are strong stimulators of laccase expression (Kajita *et al.*, 2004). The breakdown of lignocellulosic substrates from EFB by white rot fungi (Kamcharoen *et al.*, 2014; Piñeros-Castro & Velásquez-Lozano, 2014), and EFB pulp (Martín-Sampedro *et al.*, 2012) has been previously reported, but not using enzymatic extracts. Finally, our laccase activity per gram of substrate for *Dictyopanus*-LMB4 using oil palm by-products is 31.5 U.g-1 after 12 days of SSF, which is higher than the reported 7.5 U.g-1 obtained from *Pycnoporus sanguineus* in similar SSF conditions (Vikineswary *et al.*, 2006).

2.4.4. Molecular identification of selected isolates

Using extracted DNA from all three isolates showing laccase activity and the ITS1/ITS4 primers, we obtained PCR products yielding 0.7 kb fragments, correlating with previous fungi identification reports (Gardes & Bruns, 1993). Sequencing of the respective rDNA confirmed the identity of the three isolates displaying laccase activity through taxonomic identification. The *Dictyopanus*-LMB4 isolate showed around 98 % identity with five different *Dictyopanus pusillus* isolations reported at NCBI (Table 3), while the two other isolates, identified as *Pleurotus*-LMB2 and *Pleurotus*-LMB3, respectively, shared 94 and 93 % identity with the *Pleurotus* genus. These results support the usefulness of taxonomic classification during fungi sample collection and isolation selection. The *Dictyopanus* genus belongs to the *Agaricomycetes* class, and its genus is known to include species capable of

bioluminescence, which have been suggested to be linked to delignification processes through the use of secondary compounds produced during lignin degradation (Bechara, 2015). *Dictyopanus* isolates were also reported as an alternative for the pretreatment of remazol brilliant blue R (Machado *et al.*, 2005) and bamboo in ethanol production (Suhara *et al.*, 2012), further supporting the potential use of this fungus in large-scale biomass valorization.

Table 3: Homologues species with the native *D. pusillus*-LMB4 isolation

Homologues species	Query cover %	Percent of identity	Accession number
Dictyopanus sp. P-238	85	98.81	KR135361.1
Dictyopanus pusillus #8265	89	96.77	AF289061.1
Dictyopanus pusillus voucher FLAS-F-60991	87	96.69	MH016934.1
Dictyopanus pusillus voucher FLAS-F-61475	87	96.69	MH211914.1
Dictyopanus pusillus voucher FLAS-F-61126	84	97.06	MH211761.1

2.4. Conclusions

Dictyopanus-LMB4 was the native isolate selected for its high laccase activity (267 ± 18 U/mL) in the enzymatic extract obtained by SSF using EFB as substrate. To this date, this is the first report of laccase activity in *Dictyopanus* genera.

The use of low cost lignocellulosic raw materials such as EFB to obtain enzymatic extracts with laccase activity by SSF was possible. Moreover, an increase of copper and glucose concentration during SSF boosted *Dictyopanus*-LMB4 enzymatic extract laccase activity (12-fold). This rise in laccase activity by changes in SSF conditions supports the fact that those enzymes are induced in WR.

However, it would be important to determine the ligninolytic effect of the enzymatic extract from *D. pusillus*-LMB4 over lignocellulosic substrates since it could be a potential enzyme source for the pretreatment of EFB for second-generation bioethanol production.

Finally, further studies focusing on inductors for laccases and/or peroxidases could improve even more the expression of those enzymes by this native *D. pusillus*-LMB4 isolate. Also, other substrates with a lower redox potential could be tested to prove the existence of other enzymes with ligninolytic activity such as peroxidases.

3. Chapter 3: Evaluation of the delignification capacity through laccase-like activity of the enzymatic extract from *Dictyopanus pusillus*-LMB4 on oil palm by-products

3.1. Introduction

Even though cellulosic ethanol production looks like an easy alternative to reduce our dependence on fossil fuels and to reduce accumulation of agroindustry biomass, its development has faced many challenges and at the moment bioethanol production does not supply the ethanol demand. As it was described, pretreatment is the first step in the second-generation bioethanol production that exposes cellulose through lignin depolymerization. Cellulose is then hydrolyzed by cellulases, releasing fermentable sugars. An effective pretreatment ensures a good cellulose hydrolysis and thus, a successful bioethanol production. The actual cellulosic ethanol production still depends on high temperatures or pollutant solutions such as acids or bases for the pretreatment step. For instance, Abengoa, Beta Renewables, Dupont, Granbio, POET/DSM and Raizen are the main companies producing lignocellulosic ethanol, but none of those companies have implemented a lignocellulose biotechnological pretreatment (Lynd *et al.*, 2017). In fact, development of new strategies for a biotechnological pretreatment of lignocellulose with enzymes to support biofuel production in a completely green chemistry process is a crucial step for the commercialization and cost reduction of cellulosic ethanol.

The accumulated experience in the fermentation process for ethanol production has allowed the development of bioethanol industries. However, pretreatment techniques still involve chemicals and energy consuming processes, so a completely environmental friendly and efficient process for lignocellulose pretreatment is still to come to reinforce worldwide bioethanol production (Sun *et al.*, 2016).

Previous studies have described the use of basidiomycete fungi to depolymerize lignin for bioethanol production, but the procedure with ligninolytic enzymatic extracts or enzymes has not been evaluated yet. Hence, this chapter describes the use of the enzymatic extract with laccase activity obtained from *D. pusillus*-LMB4 by

SSF in the delignification process of lignocellulose residues from oil palm trees by simultaneous pretreatment and saccharification.

3.2. Materials and methods

3.2.1. Reducing sugars quantification

The increase of reducing sugars is an indirect evaluation of the delignification process that can be used to measure the effectiveness of lignocellulose pre-treatment (Saritha *et al.*, 2012; Tiwari *et al.*, 2013). Reducing sugars were quantified by the oxidation of 3,5-dinitrosalicylic acid to 3-amino,5-nitrosalicylic acid (DNS) by the reducing extremity of sugars released. The reaction was followed at 420 nm; a standard curve obtained with glucose was used to quantify the concentration of reducing sugars (Miller, 1959).

3.2.2. Total cellulolytic activity

The total cellulolytic activity consists in measuring the three enzymatic activities: endoglucanases, exoglucanases, and β-D-glucosidases, using cellulose as substrate to release reducing sugars as product. using cellulose as substrate to release reducing sugars as product. Total cellulolytic activity was quantified for the commercial cellulase solutions from *Trichoderma reesei* (Sigma-Aldrich C2730 Celluclast®, USA) by Filter Paper Units (FPU.mL-1) used in SPS by Filter Paper Units ($FPU.mL^{-1}$) in order to adjust enzymatic activity to the specific needs of the experiment. In tubes, 500 µl of commercial cellulase solutions from *T. reesei* were incubated with 1 mL of 50 mM citrate buffer at pH 4.8, and 50 mg of filter paper for 1 h, at 50 °C. The concentration of reducing sugars released was measured by the oxidation of DNS, as described above (Ghose, 1987).

3.2.2. pH profile of enzymatic extract with laccase activity from native isolation *Dictyopanus pusillus* -LMB4

In order to know the pH profile and to determine the best pH value for EE-LMB4, laccase activity was quantified at pH values from 2 to 8 (50 mM hydrochloric acid buffer for pH 2; 50 mM citric buffer for pH 3-4; 50 mM acetate buffer for pH 4.5-5,

and 50 mM phosphate buffer for pH 6-8). Laccase activity was measured using the different buffers listed and following the methodology described in section 2.2.5.1. Results represent data of experiments done in triplicate.

3.2.4. Effect of pH and temperature on laccase activity and stability

The effect of pH and temperature over time were evaluated for EE-LMB4. In order to determine the effect of pH, 1 mL of EE-LMB4 and 9 mL of selected buffers with different pH values were placed in 15 ml glass tubes. Those tubes were incubated in a water bath for 8 hours at 40°C and every hour, 100 µL were taken to measure laccase activity. pH values were based on results obtained from section 3.2.3. To evaluate the effect of temperature, 10 mL of EE-LMB4 or *Trametes versicolor* laccase (53739, Sigma-Aldrich, Canada) were placed in 15 mL glass tubes and incubated in a water bath for 7 hours at 40°C, 50°C and 60°C. Every hour, 100 µL were taken to measure laccase activity. Experiments to evaluate the effect of pH and temperature were done in triplicate, and all components (except enzymes and laccase activity test reagents) were sterilized separately at 121°C for 15 minutes and mixed under environmentally sterile conditions. Laccase activity was measure as described in section 2.2.5.1.

3.2.5. Simultaneous pretreatment and saccharification of EFB

The SPS will serve as an indirect measurement of enzymatic pretreatment over EFB. Commercial laccases or EE-LMB4 were mixed with a cellulolytic commercial cocktail in other to know the boost effect of ligninolytic enzymes over the enzymatic sacharification of EFB. SPS was performed in 50 mL tubes containing 1.5 g EFB, 16 mL of 50 mM acetate buffer at pH 4.5 with either EE-LMB4 or the commercial laccase from *T. versicolor* (53739, Sigma-Aldrich, Canada) in the presence of cellulases from *T. reesei* (Sigma Aldrich C2730 Celluclast®). SPS conditions, pH, EFB quantity and temperature, were evaluated during a previous study (Master's thesis: "Hidrólisis enzimática de subproductos lignocelulósicos generados durante el beneficio de la palma aceitera para la obtención de jarabes fermentables" performed at mycology lab at Universidad Industrial de Santander, Colombia) and used to characterize the

enzymatic extract. For the reaction mixture, both laccase and cellulase sources were added in a volume of 2 mL to reach a final concentration of 25 $U.L^{-1}$ and 50 $FPU.mL^{-1}$, respectively. Tubes were incubated at 40°C for 72 hours. The saccharification process was evaluated by the production of reducing sugars, measured by a DNS assay (section 3.2.1). Results were reported as the quantity of produced reducing sugar per gram of EFB used as substrate (g.g-1). SPS assays were performed in triplicate and all components (except enzymes) were sterilized separately at 121°C for 15 minutes and mixed under environmentally sterile conditions.

SPS of EFB was conducted in accordance with an Analysis of variance (ANOVA) to evaluate significant variables in the process. Five independent variables were evaluated: pH (3 to 5) using either 50 mM acetate buffer (pH 3 and 4) or 50 mM citrate buffer (pH 5), temperature (25, 35 and 45 °C), copper concentration (1, 3 and 5 mM), laccase (100, 200 and 300 $U.L^{-1}$) and cellulase (50, 100, 150 $FPU.mL^{-1}$) activities. Results represent four independent experiments and were analyzed by Multiple-Variable Analysis (Correlations) using the Statgraphics Centurion XVII software.

3.3. Results and discussions

3.2.1. pH profile of enzymatic extract with laccase activity from native *Dictyopanus pusillus*-LMB4 isolation

pH profile characterization of EE-LMB4 showed that pH values between 3 and 5 provided the highest laccase activity, with a maximum activity at pH 3 (Figure 16). This pH range corresponds to other typical optimal pH values of fungal laccases (Baldrian, 2006). Even though the optimal pH found for EE-LMB4 corresponds to what is expected for fungal laccases, it would be desirable to have a laccase activity close to neutral pH values, since acidic conditions tend to favor faster laccase enzyme denaturation and potential industrial applications might require higher pH (Agrawal *et al.*, 2018).

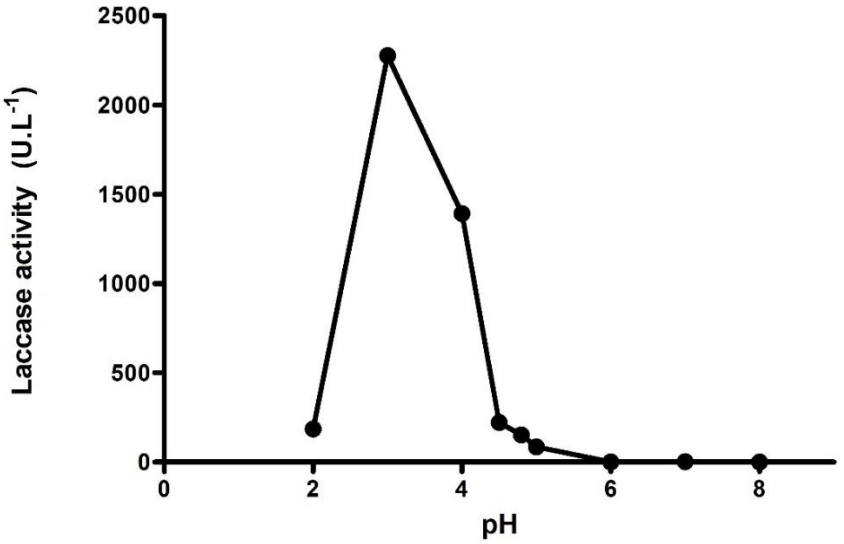


Figure 16. Laccase activity of *D. pusillus-LMB4* crude enzymatic extract at different pH. Laccase pH profile of EE-LMB4 was evaluated at room temperature using ABTS as substrate. A pH range from 2 to 8 was used as was described at section 3.2.3.

EE-LMB4 at pH values of 4 and 5 show a similar stability tendency, staying stable during the first 3 hours and losing 25% ± 0.36 and 7% ± 0.47 of its activity, respectively, after 4 hours of incubation. Moreover, at these pH values, the maximum decrease of laccase activity was 40% ± 0.43 and 38% ± 0.26 after 8 incubation hours. However, the stability of EE-LMB4 at pH 3 was lower, losing 30% ±1.12 and 75% ± 0.21 of laccase activity after the first and second hour of incubation, respectively and finally losing most of its activity at the fifth hour of incubation (Figure 17). Otherwise, thermal stability of EE-LMB4 was found to be quite significant, with reduced activity only observed at 60°C (46% ± 11.92 of activity loss after 6 hours of incubation). This behavior is quite different from that observed with the *T. versicolor* commercial laccase under the same experimental conditions, showing 28% ± 3.6 and 87% ± 1.66 of activity loss after a 6h incubation at 50°C and 60°C, respectively (Figure 18). Thus, *D. pusillus*-LMB4 appears to encode for laccase(s) with enhanced thermostability and higher tolerance to pH values around four. However, long incubation of EE-LMB4 at low pH resulted in an important activity loss.

Previous studies have shown that a laccase from *Physisporinus rivulosus* remained stable at 50°C with optimal activity at pH 3.5 (Hildén *et al.*, 2013). Similarly, a laccase from *Trametes trogii* was shown to sustain temperatures up to 75°C,

although only for short 5-min incubations (Yan *et al.*, 2015). Our results suggest that the laccase activity from *D. pusillus*-LMB4 extract has higher tolerance to acidic and thermally induced perturbations than previously identified fungal laccases. However, it would be important to identify the enzymes responsible for this activity in EE-LMB 4 to confirm thermal stability and acidity tolerance of those proteins.

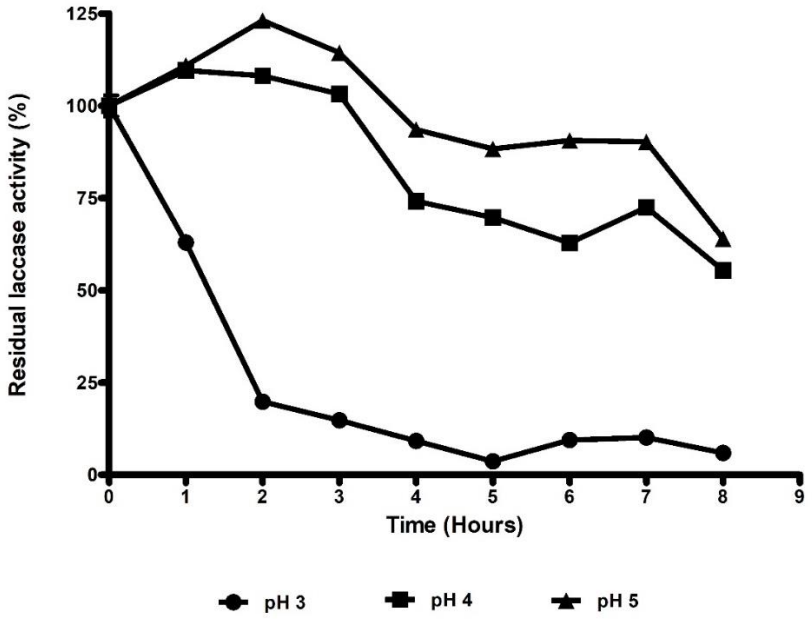


Figure 17. pH stability of crude enzymatic extract from *D. pusillus*.
pH stability assay laccase activity was evaluated at 40°C under different pH conditions (circles pH 3, squares pH 4 and triangles pH 5).

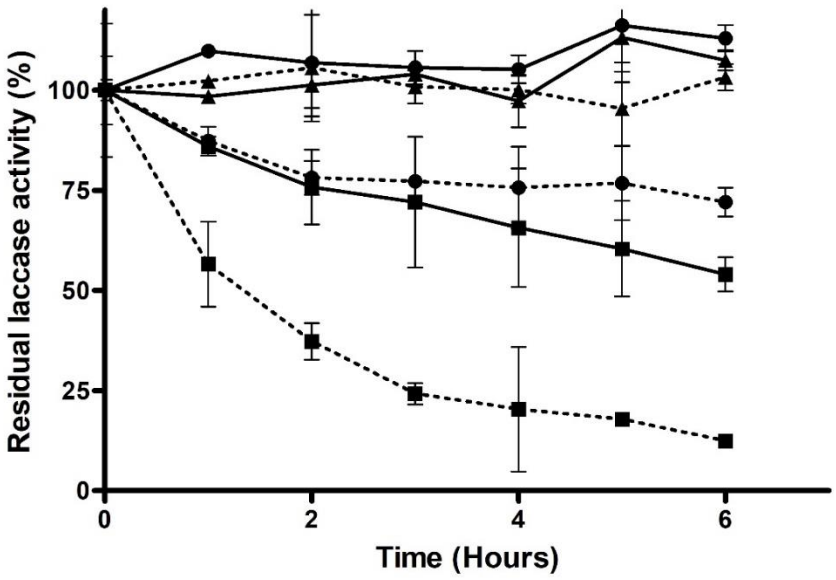


Figure 18. Thermal stability of crude enzymatic extract from *D. pusillus*.
Laccase enzymatic activity was evaluated after different temperatures 40°C (triangles), 50°C (circles), and 60°C (squares). Solid lines represent the crude EE-LMB4 while dashed lines represent the commercial laccase from *T. versicolor*, 53739.

3.3.3. Simultaneous pretreatment and saccharification of EFB

Reducing sugar release was observed when the cellulolytic enzymatic extract from *T. reesei* was used alone (20.84 ± 0.7 $g.g^{-1}$). Higher reducing sugar release from EFB was observed when the cellulolytic enzymatic extract from *T. reesei* was used with the commercial laccase enzyme from *T. versicolor* (46.47 ± 5.9 $g.g^{-1}$) or the EE-LMB4 (44.80 ± 5.21 $g.g^{-1}$), confirming that ligninolytic enzymes such as laccases favor cellulose hydrolysis, as previously shown (Davidi *et al.*, 2016; Mukhopadhyay *et al.*, 2011). Moreover, it suggests that a mix of cellulolytic and ligninolytic enzymes enhances the release of reducing sugars, offering advantages as time reduction in the pretreatment and saccharification as well as elimination of hydrolytic process inhibitors (Dhiman *et al.*, 2015). Enzymatic SPS makes the process easier, reducing energy consumption, due to the unification of two steps (pretreatment and hydrolysis) during bioethanol production, while improving bioethanol production trough elimination of fermentation inhibitors. However, production of reducing sugars was not significantly different when the commercial

laccase from *T. versicolor* or EE-LMB4 were mixed with the cellulolytic enzymatic extract from *T. reesei* (Figure 19).

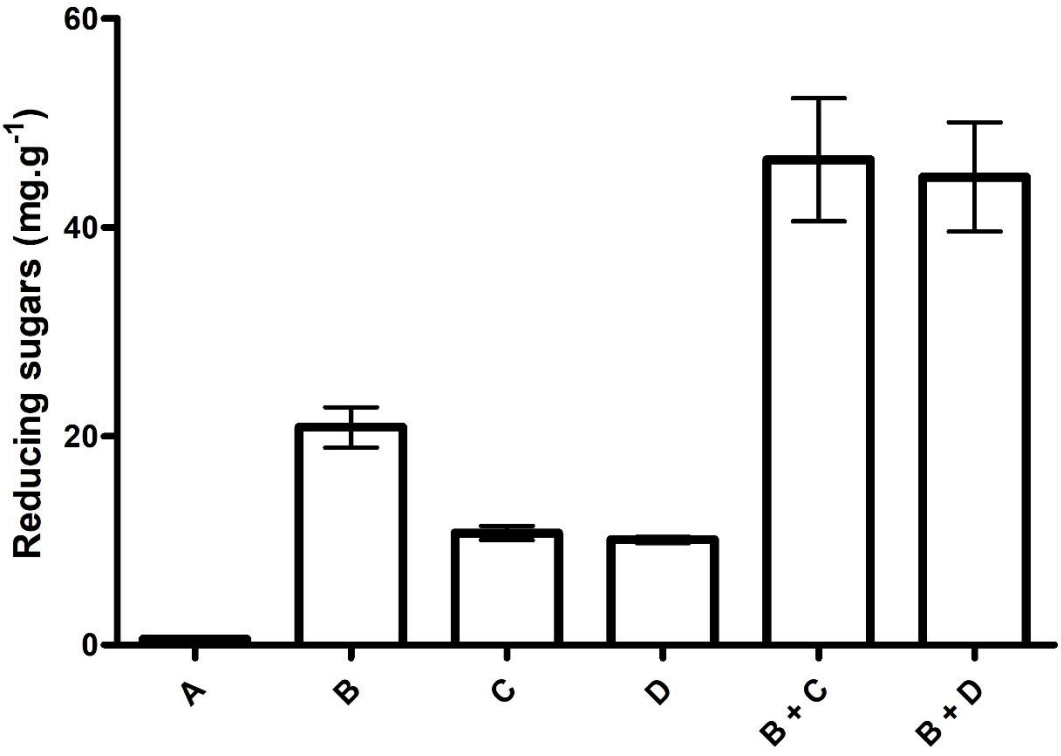


Figure 19. Comparative production of reducing sugars using ligninolytic and cellulolytic enzymes.

Comparative production of reducing sugars from EFB through SPS performed at 40°C during 72h as described in section 3.2.5. A set of experiments were performed in absence of enzymes to assess basal quantity of reducing sugars (A) and the contribution of each enzyme was evaluated by performing the assay in the presence of the cellulolytic extract from *T. reesei* alone (B), with a mixture of the cellulolytic extract and a commercial laccase from *T. versicolor* (C) or the crude EE-LMB4 (D). Reducing sugars quantification was evaluated by oxidation of DNS using glucose as normalization standard and results are expressed as mg of reducing sugar per gram of EFB.

To identify the dominant variables affecting the reducing sugar release, we compared the effect of pH, temperature, copper concentration, and the concentration of laccase ($U.L^{-1}$) and cellulase (FPU) by an ANOVA analysis (P value < 0.05 with a confidence level of 95%). Results were depicted with Pareto charts for SPS carried with laccase from *T. versicolor* (Figure 20a) or with the EE-LMB4 (Figure 20b). In both cases, pH is the dominant variable, followed in order by temperature

and copper concentration. In fact, high pH values and temperatures up to 45˚C seem to promote reducing sugar release by SPS. These results are in agreement with the previous stability experiments, where it was observed that the activity of the enzymatic extract was compromised at pH lower than 4. And it has been proven that laccase activity at neutral pH in a biotechnological process is a desirable characteristic since lower pH values increase enzyme degradation (Margot *et al.*, 2013).

It is worth mentioning that for SPS with the commercial laccase, the cellulase is the third most important factor (Figure 20a), while on the other hand, for the EE-LMB4 it is not (Figure 20b). The requirement of a cellulase activity in the case of the commercial laccase to compete with the results obtained from the EE-LMB4, considering the fact that production of ligninolytic enzymes is often accompanied by cellulolytic enzymes production in basidiomycete fungi during wood decay processes (Presley & Schilling, 2017; Zhang *et al.*, 2016), highlights the appeal of *D. pusillus*-LMB4 as an efficient, accessible and cheap source of relevant biotechnological assets in the field of delignification processes. However, cellulolytic activity of enzymatic extracts was not measured during this project. The cellulolytic activity of EE-LMB4 is currently being evaluated as part of the Master's degree research project entitled "Evaluación del efecto de enzimas lignocelulolíticas de origen fúngico sobre la cáscara de mazorca de cacao en la producción de etanol de segunda generación" at mycology lab at Universidad Industrial de Santander, Colombia. Those results will further complete the characterization of this enzymatic extract.

The highest reducing sugar concentration for the EE-LMB4 was 56.71 ± 21.85 $g.g^{-1}$ at a pH value of 4 at 45°C and with a 2:1 ratio of laccase to cellulase enzymatic units. For the commercial laccase from *T. versicolor*, reducing sugar production reached 56.98 ± 19.58 $g.g^{-1}$ in the same conditions. These results confirm that the crude EE-LMB4 exhibits similar ligninolytic efficiency than the purified commercial laccase from *T. versicolor*. It is important to highlight that results were obtained for an unpurified enzymatic extract in comparison with a purified commercial laccase, suggesting potential high enzymatic efficiency for EE-LMB4. We want to point out

that the EFB is a good lignocellulose source to obtain reducing sugars, due that during the palm oil extraction, the palm oil bunches have a previous "sterilization" process (steam vapor treatment at 100-140°C for 40 minutes) to obtain the oil palm fruits from the bunch, which could be considered as a pretreatment (Liew *et al.*, 2015). Such first pretreatment might improve the later delignification process performed by enzymes.

Even though negative effects were not significant, high copper concentrations and laccase units were the common factors between commercial laccase from *T. versicolor* and crude EE-LMB4. The negative effect regarding increasing laccase units on the reducing sugar production could be attributed to a possible agglomeration or dimerization of laccase enzymes. As for copper concentration, its presence is essential for laccase activity, and low copper concentration are enough to increase the laccase activity. Hence, above a certain concentration, increasing the content of copper will not be beneficial.

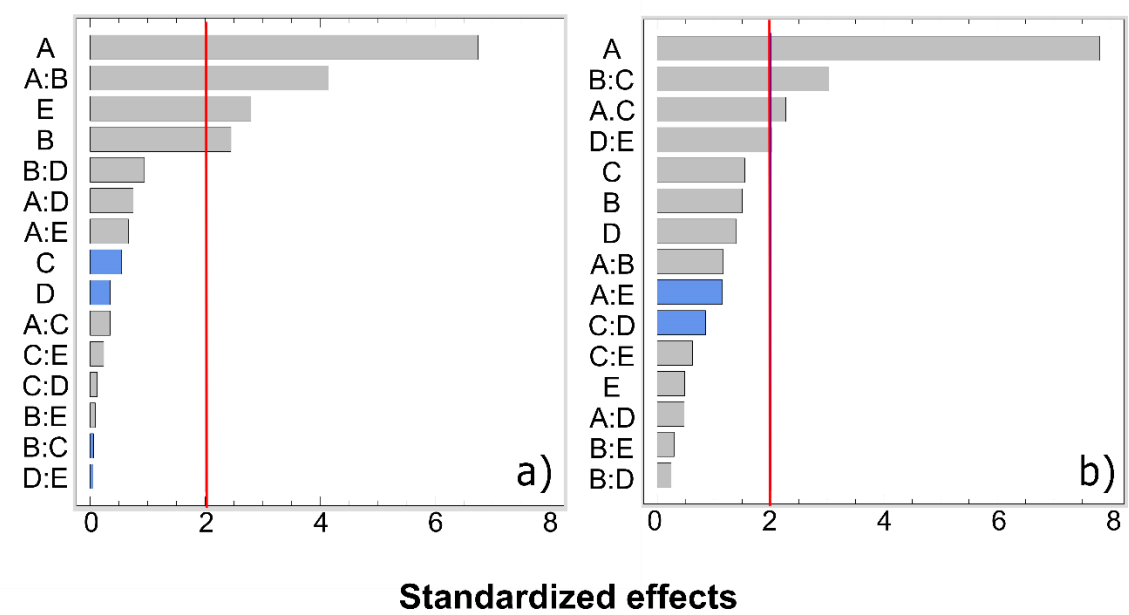


Figure 20. Pareto charts from ANOVA analysis for commercial and *D. pusillus* laccases. a) Cellulolytic extract from *T. reesei* mixed with commercial laccase from *T. versicolor,* b) Cellulolytic extract from *T. reesei* mixed with EE-LMB4 Parameters: A, pH; B, temperature; C, copper concentration; D, $U.L^{-1}$ of laccase and E, FPU of cellulase. The red line represents the statistically significant parameters in the SPS with a 95% confidence. Grey and blue bars are the positive and negative effects, respectively.

3.4. Conclusions

Results obtained throughout experiments described in this chapter aimed to evaluate the delignification capacity of EE-LMB4 on oil palm EFB. Laccase activity, assessed using ABTS oxidation, for the enzymatic extract was observed at pH values between 3 and 5. The optimal pH value was 3, but this condition is associated to loss of laccase activity over time for EE-LMB4, with a 30% and 75% drop after the first and second hours of incubation, respectively. These results rather support usage of EE-LMB4 laccase activity at higher pH values, between 4 and 5.

When thermal stability was compared between EE-LMB4 and a commercial laccase from *T. versicolor*, it was observed that the *D. pusillus* extract was able to maintain its enzymatic activity after 6 hours of incubation at 50°C while the commercial laccase had lost 28% of its activity under the same conditions.

Delignification capacity was assayed through reducing sugar release upon SPS of EFB. Using a cellulolytic commercial cocktail from *T. reesei*, EE-LMB4 and a commercial laccase from *T. versicolor* were evaluated. Results showed that both enzyme solutions were able to induce an increase of around 55% of reducing sugars release. Furthermore, comparison of SPS parameters (pH, temperature, copper concentration and concentration of enzymes) revealed that efficiency in reducing sugar release was influenced similarly when using the commercial laccase or EE-LMB4. However, it is important to highlight that EE-LMB4 is not a purified enzyme, suggesting that further purification and characterization could lead to enhance efficiency of SPS using oil palm EFB. Hence, purification and identification of laccase enzymes and/or isoenzymes from *D. pusillus*-LMB4 could reveal thermostable and low pH tolerant proteins able to perform efficiently delignification of EFB or lignin rich substrates from other agroindustry such as bagasse cane or corn residues.

4. Chapter 4: Characterization of a native enzymatic extract with ligninolytic activity from *Dictyopanus pusillus*

4.1. Introduction

Fungal laccases have been documented and identified many years ago. The first report of laccase activity from fungi exudate was made by the maceration and purification of fruit bodies belonging to *Russula delica* (Bertrand, 1896); but the first partial gene reported for a fungal laccase was from the *Neurospora crassa* (Germann & Lerch, 1986). And studies on *Polyporus versicolor* now called *Trametes versicolor* gave rise to the first fungal laccase crystal structure (Briving *et al.*, 1980). Over the last ten years, laccases have gained interest in industrial applications related to food, pharmaceutical purposes, biofuels, organic synthesis, among others (Mate & Alcalde, 2017). Incorporation of laccase enzymes within the industry depends on its low-cost production and optimal enzymatic catalytic properties according to pH conditions, organic solvent tolerance, temperature and redox potential needed for a given application; unfortunately, such properties are usually not naturally found in only one enzyme, thus the need for protein engineering (Robert *et al.*, 2011).

As it was observed from the results of the previous chapter, the enzymatic extract from *D. pusillus*-LMB4 seem to possess attractive properties including thermostability, resistance to low pH and the ability to enhance the release of reducing sugars through SPS using oil palm EFB as substrate. However, enzymes responsible of this activity need to be further characterized and identified in order to foresee a possible industrial application. Thus, this chapter describes the partial characterization of ligninolytic enzymes from a native *D. pusillus*-LMB4 isolate using gel electrophoresis, protein separation by chromatography and mass spectroscopy in an attempt to identify ligninolytic enzymes composing this mixture.

4.2. Materials and methods

4.2.1. Production of crude enzymatic extract with laccase activity from the native *D. pusillus*-LMB4 isolation

EE-LMB4 was produced by SSF with a C/N ratio of 19 and 5 mM copper as described in section 2.2.4. Thirty flasks were inoculated using cultures of native *D. pusillus*-LMB4 isolation. SSF was stopped after 28 days of growth and EE-LMB4 was extracted following the methodology of section 2.2.5. Laccase activity was checked for each flask enzymatic extract and all crude extracts showing activity were pooled. The pooled extract was concentrated by ultrafiltration through a 30 kDa pore size membrane (Amicon; Millipore, USA). Production of EE-LMB4 was done at Mycology lab, Universidad Industrial de Santander, Colombia.

4.2.2. SDS-PAGE and zymogram analyses

SDS-PAGE and zymogram analyses were performed to characterize the crude enzymatic extract showing the highest ligninolytic activity. The crude EE-LMB4 was concentrated by ultrafiltration through a 10 kDa pore size membrane (Amicon; Millipore, Canada). SDS-PAGE was performed on Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, Canada) and protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Unstained Broad-Range SDS-PAGE Standards between 6.5 and 200 kDa (Biorad, Canada) were used as molecular weight markers. To perform zymogram analyses, the concentrated crude EE-LMB4 was run on a Native-PAGE and protein bands harboring laccase activity were revealed by incubating the gel in a solution of 20 mM ABTS in 50 mM acetate buffer pH 4.5. Under these conditions, bands with laccase activity induce a color change. A commercial laccase from *T. versicolor*, 53739 was used as positive control, while hydrolytic commercial cellulase BP-99 from Tritex Inc. And α-amylase A2771 (Sigma-Aldrich, Canada) were used as negative controls, for zymogram analysis (Karp *et al.*, 2012).

4.2.3. *D. pusillus*-LMB4 enzymatic extract purification

The EE-LMB4 obtained by SSF as was described at section 4.2.1. and used to identify putative laccase enzymes. The enzymatic extract was dialyzed and concentrated by ultrafiltration using a 10 kDa pore size membrane (Amicon; Millipore) and passed through a 0.2 µm filter. Fast protein liquid chromatography (FPLC) was used to separate proteins from the enzymatic extract. The sample was loaded onto a hitrap Q FF anion exchange column, pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.4) to charge the column positively and favor protein adherence to the sepharose Q stationary phase. Elution followed by two steps linear gradient of 1 M NaCl: 0% to 50% in 75 minutes and 50% to 100% in 25 minutes. Fractions were collected every minute and monitored by absorbance at 280 nm to evaluate protein concentration (Camarero *et al.*, 2012).

Collected fractions obtained from FPLC were tested for laccase activity by ABTS oxidation (section 2.2.5.1.) And were submitted to SDS-PAGE to correlate protein bands intensity and molecular weight with laccase activity.

4.2.4. Mass spectrometry sequencing of putative laccase enzymes from *D. pusillus*-LMB4

All fractions with laccase activity identified (section 4.2.3.), were pooled, concentrated and separated by SDS-PAGE. Main protein bands were cut and destained overnight with a 50% methanol solution with agitation in a 1.5 mL tube. Gel bands were dehydrated with 400 µl of 100% acetonitrile for 10 minutes, excess liquid was removed, and the gel piece was dried in a vacuum centrifuge. Dithiothreitol (100 µL, 10 mM) was added to the gel piece and incubated 45 min at 55°C to break disulfide bonds. Excess liquid was removed, 100 µL of 55 mM iodoacetamide was added and incubated 30 min at room temperature in the dark, to avoid disulfide bond regeneration. The gel was washed twice with MilliQ water for 15 minutes with agitation and dehydrated again. A 20 µg/mL trypsin solution prepared in 25 mM ammonium bicarbonate was added to the 1.5 mL tube, covering all the gel. After a 1h incubation on ice, the liquid was replaced with a 25 mM NH_4HCO_3 solution and the gel band was kept at 37 °C overnight. The supernatant

was transferred into a new tube, the gel was washed twice with a fresh trypsin solution during 20 minutes with agitation at room temperature, and the two new supernatants were mixed with the first one. This final pool was vacuum dried and resuspended in water containing 0.1 % trifluoroacetic acid. The sample was then loaded onto a C_{18} Sep-Pak cartridge (Waters, Canada) for desalting and eluted with a solution of 50% acetonitrile/H_2O 0.1% formic acid (Gundry *et al.*, 2010).

The peptide containing solution obtained from the selected bands were analyzed to find the protein fingerprint (Henzel *et al.*, 2003). Hence, peptides obtained from trypsin digestion were separated according to their hydrophobicity using a Zorbax 300SB C_{18} 300Å, 5µ, 150 x 0.3 mm column connected to an Agilent 1100 Series nano-HPLC system with 0.1% formic acid and 90% acetonitrile in water as mobile phases. Sample ionization was obtained by electrospray with an Applied Biosystems Q-Trap 2000 mass spectrometer connected to the Agilent liquid chromatography system (LC-MS/MS). MASCOT server was used to compare obtained peak lists with fungal Protein Data Bank (PDB) database and identify possible peptide sequences and known proteins (Henzel *et al.*, 2003; Thiede *et al.*, 2005). Moreover, all identified peptides were checked with the Position-Specific Iterated BLAST (PSI-BLAST) tool at NCBI server using PDB database to select peptides with ligninolytic enzyme homology (Garg *et al.*, 2012).

Peptides with high homology and number of matches with ligninolytic fungal enzymes as evaluated with the PSI-BLAST tool analysis were used to construct a putative laccase primary structure. Peptide sequences were organized in the theoretical protein primary structure in accordance with corresponding amino acid numbering found with the protein-BLAST analysis. A new theoretical structure showing the highest homology with other laccases reported at PDB was designated as a putative laccase sequence from *D.* pusillus-LMB4 (Figure 21).

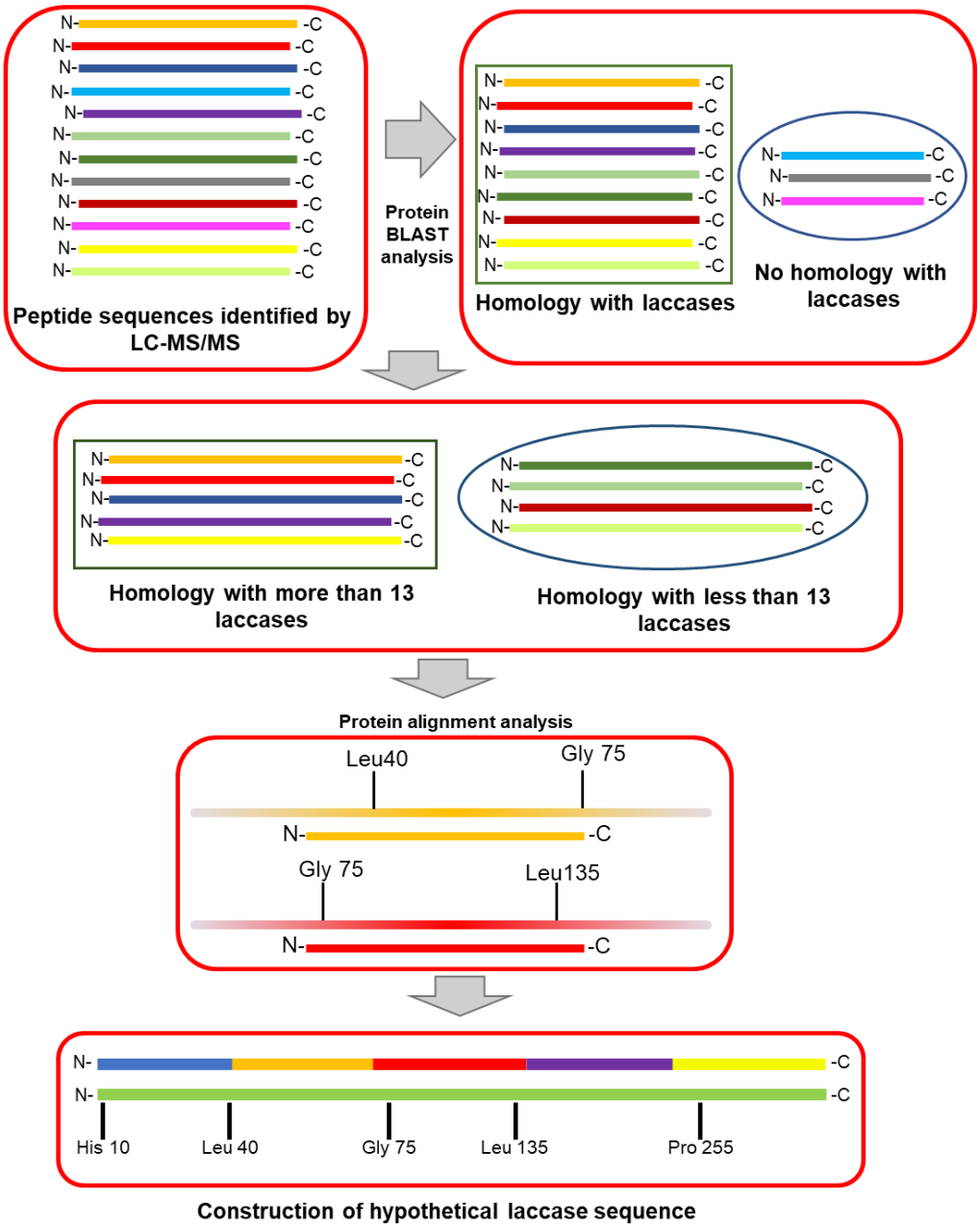


Figure 21. Process scheme to obtain a theoretical laccase protein sequence.

Peptides with a laccase homology according to BLASTp analysis were selected and then sorted in regards with match numbers. From the protein BLAST alignment analysis, peptides were organized according to the corresponding amino acid numbering to generate a theoretical laccase primary structure.

4.3. Results and discussion

4.3.1. SDS-PAGE and zymogram analyses

SDS-PAGE analysis of crude EE-LMB4 showed multiple bands, with a major protein species observed between 50 and 75 kDa (Figure 22a). This molecular weight correlates with several homologous laccases isolated from basidiomycete fungi, with molecular weights from 43 to 90 kDa and average of 66 kDa (Baldrian, 2006). Moreover, our zymogram analysis confirmed the existence of at least one main oxido-reduction enzyme exhibiting ABTS oxidation in the EE-LMB4. crude extract of *D. pusillus*-LMB4. In fact, oxidation of ABTS produced one spot with the EE-LMB4 whereas the *T. versicolor* laccase used as a positive control (Figure 22b) gave rise to two major spots on the zymogram analysis, that can be attributed to the dimerization or multimerization of laccase enzymes into the pure solution used. Negative controls commercial cellulase BP-99 and α-amylase A2771 does not show any oxidation spot. Nevertheless, these analyses cannot rule out the presence of laccase isoforms since a very weak band coloration was obtained through SDS-PAGE analysis, precluding proper band definition. Similar results were reported for an enzymatic extract with low protein concentration from the ligninolytic mushroom *Lenzites elegans*. In fact, SDS-PAGE did not show defined protein bands while zymogram analysis showed a clear green spot produced by ABTS oxidation (Pandey *et al.*, 2018).

Zymogram results are a strong probe to confirm the existence of at least one oxidative enzyme, which could be a laccase, in the EE-LMB4 obtained by SSF. Moreover, the purple spot intensity obtained with this enzymatic extract is comparable to what was observed with the commercial laccase from *T. versicolor*, suggesting similar activity levels. Thus, enzymatic extract purification was pursued despite the low protein concentration.

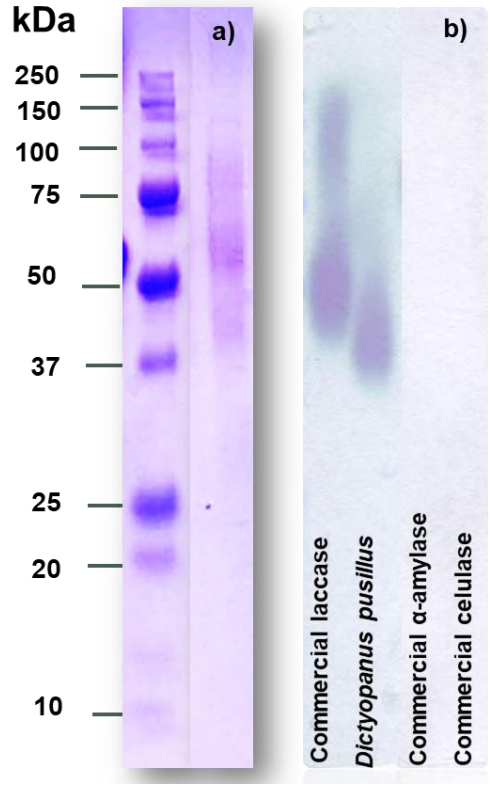


Figure 22. SDS-PAGE and zymogram of *D. pusillus*-LMB4 enzymatic extract showing laccase activity.

The EE-LMB4, obtained from SSF using lignocellulose from oil palm trees, was analyzed by a) SDS-PAGE using Coomassie blue staining, b) ABTS zymogram gel analysis using a commercial laccase from *T. versicolor* as positive activity control and commercial cellulase BP-99 from and α-amylase A2771 as negative control.

4.3.2. Purification of *D. pusillus*-LMB4 enzymatic extract

During the FPLC purification of EE-LMB4, only fractions collected between 20-28% of the linear NaCl gradient (fractions 30 to 45 minutes of elution), showed a low increase in the UV-signal at 280 nm, and a high peak at 75% of the NaCl gradient was observed which most probably corresponded to absorbance of enzymatic extract components such as pigments from the EFB (Figure 23 and 24). SDS-PAGE of samples collected during various steps of the purification and fractions from 30 to 45 minutes of the elution gradient was performed along with ABTS oxidation activity test. Only fractions from 30 to 45 minutes of the elution gradient demonstrated ABTS oxidation activity (figure 25 and 26).

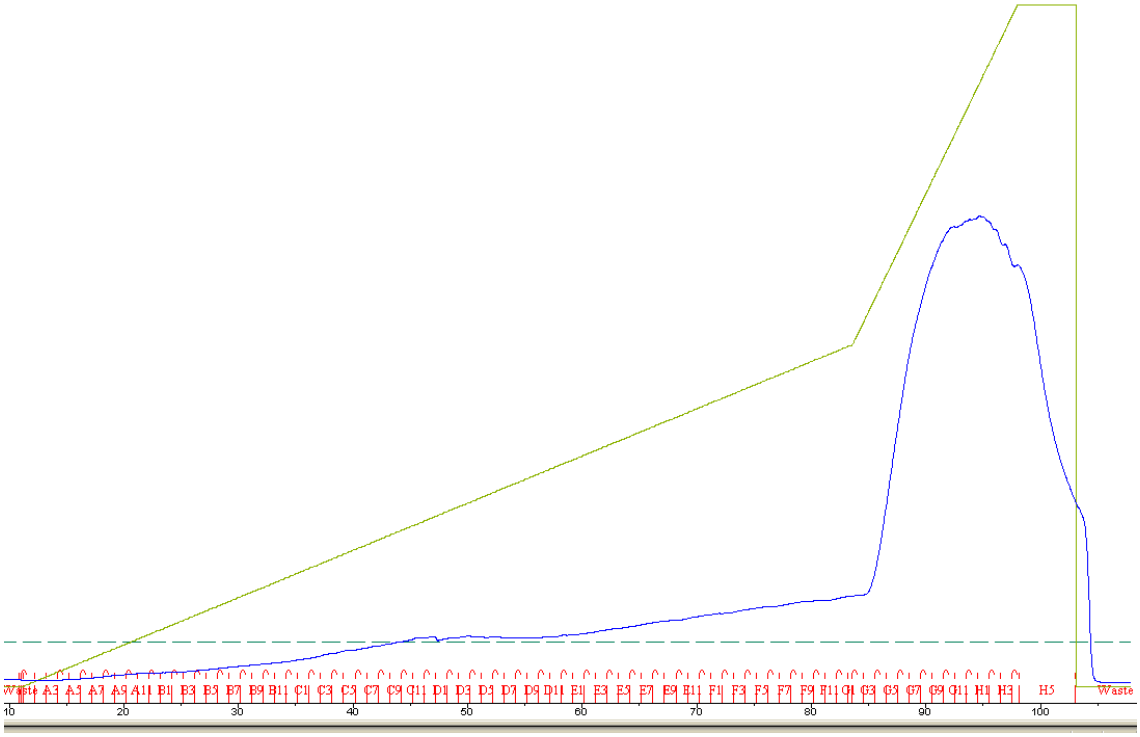


Figure 23. FPLC chromatogram of *D. pusillus*-LMB4 crude enzymatic extract.

The blue line represents absorbance at 280 nm while the green line depicts NaCl gradient. The first NaCl gradient ramp (0-50%) shows a low absorbance peaks corresponding to higher laccase activity but the second and more rapid NaCl gradient ramp shows a huge peak that does not show laccase activity.

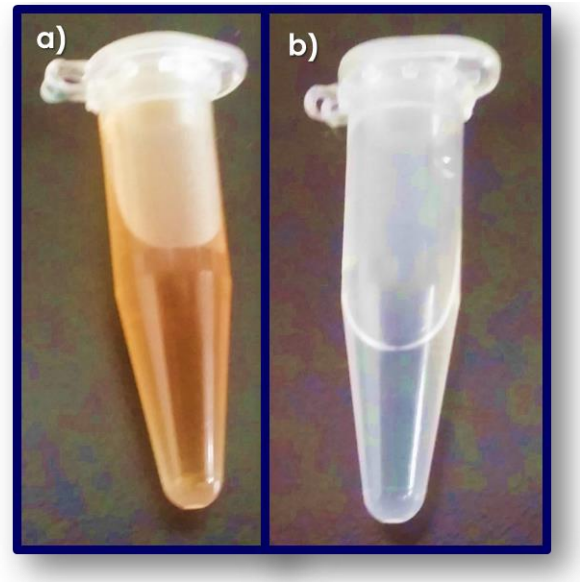


Figure 24. Appearance of *D. pusillus*-LMB4 enzymatic extracts with laccase activity.

a) Enzymatic extract before FPLC purification, b) pooled fractions with laccase activity collected from FPLC purification.

SDS-PAGE of the fractions from 30 to 40 minutes, showed three main protein bands of approximately 55, 60 and 80 kDa for fractions 30 to 40 from the FPLC purification step. Noteworthy, a 55 kDa protein band was more intense in fractions 34 to 40 and these fractions showed higher ABTS oxidation activity (Figures 25 and 26). This correlation between SDS-PAGE protein bands and laccase activity served as an indication that this 55 kDa protein could be a laccase enzyme or a pool of isoenzymes due to the fact that laccase isoenzymes have been reported to have similar molecular weights.

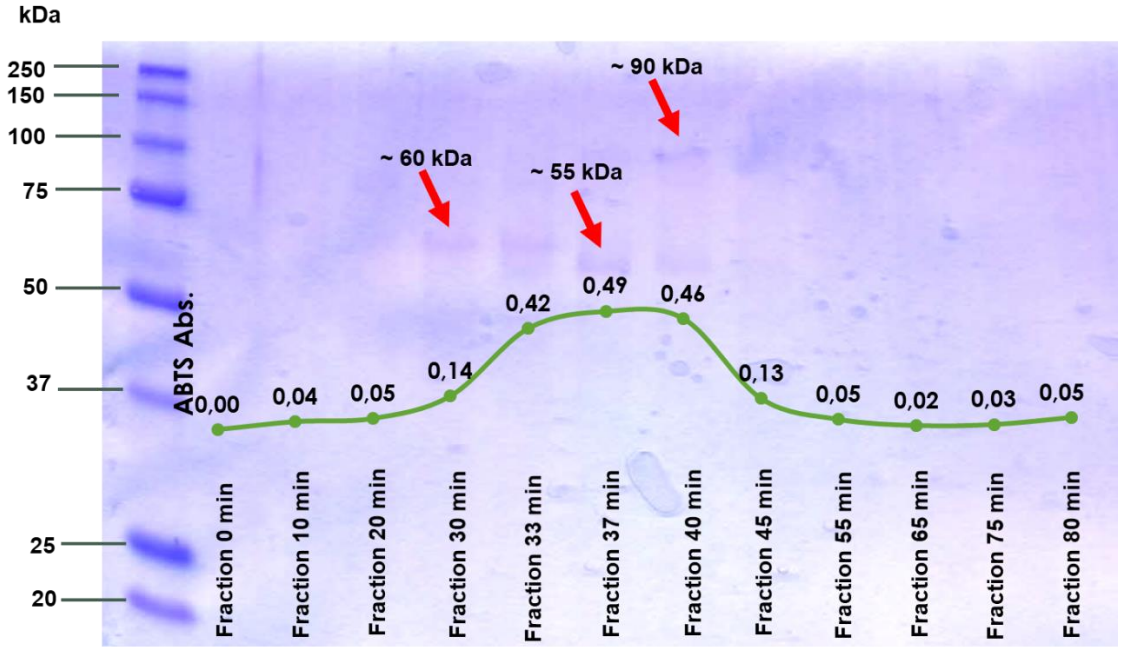


Figure 25. SDS-PAGE of fractions with laccase activity obtained by FPLC.

The presence of three bands was observed. One band with a molecular weight of ~55 kDa in fractions 33 to 40 was associated with the highest ABTS oxidation (green line).

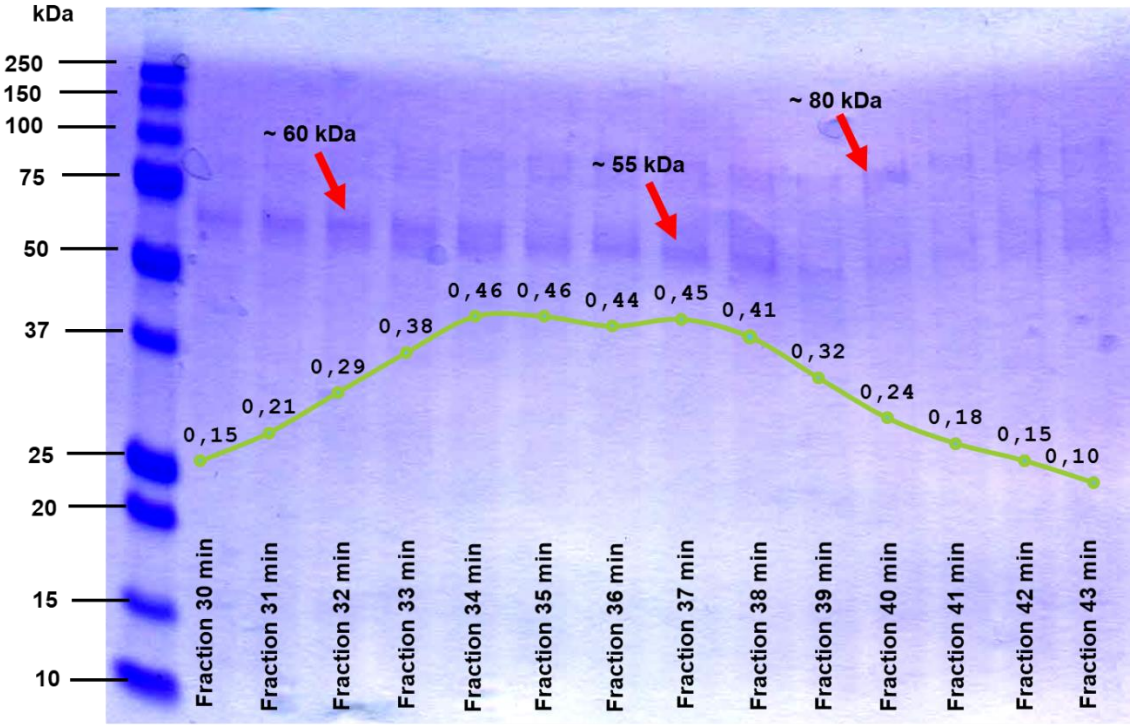


Figure 26. SDS-PAGE of fractions with higher laccase activity obtained by FPLC.

Three main bands were observed in fractions 31 to 43 were observed, with the presence of a ~55 kDa band associated with the highest ABTS oxidation (green line).

4.3.3. Identification of putative laccase enzymes from *D. pusillus*-LMB4

The 55 kDa protein band that was associated with laccase activity was selected for mass spectroscopy identification. From the ESI-MS/MS analysis, 176 peptides were identified by MASCOT server (Appendix A, Table A1). However, no relevant association between all identified peptide sequences and laccases or ligninolytic enzymes reported within NCBI was possible. We believe that the low protein concentration in the band selected or the presence of more than one protein associated to ABTS oxidation may have contributed to the poor association between peptides and ligninolytic enzymes. Also, a possible mix of isoenzymes in the single band or sample contamination during peptide extraction could be another interference that will hamper a good score for protein identification. Hence, peptides were evaluated separately with Basic Local Alignment Search Tool for proteins (BLASTp) using PDB as database and the PSI-BLAST algorithm at NCBI server.

From the 175 peptides, we found that only 144 peptides had at least one match with a laccase structure reported within PDB (Appendix A, Table A1). 18 laccase structures belonging to 13 basidiomycete fungi were identified by peptide BLAST (Table 4). The 144 peptides were organized by match numbers, and peptides with more than 13 matches were selected for building a hypothetical protein sequence. From this analysis, 32 peptides were chosen and sorted again according to frequency of amino acid position match between peptide and reported laccase sequences, to finally yield 27 sequences (Table 5). A hypothetical sequence of 506 amino acids was constructed from the final 27 peptide selected (Appendix A, figure A1). This hypothetical sequence was analyzed with BLASTp at NCBI server and results from the amino acid alignment revealed that the hypothetical sequence had a 31% homology with the crystal structure of laccase enzyme PDB accession number 2H5U from *Cerrena maxima (Lyashenko et al., 2006).*

Table 4. Fungi and laccase enzymes identified in NCBI database by peptide homology from sequences obtained from LC-MS/MS analysis of *D. pusillus*-LMB4 enzymatic extract with laccase activity.

Fungi at NCBI	Number of laccase enzymes matching identified peptides	Accession Number	Reference
Coprinus cinereus	2	1HFU_A, 1A65	(Ducros *et al.*, 1998)
Trametes versicolor	2	1KYA_A, 1GYC_A	(Bertrand *et al.*, 2002), (Antorini *et al.*, 2002)
Rigidoporus lignosus	1	1V10_A	(Garavaglia *et al.*, 2004)
Cerrena maxima	2	2H5U_A, 3DIV_A	(Lyashenko *et al.*, 2006)
Trametes trogii	1	2HRG_A	(Matera *et al.*, 2008)
Coriolus zonatus	1	2HZH_A	---
Lentinus tigrinus	1	2QT6_A	(Ferraroni *et al.*, 2007)

Pycnoporus cinnabarinus	1	2XYB_A	(Antorini *et al.*, 2002)
Trametes hirsuta	2	3FPX_A	(Pegasova *et al.*, 2003), (Polyakov *et al.*, 2009)
Steccherinum ochraceum	1	3T6V_A	(Ferraroni *et al.*, 2012)
Lentinus sp.	1	3X1B_A	---
Coriolopsis gallica	2	4A2D_A	(De la Mora *et al.*, 2012)
Coriolopsis caperata	1	4JHU_A	(Glazunova *et al.*, 2015)

Table 5. Peptides from the *D. pusillus*-LMB4 enzymatic extract with laccase activity selected to build the hypothetical laccase sequence.

Peptides were selected according to matching frequencies with known laccases. Assigned scores correspond to BLASTp results.

Peptide selected	Assigned score
EGEWIQCGVDDEGPGISAELLPR	85
FWYEKNLTTEEIADVESR	107
SYQSHTRNSLVQDPK	108
GLPLPSHPSTGGMLR	110
GVPHVLVMMCDGLGHGPLAAR	117
IAQMVDPADPEAGKLQVNAAEITPVK	129
GTRPLPLSDGCASLRQASEYNSDDR	135
EGEADATTSTK	165
VVADQQLIADTFLK	166
HSHHLWGIAQTGAVADQQGQIAGLGR	173
RNGEGLQYAVVPVTDSK	185
KIVGAILLVSLICLGFSDSVK	200
NPPLAEALLSGDLVSLVLVCYR	200
FIDSGDPLLVGEQVRACDR	259
LHGQMPNSAIIR	275
KLSTALALMGDPAVVYLDEPTTGMDPGAR	285
FRCVLAVAAPGQQTQTFSGSCEGR	315
DPSRIPLALAQHLLR	362
RADPALYAAP	385

VTAPEPGQAPSETSK	386
DETPFTGDNHTTTELANAIR	430
RVGETLMVGDDVTVTVLGVK	433
VVRFYDGGVTIR	436
QKALADCKPEPNYNGGPWFAPSYK	442
YNGSSLEEGFPQK	459
DALTPDAVALAESKR	464
LSAVGYGEDKPIATNNTSAGR	475

Although it was not possible to identify at least one complete laccase enzyme from the identified peptides by mass spectroscopy, the few peptides identified have been shown to be related to several basidiomycete laccase enzyme structures as outlined in Appendix 1, Table A1. Thus, the protein(s) extracted from the 55 kDa gel band and giving rise to ABTS oxidation could correspond to laccase enzymes. However, the fact that *D. pusillus* genomic and proteomic information are at the moment non-existent definitely hampers precise enzyme identification and our results suggest that this fungus expresses at least one laccase that differs from other known basidiomycete enzymes. Hence, these newly obtained data could lead to further exploration of *D. pusillus* genome to discover new enzymes involved in the delignification process.

Finally, it is important to point out that genomic and proteomic data is increasing rapidly with technological advancements. Performing once again this analysis with up-to-date PDB databases at NCBI server could reveal new links between *D. pusillus*-LMB4 identified peptide sequences and newly reported enzymes.

4.4. Conclusions

Purification and characterization of EE-LMB4 was attempted but the fact that chromatography did not result on the isolation of single protein bands on SDS-PAGE rendered identification of a ligninolytic enzyme difficult. However, zymogram analysis showed an ABTS oxidation for EE-LMB4 similar in intensity to the commercial laccase enzyme and the observed pattern suggests the existence of at least one oxido-reducing enzyme.

Although EE-LMB4 purification revealed more than one protein specie, collected fractions were tested for ABTS oxidation and from the positive ones, it was possible to obtain peptide sequences from LC-MS/MS analysis. However, comparison of these peptides with databases did not yield protein identification but this could be caused by the fact that the oxido-reducing enzyme from EE-LMB4 is not reported yet.

Even though, the information obtained from this chapter did not produce a confirmation about the existence of at least one laccase or another ligninolytic enzyme with ABTS oxidation activity from *D. pusillus*, it was possible to obtain a partial putative laccase enzyme sequence that could be useful for the search of possible *D. pusillus* codifying genes. However, because the genome of *Dictyopanus* genera is not yet sequenced, it was not possible to correlate this new information with previous information reported in protein or genomic databases. Thus, other means should be considered to identify proteins with delignification activity, such as genomic exploration or whole genome sequencing, to allow a correlation with information obtained in this chapter regarding ligninolytic enzymes from *D. pusillus*-LMB4.

Chapter 5: Genomic exploration of *D. pusillus*-LMB4

5.1. Introduction

Since the first report of fungus as ligninolytic agents, the exploration of new fungi has been mainly centered on acquisition of enzymatic extracts from fermentation over lignocellulose substrates or synthetic culture media. Likewise, secretome and genome studies from basidiomycete fungi involved in wood decay has been focused on the search of cellulases more than ligninases (Guo *et al.*, 2018; Janusz *et al.*, 2017). *Phanerochaete chrysosporium, P. carnosa, Ganoderma lucidum, Pleurotus sp., Ceriporiopsis subvermispora, Trametes sp.,* and *Serpula lacrymans* constitute the reported ligninolytic enzyme sources for potential biotechnological processes (Alfaro *et al.*, 2014). One limitation to exploration of new ligninolytic enzyme sources has been the high amount of time for secretome and genome investigations combined with scarce information on those fungi. However, advances in technology have now reduced sequencing time and cost, which has triggered the publication of whole genomes, including drafts from basidiomycetes (van Dijk *et al.*, 2014; Vincent *et al.*, 2017).

New data from native ligninolytic enzymes can surely facilitate exploration, production, and incorporation of ligninolytic enzymes in second-generation ethanol production. Hence, new genome engineering tools will improve the incorporation of low-cost enzymes in biotechnological lignocellulosic ethanol industries. (Binod *et al.*, 2010; Ulaganathan *et al.*, 2017). In fact, genomic studies have led the way to identification and characterization of ligninolytic enzymes from species that had already been recognized useful for lignocellulose pretreatment. Indeed, *Flammulina velutipes* and *Flammulina elastica* had been used to improve cellulose degradation and ethanol fermentation, but their whole genome sequencing have allowed the identification of cellulases, hemicellulases, and ligninases useful for second-generation ethanol production but also to better understand basidiomycete fungi processes for pretreatment of lignocellulose (Mizuno *et al.*, 2009a; Mizuno *et al.*, 2009b; Park *et al.*, 2014). Beside bioethanol production, whole genome sequencing of ligninolytic basidiomycete fungi has been useful to identify new approaches for desired products using biocatalytic methods. As such, enzymes identified from the

basidiomycete *Omphalotus olearius* were found to endorse novel terpene biosynthetic routes leading to compounds with new bioactivities (Wawrzyn *et al.*, 2012).

This chapter describes the genomic exploration of *D. pusillus*-LMB4 genome following three different approaches: 1) genome walking, 2) cDNA library construction and 3) whole genome sequencing. Genome walking was based on peptide sequences obtained by mass spectroscopy, described in the chapter 4, and with degenerate primers designed from peptide sequences with a similarity with a known laccase. The cDNA library construction was elaborated to unveil possible laccase enzyme codifying sequences. Fortunately, massive sequencing can now be accomplished at lower cost and shorter times, making this technique a successful tool to explore not only the laccase enzymes that was recognized in the *D. pusillus*-LMB4 native isolation, but other relevant enzymes.

5.2. Material and methods

5.2.1. Laccase gene exploration by genome walking

Genome walking is a technique that allows to determine DNA sequences positioned upstream and downstream of a starting known DNA sequence. In the context of this project, a degenerate DNA sequence based on the theoretical laccase protein elaborated from LC-MS/MS results and BLAST analyses (Section 4.3.3) was used as a putative starting point to identify laccase genes from *D. pusillus*-LMB4 genome (Figure 27) (Garg *et al.*, 2012).

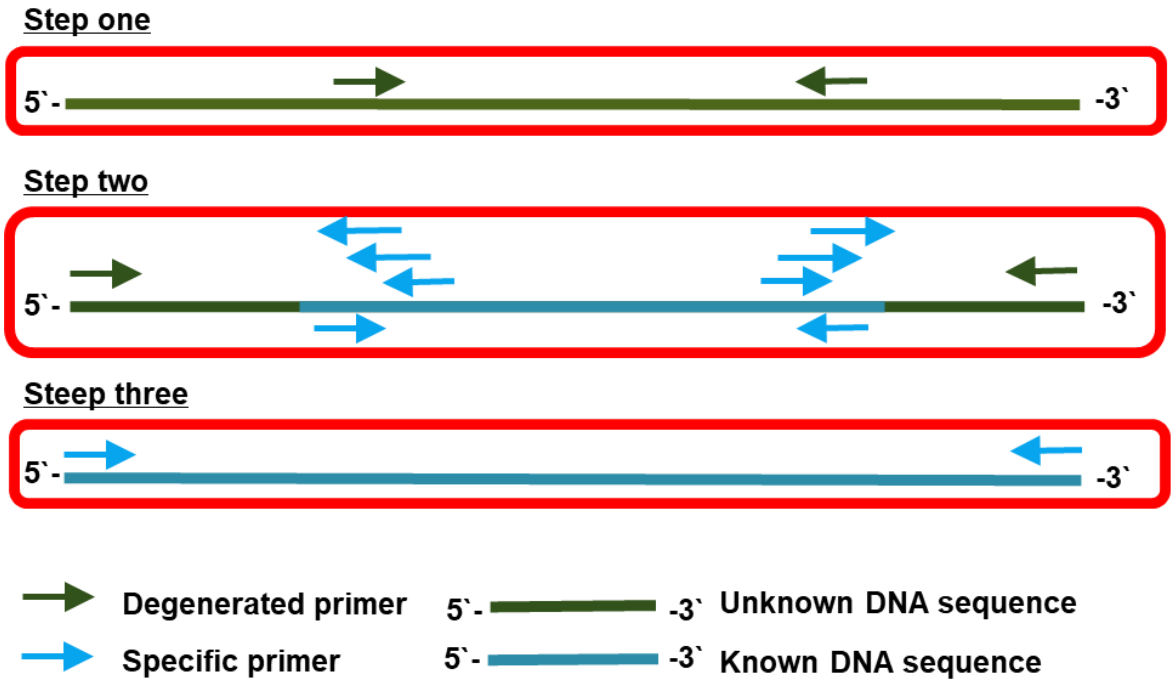


Figure 27. Scheme of genome walking strategy.

Arrows represent the primer position into an unknown gene. Lines represent a gene section into the genome. Green for degenerate primers and unknow DNA, blue for specific primers and known DNA.

This selected theoretical laccase structure (Section 4.3.3) was aligned with the *C. maxima* laccase protein sequence identified with PDB accession number 2H5U and used to design a first set of primers aiming at better defining this region. The alignment revealed a 31% homology between both sequences (Figure 28). Those similar sequences were used to design the first group of degenerate primer pairs (Table 6).

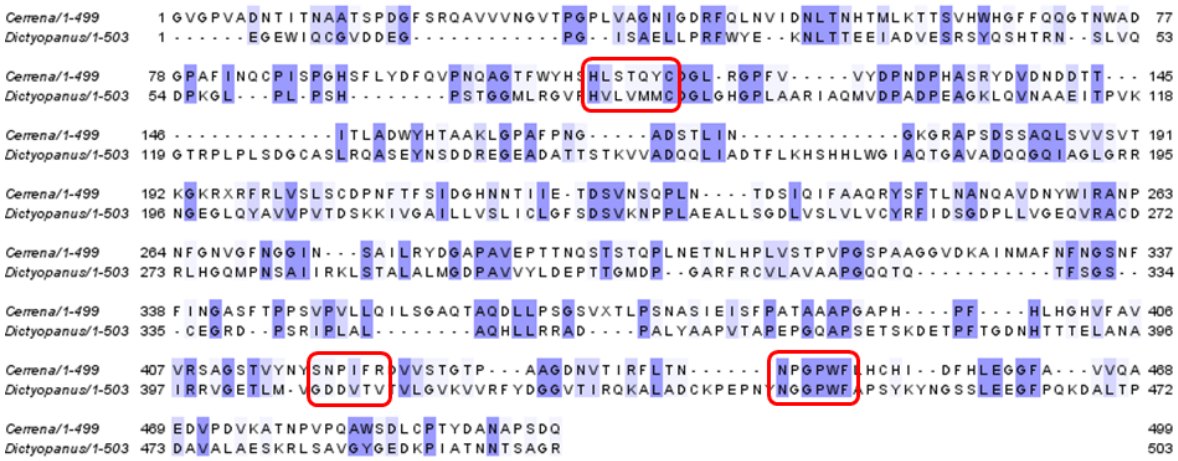


Figure 28. Alignment between laccase 2H5U from *C. maxima* and hypothetical protein laccase sequence from *D. pusillus*-LMB4.

Blue boxes show the similar amino acids positions between the two sequences. Red squares show the sequences used to design the degenerate primers.

Table 6.Designed primers based on the hypothetical laccase protein sequence elaborated from peptides identified during LC-MS/MS analysis.

Code	Primers 5´- 3´
Lac-pep-1A	CAYGTNYVNGTNATGATGTGYGAY
Lac-pep-2B	GGYGAYGAYGTNACNGTN
Lac-pep-3B	AAYGGNGGNCCNTGGTTY

Then, another pool of primers was designed based on 266 laccase gene sequences from basidiomycete fungi reported at NCBI. More precisely, these 266 gene sequences were downloaded, aligned and dissimilar sequences were eliminated. Similarity, 70 laccase gene sequences and consensus regions were identified and used to design the second group of degenerate primer pairs (Table 7). Multiple sequence alignments were performed with multiple Sequence Comparison by Log-Expectation (MUSCLE) software from the European Bioinformatics Institute (EBI).

Table 7. Designed primers based in reported laccase alignments.

Code	Primers 5´- 3´
Lac-1A	GCAYTGGCAYGGNHTNTT
Lac–1B	AANADNCCRTGCCARTGC
Lac-2A	GGNACNTWYTGGTAYCAYWSNCA
Lac-2B	TGNSWRTGRTACCARWANGTNCC
Lac-3A	CCNCKNANNCCRTCRCARTAYTG
Lac-3B	CARTAYTGYGAYGGNNNTNMGNGG
Lac-4A	CGNCCNHNNCCRTTRATNAR
Lac-4B	YTNATYAAYGGNNDNGGNCG
Lac-5A	GYCAYATHGANYBBCAYYTNV
Lac-5B	BNARRTGVVRNTCDATRTGRC

DNA from *D. pusillus*-LMB4 was obtained as described previously (Section 2.2.6.). Temperature ramping based on primer melting points was used to select the best hybridization parameters for degenerate primer pairs and DNA sequences were amplified through degenerate PCR using the iProof™ High-Fidelity DNA Polymerase kit (Bio-Rad, Canada). Standardized PCR conditions for the two groups of primers were: 98°C for 5 min, followed by 35 cycles of 98°C for 1 min, 51°C for 1 min, and finally 3 min at 72°C.

PCR products were displayed on agarose gel, stained with GelRed® (Biotium, USA) and viewed by UV light with 1 Kb Plus DNA Ladder (Thermo-Fisher Scientific, Canada) used to determine band size. All significant bands observed on the PCR product gel were extracted, purified and sequenced by the Sanger method using the same degenerate primers employed to obtain the PCR products. Obtained sequences were compared to the nucleotide database from NCBI to identify homology with other laccase genes, supporting the idea that amplified sequences might correspond to a putative ligninolytic enzyme gene.

Overall, the DNA sequence obtained by genome walking was extracted by combining degenerate primers, Thermal Asymmetric Interlaced (TAIL) (TAIL-PCR) (Yang *et al.*, 2014) and Arbitrary Primed (AP) (AP-PCR) (Liang *et al.*, 2008) PCR techniques. The whole sequence obtained was finally analyzed to identify possible

homologue laccase genes by Basic Local Alignment Search Tool for nucleotides BLASTn at NCBI.

5.2.2.*Dictyopanus pusillus*-LMB4 cDNA library construction

cDNA synthesis was performed at the mycology lab from Universidad Industrial de Santander, Colombia, since Colombian legislation does not allow native species to be brought in another country, to protect the biodiversity and natural resources.

Trizol (Invitrogen, Canada) and iscript Select cDNA Synthesis Kit (Biorad, Canada) were used to extract total RNA and synthesize cDNA from *D. pusillus*-LMB4. Briefly, mycelium was grown on wheat bran extract to favor expression of ligninolytic enzyme mRNA. In sterile conditions, trizol reagent (1 mL), mycelium (100 mg) and glass pearls were mixed and incubated for 5 minutes at room temperature to break fungal cell walls and to allow dissociation of nucleoprotein complexes. Then, phenol (0,2 mL) was added, the solution mixed by hand and let to stand 3 minutes at room temperature. The mixture was centrifuged for 15 minutes at 12 000 g at 4°C, and RNA was collected in the colorless upper aqueous phase. RNA was precipitated by adding isopropyl alcohol and centrifuged for 10 minutes at 9 000 g at 4°C. The RNA pellet was resuspended and washed with 75% ethanol. The final RNA pellet was air dried and resuspended with RNAse-free PECT treated water.

RNA concentration was determined by UV-VIS spectrophotometry and the quality of the extracted RNA was assessed by the observation of two strong ribosomal RNA bands and no degradation pattern on agarose gel electrophoresis. Iscript™ Select cDNA Synthesis Kit (Bio-Rad, Canada) was used to synthetize the cDNA from the extracted mRNA following the supplier's recommendations and products were cleaned with a Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Canada). RNA was also extracted from *P. pastoris* strain to validate the procedure and actin was used as a housekeeping gene for *D. pusillus*-LMB4 and *P. pastoris* cDNA.

The clonejet PCR Cloning Kit (Thermo-Fisher, Canada) was used to insert cDNA fragments from *D. pusillus*-LMB4 into the blunt linearized pJET1.2 cloning vector (Appendix A, Figure A2) using the Blunt-End Cloning Protocol from the supplier.

Briefly, cDNA and cloning vector were ligated using T4 DNA ligase in the recommended buffer at room temperature for 5 minutes. Resulting vectors were used to transform *E. coli* Rosetta DE3 and *E. coli* DH5α competent cells by electroporation and heat shock, respectively. Transformed cells were plated on Lysogenic Broth (LB) agar with carbenicillin at 50 $mg.mL^{-1}$ and incubated overnight at 37°C. Each colony was grown individually in 2 mL of LB medium with carbenicillin at 50 $mg.mL^{-1}$ and incubated overnight at 37°C in order to extract plasmids using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio-Basic, Canada). Isolated plasmids were kept at 4°C until further use.

cDNA fragments were amplified from the pJET1.2/blunt cloning vector by PCR, using the forward sequencing primer 5'-CGACTCACTATAGGGAGAGCGGC-3' and reverse sequencing primer 5'-AAGAACATCGATTTTCCATGGCAG-3' as suggested by clonejet PCR Cloning Kit. PCR conditions were 98°C for 5 min, followed by 35 cycles of 98°C for 1 min, 54°C for 1 min, and finally 10 min at 72°C. PCR products were loaded onto 1% agarose gels, separated by electrophoresis, stained with GelRed® (Biotium, USA) and viewed by UV light to visualize PCR product bands. PCR products were also cleaned using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Canada).

pD912-AKS expression vector (Appendix A, Figure A3) was selected to prepare a heterologous protein expression system in Pichia pastoris PPS-9011 (BG11, aox1δ (muts)). AKS vector have an AKS–Alpha-factor_kex_ste signal peptide to facilitate protein expression under the control either of an inducible (AOX) or constitutive (GAP) promoter and zeocin resistance gene (Atum, USA). P. pastoris PPS-9011 strain and AKS vector were a generous gift of Jean-François Lemay from Centre National en électrochimie et en Technologies Environnementales (CNETE), Shawinigan, Canada. Overexpression of enzymes using this strain and vector system was evaluated and standardize by the CNETE laboratory.

A Gibson Assembly cloning kit (New England Biolabs, Canada) was used to insert cDNA fragments recovered from the pJET1.2/blunt cloning vector into the linearized AKS expression vector. Each one of these primers was designed to expose an extra DNA region that ensures overlapping and a successful assembly

following the Gibson reaction. The reaction was performed by mixing 1 µl of the AKS linearized expression vector, 3 µl of cDNA fragments, 10 µl of Gibson Assembly Master Mix 2X and 6 µl of DEPC-treated water followed by an 1h incubation at 50°C. The reaction was stopped in an ice bath and 2 µl of the last reaction were used to transform NEB 5-alpha *E. coli* competent cells through a heat shock procedure.

Transformed cells were added to 950 µl of SOC broth, incubated at 37°C for one hour at 250 rpm and plated on LB low salt media with zeocin (1 $mg.mL^{-1}$) to pursue incubation for 24 hours in the dark. Colonies obtained in each plate by the Gibson assembly were recovered using 2 mL of LB low salt broth and glass pearls, and plasmids were extracted with EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio-Basic, Canada). 20 µg of plasmid obtained by Gibson assembly was mixed with 50 µl of *P. pastoris* PPS-9011 competent cells in a pre-chilled electroporation cuvette and submitted to 1500 V. Cold sorbitol (1 M, 500µl) was used to transfer cells from the cuvette to sterile tubes containing 500 µl of YDP broth (1% yeast extract, 2% peptone, 2% glucose) and tubes were incubated at 30°C for 2 hours. Finally, 100 µl of transformed cells were plated on Yeast Extract Peptone Dextrose Medium (YDPS) agar (1% yeast extract, 2% peptone, 2% glucose, 1M sorbitol, 1.5% agar) with zeocin (1 $mg.mL^{-1}$) and incubated for 3 days in the dark.

5.2.3. cDNA library screening

The screening method was elaborated following recommendations from Atum (USA). Each colony of *P. pastoris* obtained from the cDNA library on YDPS was plated on ABTS supplemented Buffered Methanol-complex Medium (BMMY) agar plates (1% yeast extract, 2 % peptone, 13.4 $g.L^{-1}$ yeast nitrogen base without amino acids, 0.004 $mg.L^{-1}$ biotin, 1% methanol, 1.5% agar 0.1% ABTS) using zeocin (1 $mg.mL^{-1}$) for selection. Laccase positive colonies showed a green halo due to ABTS oxidation within the BMMY culture media. Additionally, each colony was transferred to sterile tubes containing 2.5 mL of BMMY broth with zeocin at 1 $mg.mL^{-1}$, and tubes were incubated for 2 days at 28°C and 250 rpm. Once completed, 25 µl of methanol was added every day for 3 days. After 5 days, the culture was assayed for laccase

activity through the ABTS oxidation assay described in section 2.2.5.1. (Antošová & Sychrová, 2016).

5.2.4. Whole genome sequencing of *Dictyopanus pusillus*-LMB4

From the different approaches platforms available to perform whole genome sequencing, the PacBio® sequel system from Pacific Biosystems, that uses a proprietary Single Molecule Real-Time (SMRT) technology, was chosen. PacBio SMRT (third-generation DNA sequencing) was selected due to its advantages over first- and second-generation DNA sequencing approaches such as Sanger or Illumina technologies. Among the advantages of PacBio SMRT, sequence detection of single copy molecules per DNA template, real time detection of sequenced base pairs and production of long reads that can exceed 10 kb in length (Heather & Chain, 2016).

Sequenced DNA was obtained from a D. pusillus-LMB4 mycelium grown on Potato Dextrose Agar (PDA), to avoid cross contamination that can occur using crude extracts such as wheat bran extract. Fifteen colonies of 15 days of growth on PDA were used to extract genomic DNA (gDNA) following a high salt phenol chloroform cleanup protocol recommended by PacBio® systems. Briefly, 0.5 g of mycelium was placed in a tube with a lysis solution (0.1 M NaCl, Tris-HCl pH 8, 5% SDS) and 0.5 mm diameter glass beads, and shaken until mycelium was broken. Cellular debris were pelleted by centrifugation at 11 000 g for 10 minutes and supernatant was mixed in the same proportion with a phenol-chloroform-isoamyl alcohol solution (25:24:1). The mixture was centrifuged at 11 000 g for 5 minutes, the upper aqueous phase was mixed once more in the same proportion with a chloroform-isoamyl alcohol solution (24:1). After a final centrifugation at 14 000 g for 10 minutes, the aqueous fraction was collected, and gDNA was precipitated and washed with ethanol. The DNA pellet was dissolved in DEPC-treated DNAse-free water.

The genomic DNA from *D. pusillus*-LMB4 was sequenced using five SMRT cells on a Pacific Biosciences RS II system at the Génome Québec Innovation Centre (McGill University, Montreal, Canada).

5.2.5. *De novo* genome assembly of *Dictyopanus pusillus*-LMB4

The resulting sequencing reads were *de novo* assembled in contiguous sequences using Canu software version 1.7 (Koren *et al.*, 2017). The heterozygous genome was then reduced using Redundans version 0.14a (Pryszcz & Gabaldón, 2016). Protein encoding genes were predicted with webaugustus (Hoff & Stanke, 2013) using *Laccaria bicolor* as a training dataset. The resulting predicted gene sequences were annotated using the webserver of eggNOG-mapper (Huerta-Cepas *et al.*, 2017).

5.2.6. Ligninolytic laccase enzyme annotations of *D. pusillus*-LMB4 genome draft

Fifty-five laccase protein sequences reported from *S. hirsutum*, *T. versicolor, P. strigosozonata*, and *D. squalens* species *(Floudas et al., 2012)* were used to identify putative laccase enzymes in the translated genome draft from *D. Pusillus*-LMB4, based on similarities between *D. pusillus*-LMB4 partial genome sequences and NCBI DNA information related to those species.

Each putative protein laccase sequence found was submitted to the Basic Local Alignment Search Tool for proteins BLASTp tool from NCBI server to find a correlation with other laccase enzymes reported within PDB and identify new consensus regions. Moreover, the four conserved copper-binding motifs, *i.e.* Cu1 (HWHGFFQ), Cu2 (HSHLSTQ), Cu3 (HPFHLHG), and Cu4 (HCHIDFHL) (Kumar *et al.*, 2003), were searched into these putative protein sequences. Also, the sequences corresponding to putative laccases were further analyzed using interproscan (Jones *et al.*, 2014) to verify the presence of multicopper oxidase signatures (PS00079 and PS00080 Prosite entries, ExPASy Bioinformatics Resource Portal) and Cu-oxidase Pfam domains (PF00394, PF07731, and PF07732 entries) (Moreno *et al.*, 2017). Comparisons with the Laccase and Multicopper Oxidase Engineering Database (Sirim *et al.*, 2011) was also used to validate that the identified sequences were laccases.

5.2.7. Homology modeling for putative laccases of D. pusillus-LMB4

Protein prediction models and Global Model Quality Estimation (GMQE) for each putative laccase sequence were built and calculated respectively using SWISS-MODEL tool from ExPASy Bioinformatics Resource Portal. Predicted models obtained for a putative protein laccase sequence were compared to those of similar laccases using USCF Chimera software. Finally, putative protein laccase sequences were aligned by the MUSCLE software from EMBL-EBI. Prediction models and alignments were then used to evaluate similarities between identified sequences in order to possibly identify isoenzymes.

5.2.8. DNA amplification of laccase enzymes annotated of *D. pusillus*-LMB4 genome draft

For laccase gene amplification of *D. pusillus*-LMB4 DNA, three sequences were selected, based on the structural comparison between putative laccase structural models and reported laccase enzyme structures (Sections 5.2.6 and 5.2.7.). For each reported laccase enzyme structures, the highest similar *D. pusillus*-LMB4 laccase sequence with the protein model was selected. Finally, for each putative laccase sequence selected a pair of specific primers was designed.

DNA from *D. pusillus*-LMB4 was obtained as described previously (Section 2.2.6). PCR products were displayed on agarose gel, stained with GelRed® (Biotium, USA) and viewed by UV light with 1 Kb Plus DNA ladder (Thermo-Fisher Scientific, Canada) used to determine band size. The observed gel bands were extracted, purified and sequenced by the Sanger method using primers employed to obtain the PCR products. Sequences were compared to the nucleotide database from NCBI using BLASTn, BLASTx and tBLASTx to identify homology with other laccase gene, protein and translated nucleotides, respectively.

Finally, the laccase sequences obtained from the *D. pusillus*-LMB4 genome were aligned with the genome walking sequence to evaluate coverage and homology between those putative laccase genes using the MUSCLE software from EMBL-EBI.

5.3. Results and discussions

5.3.1. Laccase gene exploration by genome walking

Agarose gel electrophoresis of PCR products revealed multiple bands between 0.4 and 2.0 kb with the first group of primers (Table 3) as obtained using forward primer Lac-pep-1A and reverse primer Lac-pep-3 (Figure 29). Once purified, these PCR product bands allowed to obtain two sequences of 866 bp and 838 bp, from which a new primer pair was designed and termed Lac-1A and Lac-2B (Table 4). Using these, 2 overlapping sequences were obtained from PCR amplification that reached an overall length of 1704 bp.

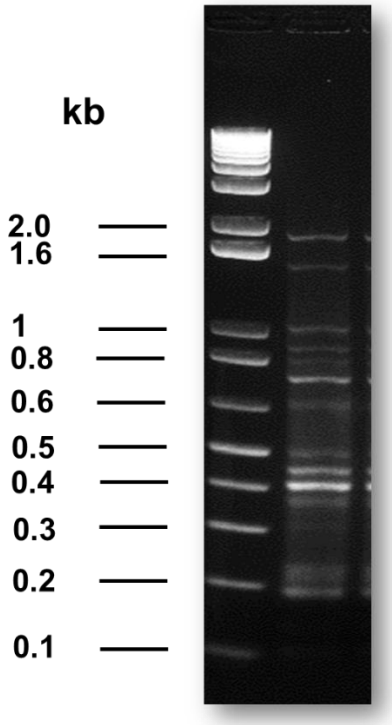


Figure 29. Degenerate PCR products analysis.
Agarose gel electrophoresis of PCR products obtained using Lac-pep-1A and Lac-pep-3B degenerate pair of primers.

The two overlapping sequences obtained were analyzed by BLASTn using the discontiguous megaBLAST algorithm appropriate for DNA sequences with dissimilarities. Comparison of the two overlapping sequences with DNA partial sequence from basidiomycete reported at NCBI highlighted similarities with the following species: *Serpula lacrymans,(Eastwood et al., 2011) Stereum hirsutum*,

Trametes versicolor, Coniophora puteana, Punctularia strigosozonata, Dichomitus squalens, Fomitiporia mediterranea, Gloeophyllum trabeum, *(Floudas et al., 2012); Schizophyllum commune, (Ohm et al., 2010); Postia placenta (Gaskell et al., 2017); Trametes hirsuta (Pavlov et al., 2015); Phanerochaete carnosa (Suzuki et al., 2012); Fibroporia radiculosa (Tang et al., 2012)* and *Coprinopsis cinerea (Stajich et al., 2010)* (Table 8). Interestingly, those fungi have been associated to wood decay and production of ligninolytic and cellulolytic enzymes (Figure 30). However, the sequences with similarities were labeled as hypothetical and/or unknow protein, pointing out the fact that genomic data regarding fungi is still insufficient.

Table 8: Basidiomycete fungi reported at NCBI found using a partial DNA sequence from *D. pusillus*-LMB4 by genome walking

Fungi	Description	Query cover	E-value	% identity	Number accession
Serpula lacrymans	hypothetical protein	19%	1e-13	89.06	XM_007312747.1
Stereum hirsutum	hypothetical protein	15%	4e-17	87.71	XM_007298619.1
Trametes versicolor	repeat-containing protein	15%	1e-10	69.05	XM_008034515.1
Coniophora puteana	hypothetical protein	8%	6e-8	78.75	XM_007764384.1
Punctularia strigosozonata	repeat-containing protein	13%	6e-8	77.65	XM_007378950.1
Dichomitus squalens	hypothetical protein	11%	7e-7	76.14	XM_007360442.1
Fomitiporia mediterranea	hypothetical protein	3%	1e-4	81.36	XM_007265358.1
Gloeophyllum trabeum	hypothetical protein	13	1e-3	79.03	XM_007862724.1
Schizophyllum commune	hypothetical protein	17%	5e-15	74%	XM_003038735.1

Postia placenta	hypothetical protein	12%	1e-10	68.6	XM_024481762.1
Trametes hirsuta	chromosome 1, complete sequence	23	5e-9	66.98	CP019371.1
Phanerochaete carnosa	hypothetical protein	12	6e-8	71.22	XM_007390510.1
Fibroporia radiculosa	Predicted protein	5	7e-7	76.14	XM_012329082.1
Coprinopsis cinérea	repeat-containing protein	10	9e-6	72.32	XM_001829009.1

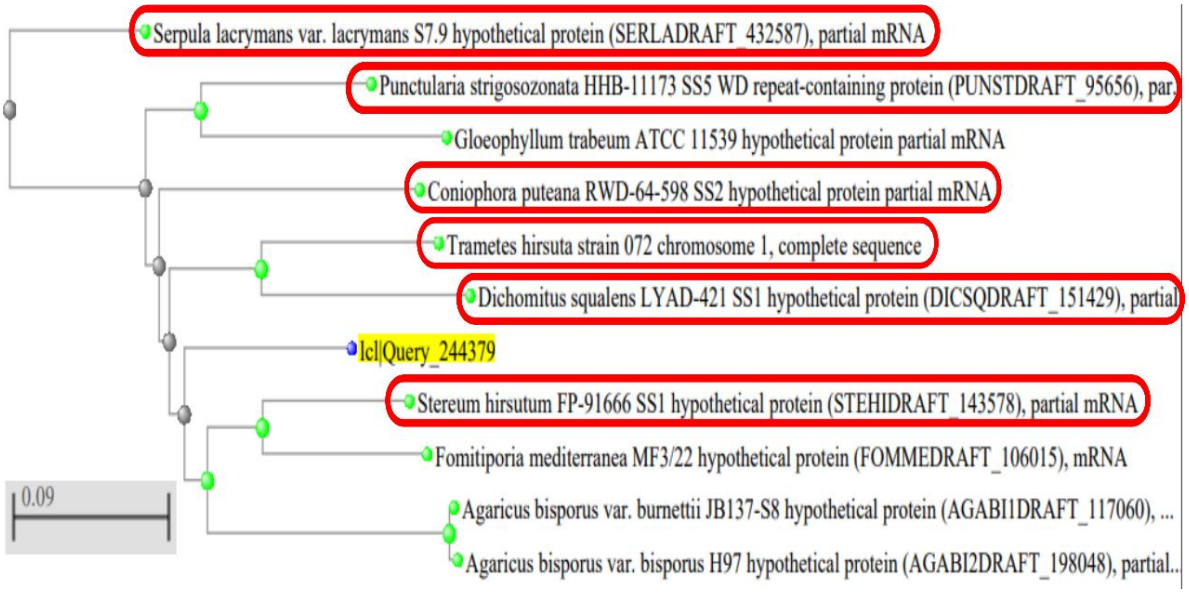


Figure 30.Preliminary taxonomic BLAST tree.

The BLAST tree was created using the two overlapping genomic DNA sequences from *D. pusillus*-LMB4 obtained from degenerate primer PCR and genomic information from ligninolytic fungi with high homology. Fungi associated to ligninolytic activity are highlighted by red squares and a putative laccase sequence is marked in yellow**.**

Upstream and downstream DNA sequences from this first genomic stretch were searched by TAIL-PCR and SP-PCR. TAIL-PCR first generated weak and multiple bands as observed through gel electrophoresis but at the end of the third cycle fewer bands were observed, one more intense than the others. This latter PCR product

was purified and sequenced using the specific primers (Table 7) used for this TAIL-PCR and a total length of 995 bp upstream was obtained, increasing the DNA sequence from *D. pusillus*-LMB4 to 2699 bp. TAIL-PCR was successful to extend the upstream DNA sequence but not on the downstream side. The AP-PCR strategy was thus used to identify sequences downstream. New specific primers (Table 9) allowed the amplification of a PCR product of approximately 3000 bp (Figure 31), (Appendix A, figure A4). These new sequence data were submitted to BLASTn analysis (Figure 32). Results showed increased association with the fungal species previously identified along with another ligninolytic fungi *Laccaria bicolor* (Martin *et al.*, 2008) but a whole laccase gene was not pointed out (Table 10).

Table 9. Specific primers based on partial sequences obtained from *D. pusillus-LMB4* genome walking.

Code	Primers 5´- 3´
Lac-Esp-1A	GAAGGGAAGGAAATGCCGGG
Lac-Esp-1B	TGGAGTAGTCTCAATGCTTG
Lac-Esp-2A	AATAGGGGCCATATATCTCA
Lac-Esp-2B	AAAATGCATTCATGTCGGGG

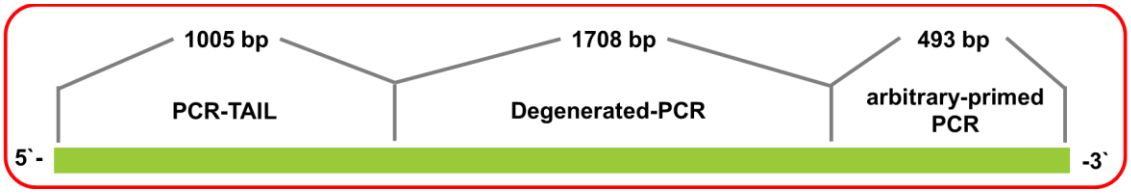


Figure 31. Scheme of genome walking results.

An intermediate sequence of 1708 base pairs were obtained using degenerate primers. This sequence was submitted to a BLASTn analysis and fungal DNA sequences with high identity were used to design degenerate primers for TAIL and arbitrary-primed PCR. 5´ and 3´ extremes of this putative gene were successfully amplified and sequenced, 1005 bp and 493 bp were added to the first sequence obtained, respectively.

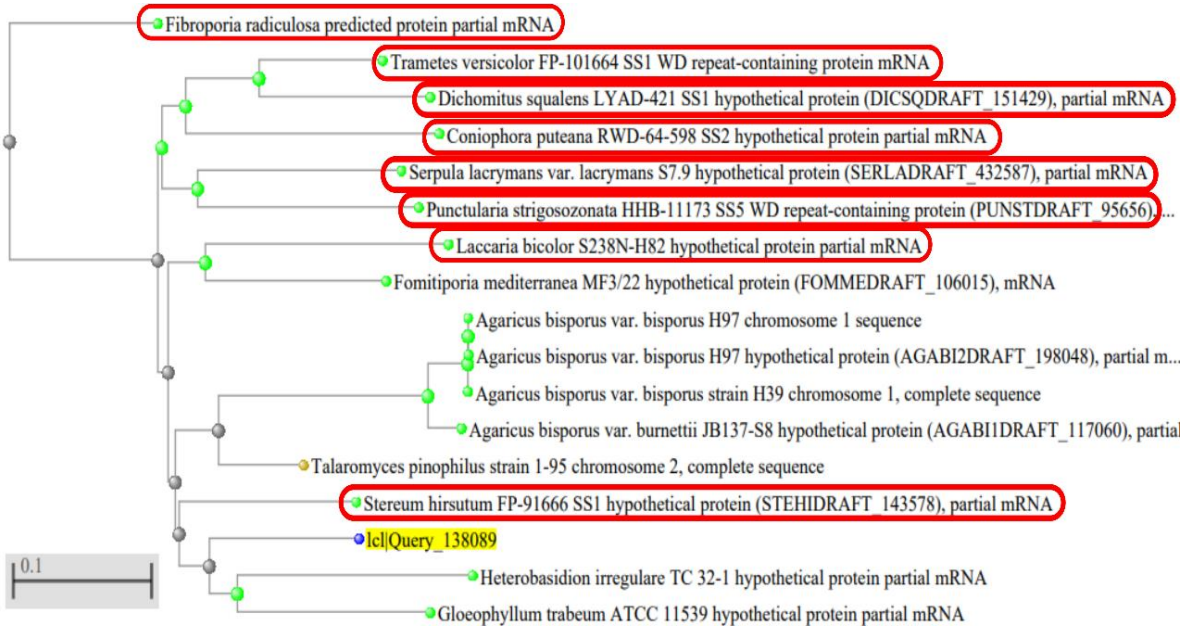


Figure 32. Taxonomic BLAST tree from whole genome walking data.

BLAST tree was created using whole *D. pusillus*-LMB4 genomic DNA sequence obtained with the genome walking approach (1708 base pairs) and genomic information from ligninolytic fungi with a high homology. Fungi associated to ligninolytic activity are highlighted by red squares and a putative laccase sequence is marked in yellow.

Table 10: Basidiomycete fungi reported at NCBI found using the final partial DNA sequence from *D. pusillus*-LMB4 by genome walking

Fungi	Description	Query cover	E-value	% identity	Number accession
Fibroporia radiculosa	Predicted protein	10%	1e-6	76.14	XM_012329082.1
Trametes versicolor	repeat-containing protein	18%	2e-10	69.05	XM_008034515.1
Dichomitus squalens	hypothetical protein	11%	1e-6	76.14	XM_007360442.1
Coniophora puteana	hypothetical protein	11%	1e-7	78.75	XM_007764384.1
Serpula lacrymans	hypothetical protein	17%	1e-12	89.06	XM_007312747.1

Punctularia strigosozonata	repeat-containing protein	15%	1e-07	76.65	XM_007378950.1
Laccaria bicolor	hypothetical protei	19%	3e-09	66.96	XM_001873926.1
Stereum hirsutum	hypothetical protein	18%	4e-16	84.71	XM_007298619.1

Ligninolytic enzyme identification from basidiomycete fungi using a walking through the genome strategy was previously successful but the fact that genomic data was available for the studied species surely increased efficiency of the method. Indeed, genomic data can help to design proper degenerate primers design as it was the case for a study regarding *Polyporus brumalis (Nakade et al., 2013).* Just as it was done for this study, previous information from basidiomycete laccases from species recognized as ligninolytic agents was used to identify new laccase enzymes through a walking genome strategy and was successful for a *Trametes trogii* isolation (Yan *et al.*, 2014). However, the walking genome strategy was not successful in our case and the lack of previous genetic data or protein sequences reported for the *Dictyopanus* genera has probably hindered the process.

Hence, walking into the genome gave a 2955 bp sequence of *D. pusillus*-LMB4 genome possibly related to ligninolytic enzymes, although a specific gene was not identified. Nevertheless, this data associates *D. pusillus*-LMB4 genome with several other ligninolytic species, information that could be used in the future for further DNA analysis of *D. pusillus*-LMB4 and for the search of lignocellulolytic enzymes from this native isolate.

5.3.2. cDNA library screening

Following the clonejet PCR Cloning kit procedure, it was possible to recover a total of 3760 colonies by heat shock transformation of *E. coli* DH5α strain while electroporation of *E. coli* Rosetta DE3 did not generate any transformed colonies. Although electroporation is more effective that the heat shock transformation, it is possible that electroporation was not successful in our case due to a low

compatibility between *E. coli* Rosetta DE3 and pJET1.2/blunt plasmid. PCR amplification of the pJET1.2/blunt vector cloned cDNA (cloneJET PCR Cloning kit) showed multiple bands between 200 and 5000 bp. These PCR products were inserted into the AKS *P. pastoris* expression vector following a Gibson reaction which yielded 921 colonies. The low quantity of colonies obtained in this last step, surely indicates that Gibson reaction optimization could be beneficial. AKS expression vectors were extracted and used for *P. pastoris* transformation, and a total of 41 colonies was obtained through this cDNA library construction procedure. *P. pastoris* colonies were recovered and inoculated in BMMY agar with ABTS, and BMMY broth, both culture media with added zeocin. Colony growth was observed in BMMY agar with ABTS and BMMY broth under methanol induction but, unfortunately, no green halo on agar plates or ABTS oxidation by culture supernatants was observed, suggesting that the *D. pusillus*-LMB4 cDNA library did not contain laccase-like genes.

For a successful cDNA library construction, important factors include: RNA extraction quality, existence of diploid genetic information which can influence the success of gene identification, previous information about the use of this expression vector, the use of a signal peptide and heterologous systems for expression of those kinds of enzymes. In our case, quality and quantity of extracted RNA could have precluded us from getting at least one laccase-like gene into the cDNA library although a gel electrophoresis analysis suggested that no RNA degradation had occurred during extraction (Figure 33). Moreover, the use of a known laccase gene as control or a specific cDNA construction kit could have improved the outcome of this technique.

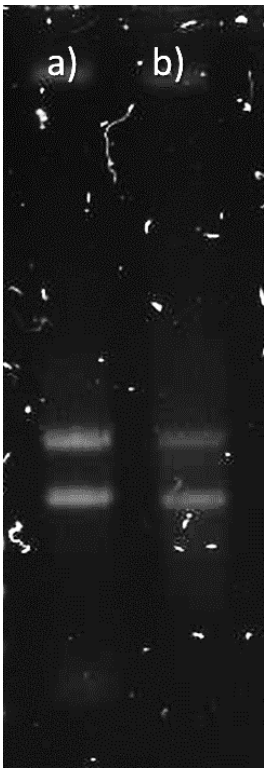


Figure 33. RNA extractions products analysis.
Agarose gel electrophoresis of RNA extraction products Two clean bands were observed which correspond to 60S and 40S rRNA subunits. A) RNA from *D. pusillus*-LMB4 b) RNA isolation from *P. pastoris*.

In fact, the number of colonies recovered from this cDNA library construction does not represent a large amount of possible proteins, especially considering that an average of 13000 proteins and average of 12 laccases were reported, through genome sequencing of *S. hirsutum*, *T. versicolor, P. strigosozonata*, and *D. squalens* species that show similar characteristics with *D. Pusillus*-LMB4 (Table 11) *(Floudas et al., 2012).* However, the percentage of positive colonies from a cDNA library screening is usually low. For instance, a *Pleurotus ostreatus* Xin831 cDNA library generated a total of 20000 *P. pastoris* transformed colonies, a quantity that covers the average number of proteins reported for basidiomycete fungi, but only 4 colonies were positive for laccase activity (Qi *et al.*, 2017). Moreover, cDNA exploration using a combination of RNA sequencing and quantitative RT-PCR resulted in the identification of only 4 potential laccases and 1 laccase isoenzyme from *Trametes hirsuta* 072 (Vasina *et al.*, 2015).

Table 11. Characteristic of basidiomycete fungi with a *D. pusillus*-LMB4 homology.

Species	Accession number	Total DNA sequence length (bp)	Number of reported proteins	Number of reported laccases
Dichomitus squalens	AEID00000000	42748430	12287	13
Punctularia strigosozonata	AEGM00000000	34171901	11540	12
Stereum hirsutum	AEGX00000000	46511623	14066	17
Trametes versicolor	AEJI00000000	44794008	14032	7

Clearly, the existence of previous genome sequencing data facilitates exploration of proteins and enzymes and it is certainly useful to identify basidiomycete fungi enzymes involved in the delignification process such as cellulases, xylanases, peroxidases and laccases. Indeed, studies on *Trametes hirsuta* 072 laccases were only able to identify a few enzymes prior to genome sequencing but this latter procedure finally brought up a complete gene family and allowed a deeper understanding of expression/transcription regulation of its ligninolytic enzymes (Moiseenko *et al.*, 2018).

In another way, the existence of several laccase enzymes in WR fungi is supported by protein evolution that those enzymes were submitted to over the years. A common ancestor for ligninolytic enzymes has been portrayed, ancestral proteins being considered as promiscuous enzymes, lacking substrate specificity. Evolution has occurred by WR fungi exposition to complex substrates consisting of lignin-based substrates of various composition, including the presence of hazardous compounds that has increased in the last centuries. In fact, this adaptative ability has been used to developed ligninolytic enzymes with improved specificity and/or efficiency towards recalcitrant compounds(Alcalde, 2015). Along with adaptation of a given enzyme to specific needs, a process that can be related to isoenzyme emergences, it is conceivable that gene duplications have occurred at given times during evolution of WR fungi in order for the microorganism to further expand its enzymatic activity repertoire (Ayuso-Fernández *et al.*, 2018).

5.3.3. *De novo* genome assembly of *Dictyopanus pusillus*-LMB4

Given the interesting ligninolytic activity of *D. pusillus*-LMB4, its DNA was sequenced by long-reads SMRT sequencing, thus allowing annotation and analysis of the encoded laccases in its genome. It was possible, after *de novo* assembly, to find a genome with a level of heterozygosity estimated at 13.53%. The reduction in homozygous genome allowed to find an assembly of 49.37 Mbp distributed in 3463 contigs (N50 = 23741 bp). A total of 16866 Coding Sequence (CDSs) (after the splicing of the 95174 annotated introns) was predicted to be encoded in the genome of *D. pusillus*. We were able to confidently annotate 13 CDSs as putative complete laccases (Table 12). Our result values are in accordance with the results reported from basidiomycete species fungi (Li *et al.*, 2018). However, it is necessary dig deeper in the new *D. pusillus*-LMB4 genome.

Table 12. Assembly of genome draft from *D. pusillus*-LMB4.

Feature	Value
Genome assembly size (Mbp)	49.37
# contigs	3463
N50	23741
GC (%)	53.08
# CDSs	16866
# introns	95174
Heterozygosity (%)	13.53

5.3.4. Ligninolytic laccase enzyme annotations of *Dictyopanus pusillus*-LMB4 genome draft

From the draft genome assembly from *D. pusillus*-LMB4 and based on previously reported laccase genes (Floudas *et al.*, 2012), it was possible to identify 13 putative laccase protein sequences encompassing 508 to 733 amino acids, a protein length expected for fungal laccases (Appendix A, figure A5). Also, two partial putative laccase sequence of less than 250 amino acids was found. All putative laccase enzymes identified had a strong structural association with the original 49 laccase sequences used as template and with another new laccase reported from the

basidiomycete fungi founded (Section 5.3.1.). Using the BLASTp tool from NCBI server, a strong association was observed (more than 45% identity) with five laccase enzymes from the following species: *Trametes trogii, Steccherinum murashkinskyi, Trametes versicolor, Trametes* Sp. Ah28-2 *and Rigidoporus lignosus*. From these homologous proteins, the 1GYC_A reported laccase from *Trametes versicolor* was the sequence most frequently associated to the identified *D. pusillus*-LMB4 putative laccases (Table 13).

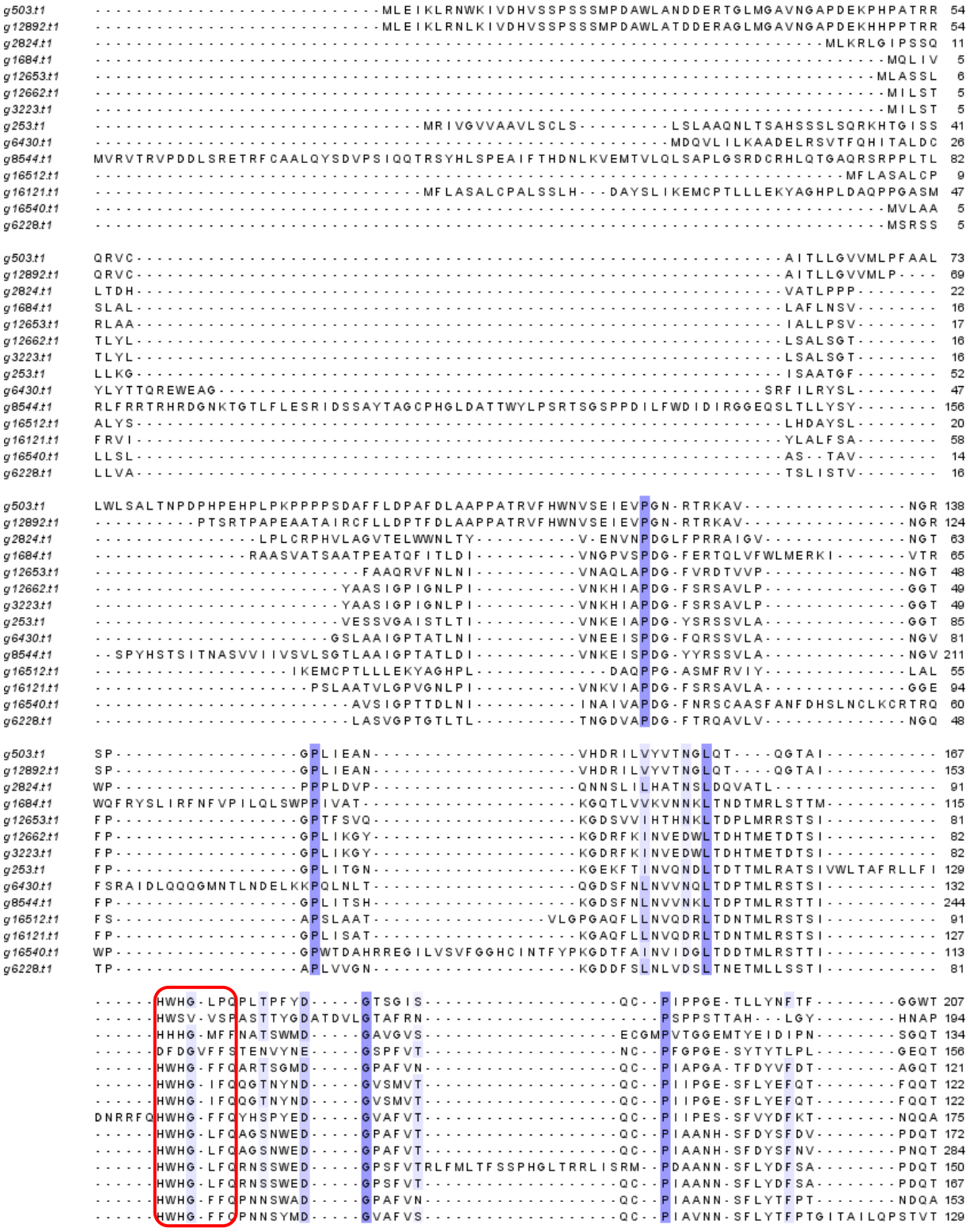
Those information lead to confirm the existence of laccase genes in the genome of *D. pusillus*-LMB4*,* associating the laccase and delignification activity described in the last chapters with those new putative sequences. Moreover, analysis of the putative laccase protein sequences indicated the presence of at least one conserved copper-binding motif in the 14 identified sequences, with the G16540.t1 sequence comprising four copper-binding motifs. Those copper-binding motifs are strongly associated with laccase enzymes and were the characteristic chosen for protein sequence alignment. Furthermore, multicopper oxidase signatures (PS00079 and PS00080) and Cu-oxidase Pfam domains (PF00394, PF07731 and PF07732) were also found in ten sequences (Table 8). Protein alignment of the 14 putative sequences showed high identity with known laccases (Table 8). Interestingly, the alignment displayed new consensus regions aside from copper-binding motifs (Figure 34). These new consensus regions could be associated with the thermotolerance and/or higher resistance to low pH conditions observed with the EE-LMB4 (Section 3.3.1), as well as with a new enzyme characteristic such as oxidation of high redox potential substrates. However, those new consensus regions have not been reported or associated yet to laccase properties found in the crude EE-LMB4. Structural studies on these *D. pusillus*-LMB4 laccases would clarify the role of these new consensus regions.

Table 13. Properties of the putative laccase protein found in the draft genome of *D. pusillus*-LMB4.

Locus tag	Length a.a.	Number of similar laccases	Multicopper oxidase signatures		Cu-oxidase Pfam domains			Copper-binding motifs				Comparison with the most homologous laccase reported				
			PS00079	PS00080	PF00394	PF07731	PF00394	Cu1	Cu2	Cu3	Cu4	Identity %	Query cover %	Protein Number accession	Organism	Reference
G253.t1	553	43	X	X	X	X	X	X	X	X		57	89	2HRG_A	*Trametes trogii*	*(Ferraroni et al., 2007; Matera et al., 2008)*
G3223.t1	535	40	X	X	X	X	X	X	X	X		57	97	5MHW_A	*Steccherinum murashkinskyi*	*(Polyakov et al., 2017)*
G5839.t1	146	40			X							65	100	1GYC_A	*Trametes versicolor*	*(Antorini et al., 2002; Piontek et al., 2002)*
G6228.t1	508	40		X	X	X	X	X		X	X	58	96	3KW7_A	*Trametes Sp. Ah28-2*	*(Ge et al., 2010)*
G6430.t1	576	40	X	X	X	X	X	X				50	90	1GYC_A	*Trametes versicolor*	*(Antorini et al., 2002; Piontek et al., 2002),*

G8544.t1	696	43	X	X	X	X	X	X	X			58	72	1GYC_A	*Trametes versicolor*	*(Antorini et al., 2002; Piontek et al., 2002)*
G12653.t1	545	43	X	X	X	X	X	X	X	X		46	99	1V10_A	*Rigidoporus lignosus*	*(Cambria et al., 2000; Garavaglia et al., 2004)*
G12662.t1	529	40	X	X	X	X	X	X	X	X		57	96	5MHW_A	*Steccherinum murashkinskyi*	*(Polyakov et al., 2017)*
G16121.t1	580	43	X	X	X	X	X	X	X	X		52	88	1GYC_A	*Trametes versicolor*	*(Antorini et al., 2002; Piontek et al., 2002)*
G16512.t1	558	40	X		X	X	X	X	X	X		58	87	1GYC_A	*Trametes versicolor*	*(Antorini et al., 2002; Piontek et al., 2002)*
G16540.t1	540	43	X	X	X	X	X	X	X	X	X	61	96	3KW7_A	*Trametes Sp. Ah28-2*	*(Ge et al., 2010)*
G503.t1	733	40			X	X	X	X				27	86	3DKH_A	*Melanocarpus albomyces*	*(Hakulinen et al., 2008)*

G1684.t1	531	40	X	X	X	X	X			X		37	93	4JHU_A	*Coriolopsis caperata*	*(Glazunova et al., 2015)*
G2824.t1	638	40	X	X	X	X	X	X	X	X		36	81	1ZPU_A	*Saccharomyces cerevisiae*	*(Taylor et al., 2005)*
G3514.t1	249	21		X								29	40	3SQR_A	*Botrytis aclada*	*(Osipov et al., 2014)*


g503.t1
g12892.t1
g2824.t1
g1684.t1
g12653.t1
g12662.t1
g3223.t1
g253.t1
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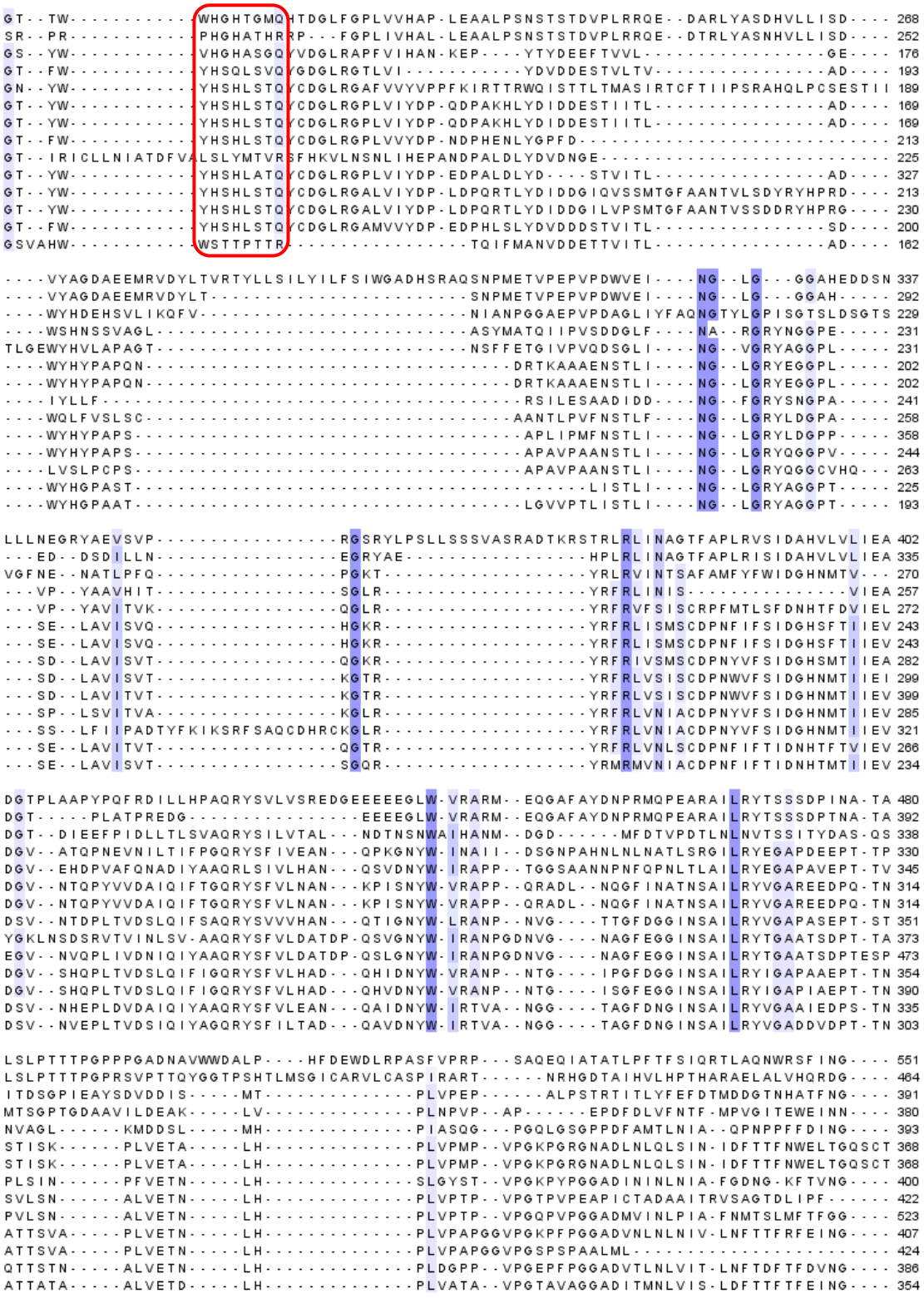
g16540.t1
g6228.t1



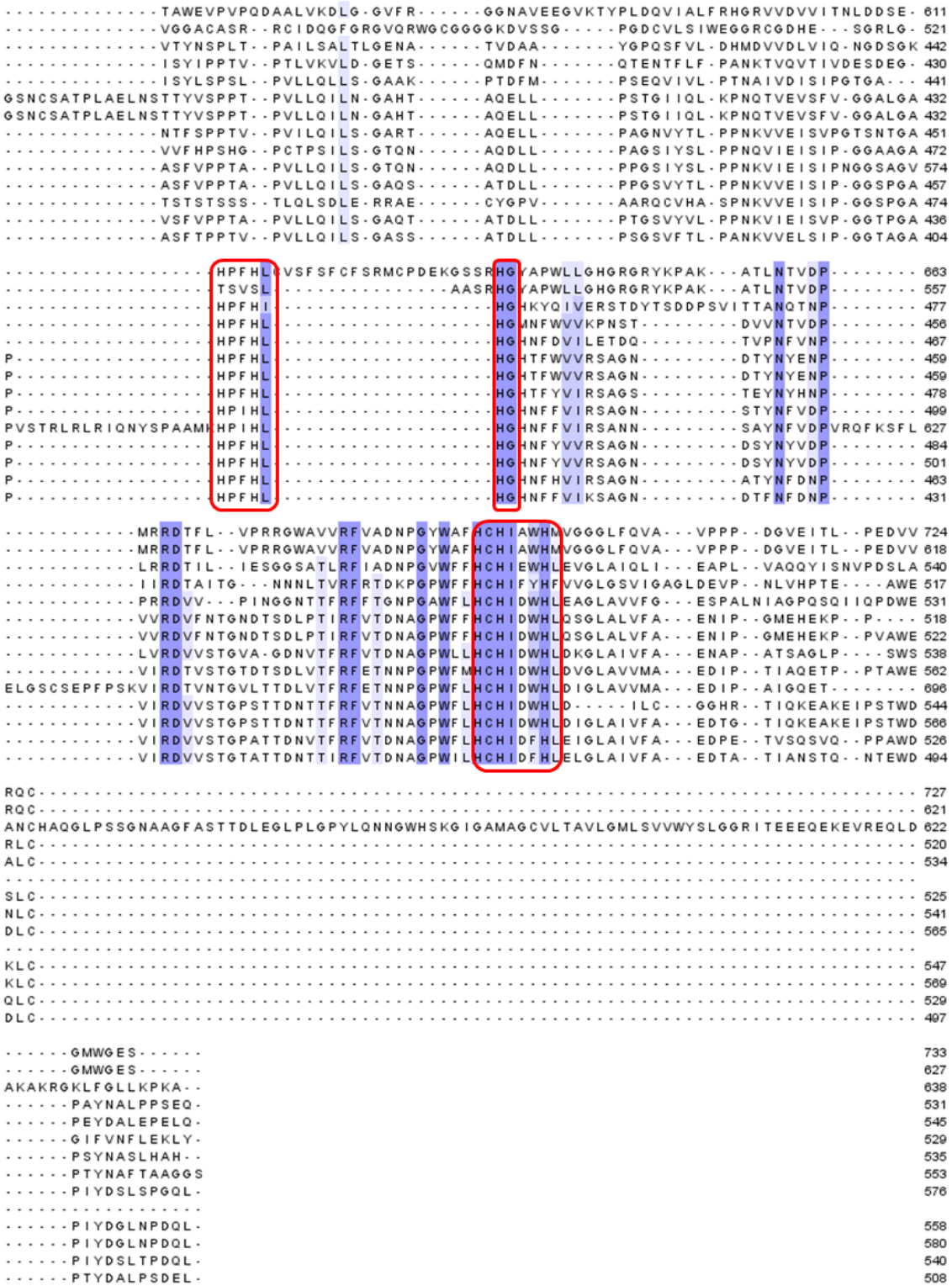
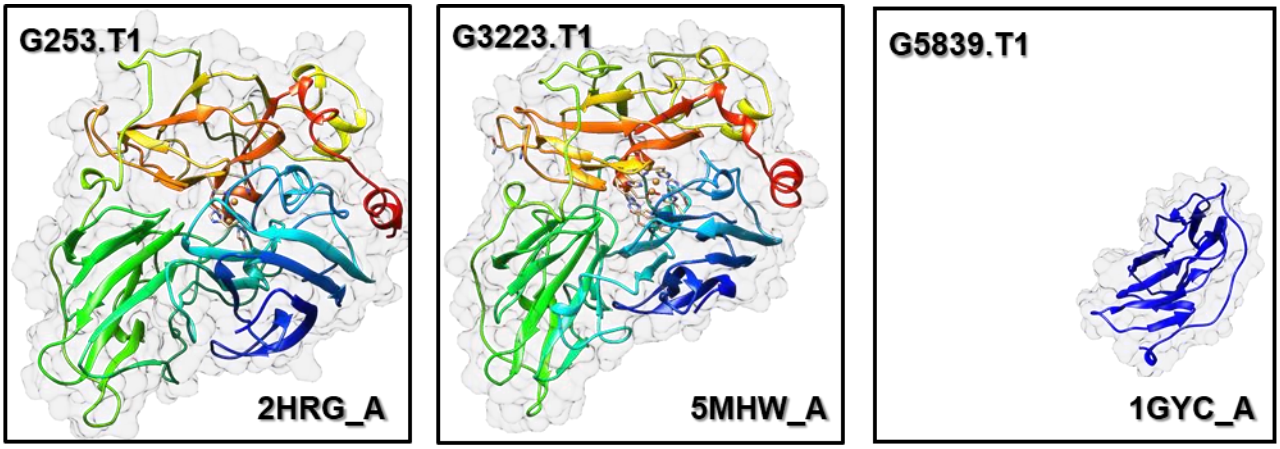


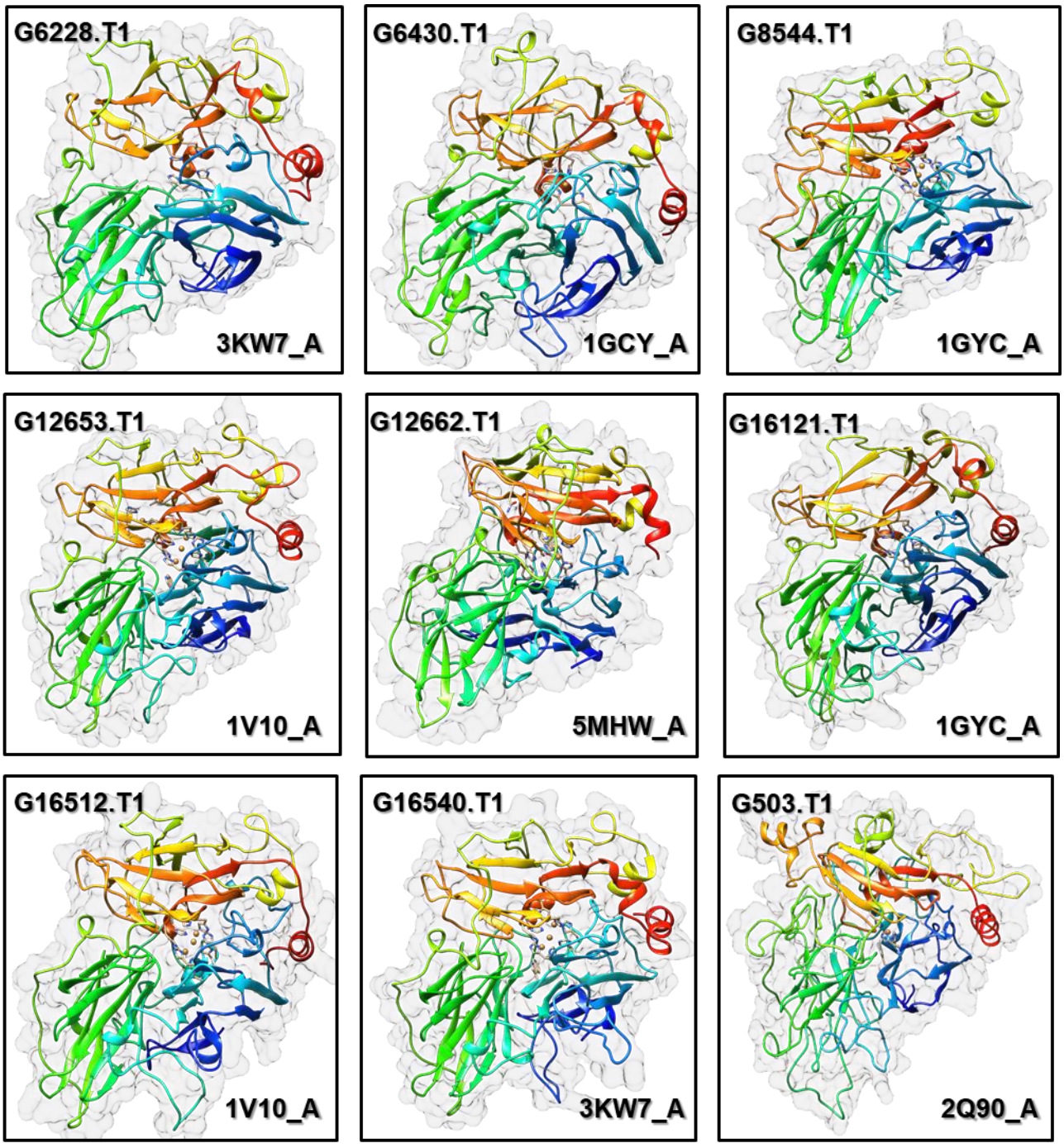
Figure 34. Alignment of putative laccase sequences from *D. pusillus*-LMB4 draft genome.

Alignment based on copper-binding motifs (shown in red boxes) displays new conserved regions highlighted in purple. Conserved copper binding motifs 1,2 and 4 are not split while the copper binding motif 3 is split into the alignment.

5.3.5. Homology modeling for putative laccases of *D. pusillus*-LMB4

Structural models of those putative laccase sequences were prepared based on PDB structures from proteins identified as having the highest identity (Table 8). Predicted structures showed high homology with reported laccase structures, including the partial sequences (Figure 35). Such similarity between established laccase structures and those predicted models emphasize the perception that predicted laccase enzymes from *D. pusillus*-LMB4 draft genome constitute possible laccase proteins. Also, these structural models suggest the existence of several laccase enzymes since putative protein models generated different protein structures. Furthermore, this reinforces the notion that more than one laccase enzyme was contained in *D. pusillus*-LMB4 enzyme isolation (section 4.2.1). GMQE scores are expressed as a number between 0 and 1 and estimate the accuracy of the tertiary structure of the resulting model. Higher GMQE values indicate higher reliability of the model as compared with crystal structures obtained from similar proteins. GMQE scores obtained from laccase model structures of *D. pusillus*-LMB4 genome draft, correspond to higher reliability and are comparable to what one would expect from experimental structures (Table 14) (Benkert *et al.*, 2011; Waterhouse *et al.*, 2018). Noteworthy, the highest GMQE values correlate with greater identity as evaluated with the BLASTp tool from NCBI server. Overall, through genome sequencing, it was possible to confirm the existence of *D. pusillus*-LMB4 encoding genes that can be responsible for the ligninolytic activity of this fungus towards EFB, resulting in production of reducing sugars that can be fermented to produce ethanol.




G6228.T1
3KW7_A
G6430.T1
1GCY_A
G8544.T1
1GYC_A
G12653.T1
1V10_A
G12662.T1
5MHW_A
G16121.T1
1GYC_A
G16512.T1
1V10_A
G16540.T1
3KW7_A
G503.T1
2Q90_A

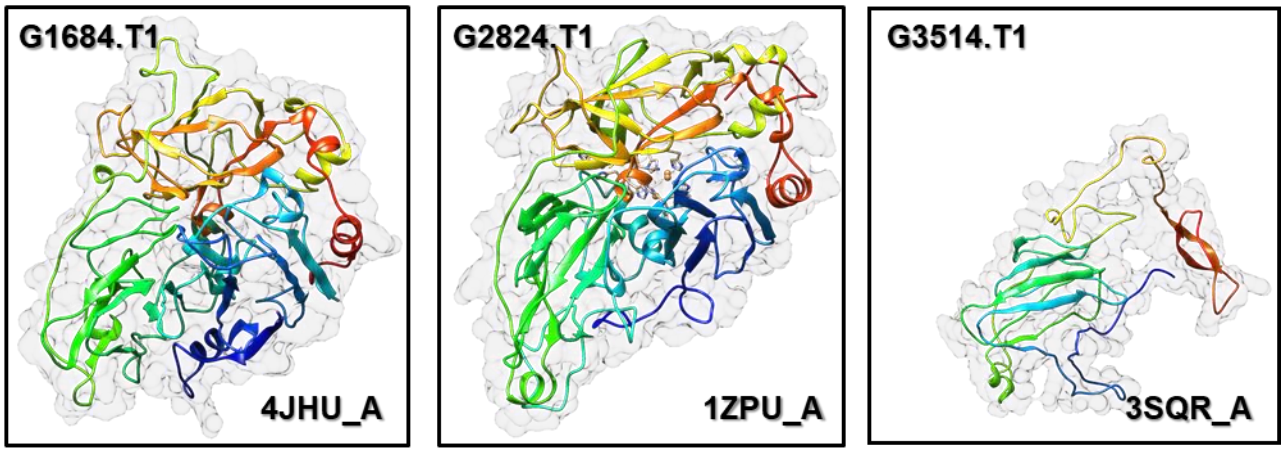


Figure 35. Predicted structures of putative laccase sequences from *D. pusillus*-LMB4 draft genome.

Laccase protein models were build using the higher identity laccase structure reported for each putative sequence. Protein accession number of structures used as models are at bottom right corner for the putative laccase sequences, annotation numbers are at the upper left corner. Structures are colored from blue to red following amino acids from N- to C-termini.

Table 14. GMQE values of predicted structures of putative laccase sequences from *D. pusillus*-LMB4 draft genome.

Locus tag	GMQE value	Protein number accession	Protein identity %
G253.t1	0.78	2HRG_A	57
G3223.t1	0.8	5MHW_A	57
G5839.t1	0.85	1GYC_A	65
G6228.t1	0.79	3KW7_A	58
G6430.t1	0.72	1GYC_A	50
G8544.t1	0.64	1GYC_A	58
G12653.t1	0.74	1V10_A	46
G12662.t1	0.79	5MHW_A	57
G16121.t1	0.71	1GYC_A	52
G16512.t1	0,72	1GYC_A	58
G16540.t1	0.79	3KW7_A	58
G503.t1	0.49	3DKH_A	61
G1684.t1	0.65	4JHU_A	27
G2824.t1	0.62	1ZPU_A	37
G3514.t1	0.46	3SQR_A	36

Comparison between putative laccase structural models by groups based on similar reported laccase enzyme structures (1GYC_A, 3KW7_A, and 5MHW_A) showed conserved structural protein core and catalytic site within each laccase structural group, as was observed for the putative laccase sequences by amino acid alignments (Figure 31). However, external loops of the putative laccase sequences display protein structural differences, reinforcing the idea of the existence of *D. pusillus*-LMB4 laccase isoenzymes (Figure 36). Nevertheless, such differences in external loop structures could also give rise to new laccase enzyme properties, including thermal stability and low pH resistance as observed with EE-LMB4 (Section 3.2.1).

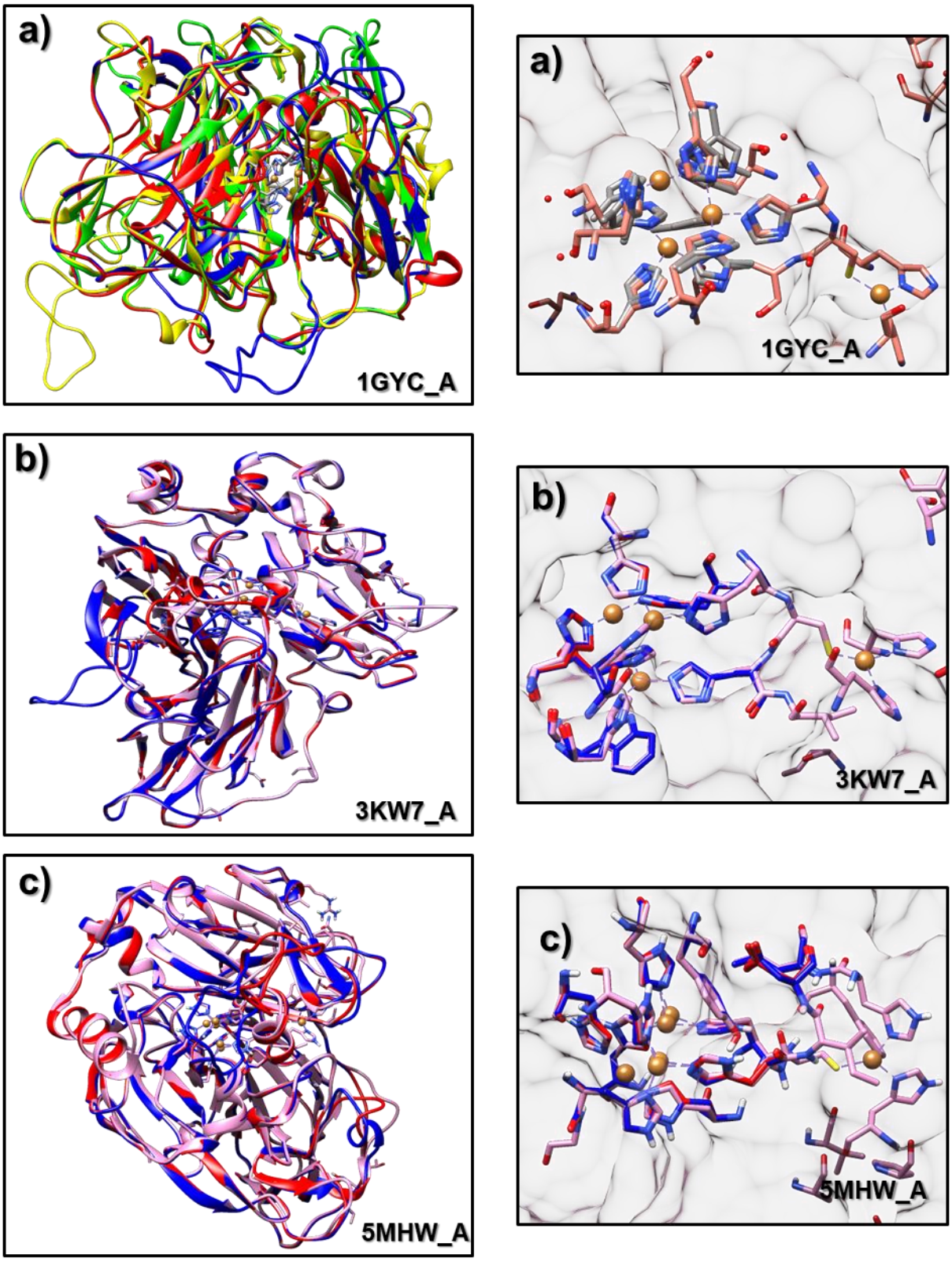


Figure 36. Structural comparison of putative laccase sequences from *D.* pusillus-LMB4 draft genome.

Left column contains complete protein structure overlays, and figures on the right side represent catalytic site comparisons. Protein accession number of structures used as models are at bottom right corner and overlapped structures are a) red G6430.t1, blue G8544.t1, yellow G16121.t1, green G16512.t1 and purple 1GYC_A (crystal structure), b) red G6228.t1, blue G16540.t1, and purple 3KW7_A (crystal structure), c) red G3223.t1, blue G12662.t1 and purple 5MHW_A (crystal structure). Copper atoms are depicted as small brown spheres.

The identification of putative laccase genes will help the incorporation of ligninolytic enzymes in the pretreatment process for lignocellulosic ethanol production. First, it could favor overexpression of ligninolytic enzymes in heterologous systems for their production at industrial scale, in order to reduce time and cost production (Antošová & Sychrová, 2016). Furthermore, putative genes or protein sequences could be engineered to improve native properties by processes such as protein enzyme evolution, so that specific enzymes can be used in defined tasks, for instance the oxidation of lignin (Plácido & Capareda, 2015).

Production of improved ligninolytic enzymes at industrial scale is most certainly the first step for a successful integration of enzymes in the pretreatment process for cellulosic ethanol production. Indeed, enzymes for lignocellulose pretreatment are the biotechnological tool that will lead second-generation bioethanol production into green chemistry processes, while helping to reduce the accumulation of lignocellulose cellulosic residues from agroindustry and reducing the use of pollutants such as acid and alkali solutions currently employed for the pretreatment process in cellulosic ethanol production.

5.3.6. DNA amplification of laccase enzymes annotated from of *Dictyopanus pusillus*-LMB4 genome draft

Pairs of specific primers were designed from the putative laccase codifying DNA sequences G12653.t1, G12662.t1 and G16540.t1, those sequences corresponding to the 1GYC_A, 3KW7_A, and 5MHW_A enzyme structure models, respectively (Table 15).

Table 15. Specific primers based on putative laccase genes from *D. pusillus*-LMB4 genome walking.

Code	Primers 5´- 3´	Expected length bp
G12653-F	ATGCTGGCCTCCTCTCTTCG	1.635
G12653-R	TCACTGAAGCTCGGGCTCGA	
G12662-F	ATGATTCTCTCGACCACTCT	1.587
G12662-R	TCAGTAGAGTTTCTCAAGGA	
G16540-F	ATGGTTTTAGCTGCTCTCCT	1.620
G16540-R	TTAAAGCTGATCCGGGGTCA	

Agarose gel electrophoresis of PCR products revealed bands for all the pairs of primers (Figure 37). Several bands were obtained from the G16540.t1 and G12653.t1 primer pairs, while a single band around 3.0 kb was obtained for G12662.t1 pair of primers. A similar band is also observed between 2.0 and 3.0 kb for the other two sequences but with less intensity. Now, a band between 2.0 and 3.0 kb overcomes the expected length based on the putative laccase protein sequences, but the extra length could be related to the presence of intron sequences into the putative laccase genes. The presence of intron sequences is common in basidiomycete laccase genes. Ligninolytic species such as *Ceriporiopsis subvermispora*, *Hypsizygus marmoreus* and *Trametes versicolor,* among others, have showed an average of 11 intron sequences for laccase enzymes with around 450 amino acids corresponding approximatively to gDNA sequences of 2.5 kb (Jönsson *et al.*, 1995; Karahanian *et al.*, 1998; Zhang *et al.*, 2015). For those reasons, it is highly probable that PCR products generated by the specific primers designed from the putative laccase sequences, could be true DNA laccase sequences from *D. pusillus*-LMB4. Future analyses of the PCR product sequences and identification of introns and exons will be necessary to identify the codifying sequence for putative laccase genes from *D. pusillus*-LMB4.

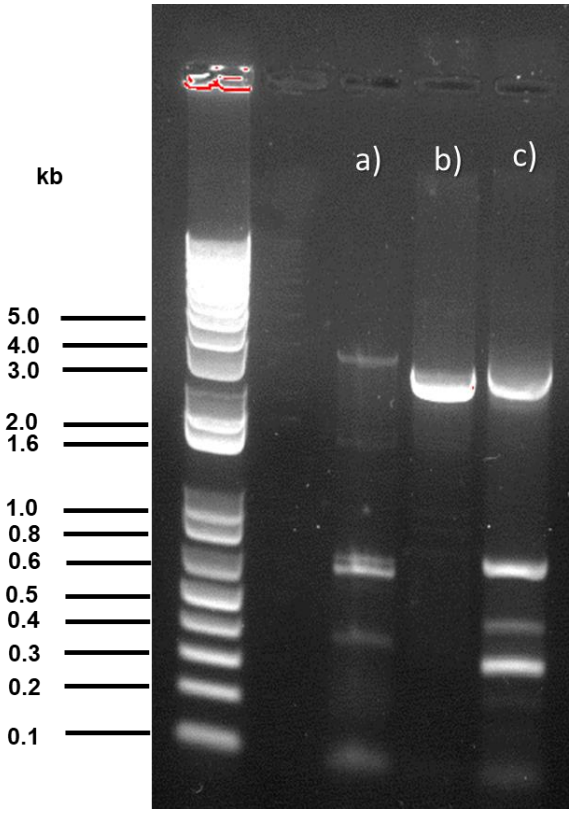


Figure 37. PCR product analysis of putative laccase genes from *D. pusillus*-LMB4 genome draft.

Agarose gel electrophoresis of PCR products obtained using specific primers designed on putative laccase sequences selected from *D. pusillus*-LMB4 A) G12653.t1, b) G12662.t1 and c) G16540.t1.

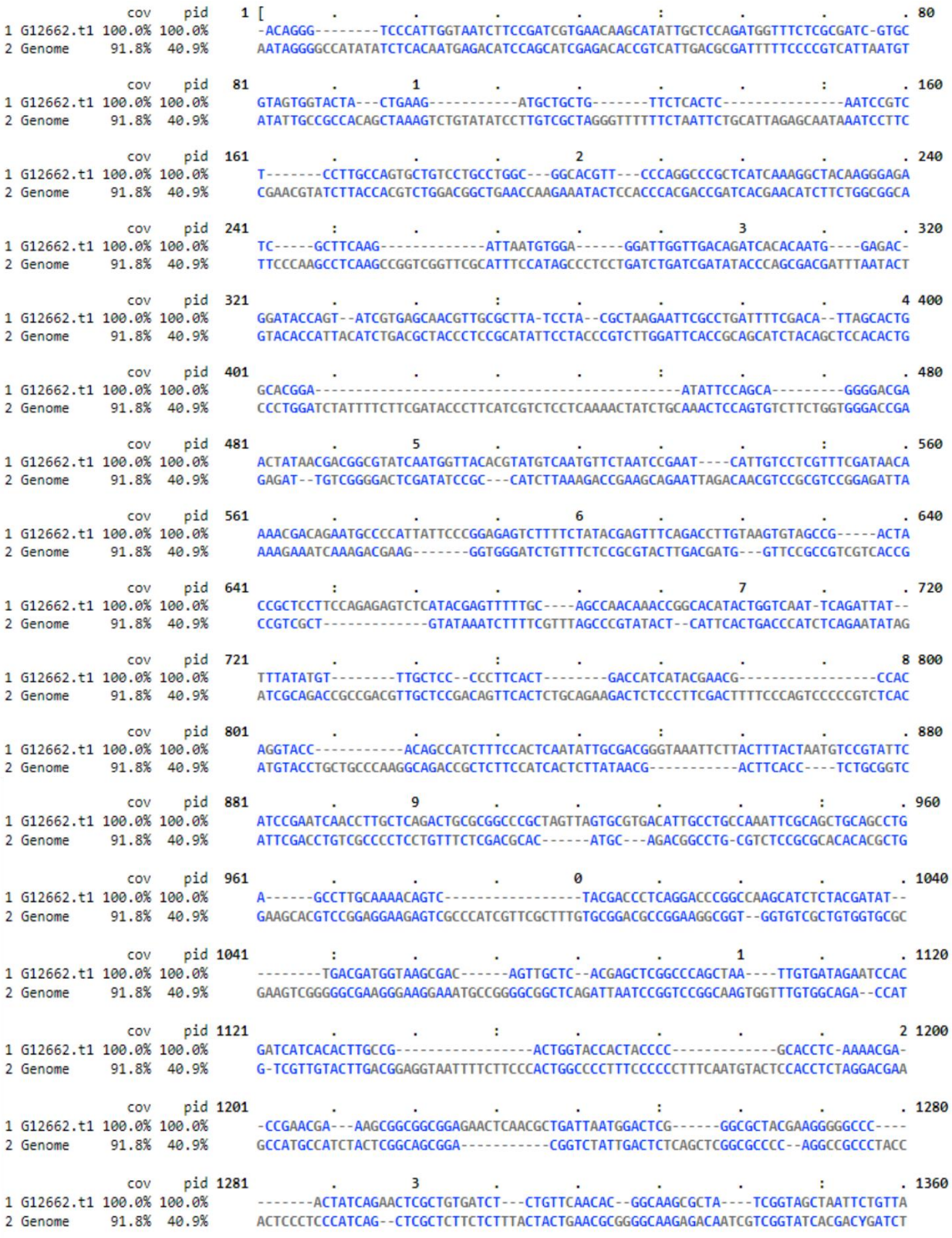
From purified PCR products, it was possible to obtain a sequence of 2415 bp using the pair of primers from the putative sequence G12662.t1. This new sequence corresponds to the fragment length observed on the agarose electrophoresis gel. Also, BLAST analysis results using this new sequence showed several similar genomic nucleotide sequences, along with translated laccase protein sequences from ligninolytic basidiomycete fungi (Table 16). Nucleotide sequence comparison showed that fungi with higher identity were *Trametes hirsuta* (Pavlov *et al.,* 2015) and *Ceriporiopsis rivulosa* (Karahanian *et al.,* 1998) with 77.27% and 85.71% of identity, respectively. Upon analysis of translated protein sequences, two reported laccase structures from *Lentinus tigrinus* (Ferraroni *et al.*, 2007) and *Steccherinum murashkinskyi (Polyakov et al., 2017),* and two protein sequences from *Peniophora lycii (Moiseenko et al., 2016)* and *Peniophora sp.* (Otero *et al.*, 2017), showed highest BLAST alignment similarities with this putative

G12662.t1 protein obtained from the nucleotide sequence. In fact, protein alignments for this putative G12662.t1 sequence and the four identified laccases from ligninolytic basidiomycete fungi showed E-values near to zero (Table 16). Such E-values indicate that matches have low background noise, especially for short length sequences (Altschul *et al.*, 2005).

Table 16: **BLAST analysis results based on the nucleotide sequence from *D. pusillus*-LMB4 obtained by PCR amplification following genome draft data.**

Accession number	Protein	Organisms	Query coverty %	Identity %	E-value
blastn (nucleotides to nucleotides)					
CP019371.1	- -	*Trametes hirsuta*	5	77.27	6e-06
JQ027727.1	- -	*Ceriporiopsis rivulosa*	5	85.71	6e-06
blastx (translated nucleotides to proteins)					
2QT6_A	Laccase	*Lentinus tigrinus*	78	36.60	1e-75
5MEW_A	Laccase 2	*Steccherinum murashkinskyi*	79	33.50	4e-73
tblastx (translated nucleotides to translated nucleotides)					
MG550091	Laccase B	*Peniophora lycii*	51	- -	1e-101
MF176137	Laccase 2	*Peniophora sp.*	53	- -	1e-103

Moreover, alignment between nucleotide sequences from genome walking and laccase G12662.t1 from the *D. pusillus*-LMB4 gives 91.8% and 43.7% of cover and homology, respectively (Figure 38). Overall, these similarities with known proteins and/or genes support the existence of a *D. pusillus*-LMB4 laccase enzyme, and most probably isoenzymes, that are responsible of the delignification capacity demonstrated with enzymatic extracts in chapter 3. The existence of laccase isoenzymes has been demonstrated in ligninolytic basidiomycetes such as *Pleurorus nebrodensis* (Yuan *et al.*, 2016), *Cyathus bulleri* (Vats & Mishra, 2018), *Trametes versicolor* (Martínez-Morales *et al.*, 2015), among others. Furthermore, the homology between those sequences uphold the results from laccase identification by FPLC and mass spectroscopy obtained in chapter 4.


cov pid 1 [. . . . : . . . 80
1 G12662.t1 100.0% 100.0% -ACAGGG--------TCCCATTGGTAATCTTCCGATCGTGAACAAGCATATTGCTCCAGATGGTTTCTCGCGATC-GTGC
2 Genome 91.8% 40.9% AATAGGGGCCATATATCTCACAATGAGACATCCAGCATCGAGACACCGTCATTGACGCGATTTTTCCCCGTCATTAATGT
cov pid 81 . 1 . . . . : . 160
1 G12662.t1 100.0% 100.0% GTAGTGGTACTA---CTGAAG-----------ATGCTGCTG-------TTCTCACTC---------------AATCCGTC
2 Genome 91.8% 40.9% ATATTGCCGCCACAGCTAAAGTCTGTATATCCTTGTCGCTAGGGTTTTTTCTAATTCTGCATTAGAGCAATAAATCCTTC
cov pid 161 . . . 2 240
1 G12662.t1 100.0% 100.0% T-------CCTTGCCAGTGCTGTCCTGCCTGGC---GGCACGTT---CCCAGGCCCGCTCATCAAAGGCTACAAGGGAGA
2 Genome 91.8% 40.9% CGAACGTATCTTACCACGTCTGGACGGCTGAACCAAGAAATACTCCACCCACGACCGATCACGAACATCTTCTGGCGGCA
cov pid 241 : . . . . 3 . . 320
1 G12662.t1 100.0% 100.0% TC-----GCTTCAAG-------------ATTAATGTGGA------GGATTGGTTGACAGATCACACAATG----GAGAC-
2 Genome 91.8% 40.9% TTCCCAAGCCTCAAGCCGGTCGGTTCGCATTTCCATAGCCCTCCTGATCTGATCGATATACCCAGCGACGATTTAATACT
cov pid 321 . . : 4 400
1 G12662.t1 100.0% 100.0% GGATACCAGT--ATCGTGAGCAACGTTGCGCTTA-TCCTA--CGCTAAGAATTCGCCTGATTTTCGACA--TTAGCACTG
2 Genome 91.8% 40.9% GTACACCATTACATCTGACGCTACCCTCCGCATATTCCTACCCGTCTTGGATTCACCGCAGCATCTACAGCTCCACACTG
cov pid 401 . . . . : . . . 480
1 G12662.t1 100.0% 100.0% GCACGGA----------ATATTCCAGCA---------GGGGACGA
2 Genome 91.8% 40.9% CCCTGGATCTATTTTCTTCGATACCCTTCATCGTCTCCTCAAAACTATCTGCAAACTCCAGTGTCTTCTGGTGGGACCGA
cov pid 481 . 5 . . . . : . 560
1 G12662.t1 100.0% 100.0% ACTATAACGACGGCGTATCAATGGTTACACGTATGTCAATGTTCTAATCCGAAT----CATTGTCCTCGTTTCGATAACA
2 Genome 91.8% 40.9% GAGAT--TGTCGGGGACTCGATATCCGC---CATCTTAAAGACCGAAGCAGAATTAGACAACGTCCGCGTCCGGAGATTA
cov pid 561 . . . 6 . . . 640
1 G12662.t1 100.0% 100.0% AAACGACAGAATGCCCCATTATTCCCGGAGAGTCTTTTCTATACGAGTTTCAGACCTTGTAAGTGTAGCCG-----ACTA
2 Genome 91.8% 40.9% AAAGAAATCAAAGACGAAG-------GGTGGGATCTGTTTCTCCGCGTACTTGACGATG---GTTCCGCCGTCGTCACCG
cov pid 641 : . . . . 7 . . 720
1 G12662.t1 100.0% 100.0% CCGCTCCTTCCAGAGAGTCTCATACGAGTTTTTGC----AGCCAACAAACCGGCACATACTGGTCAAT-TCAGATTAT--
2 Genome 91.8% 40.9% CCGTCGCT-------------GTATAAATCTTTTCGTTTAGCCCGTATACT--CATTCACTGACCCATCTCAGAATATAG
cov pid 721 . . : 8 800
1 G12662.t1 100.0% 100.0% TTTATATGT--------TTGCTCC--CCCTTCACT--------GACCATCATACGAACG-----------------CCAC
2 Genome 91.8% 40.9% ATCGCAGACCGCCGACGTTGCTCCGACAGTTCACTCTGCAGAAGACTCTCCCTTCGACTTTTCCCAGTCCCCCGTCTCAC
cov pid 801 . . . . : . . . 880
1 G12662.t1 100.0% 100.0% AGGTACC-----------ACAGCCATCTTTCCACTCAATATTGCGACGGGTAAATTCTTACTTTACTAATGTCCGTATTC
2 Genome 91.8% 40.9% ATGTACCTGCTGCCCAAGGCAGACCGCTCTTCCATCACTCTTATAACG-----------ACTTCACC----TCTGCGGTC
cov pid 881 . 9 . . . . : . 960
1 G12662.t1 100.0% 100.0% ATCCGAATCAACCTTGCTCAGACTGCGCGGCCCGCTAGTTAGTGCGTGACATTGCCTGCCAAATTCGCAGCTGCAGCCTG
2 Genome 91.8% 40.9% ATTCGACCTGTCGCCCCTCCTGTTTCTCGACGCAC------ATGC---AGACGGCCTG-CGTCTCCGCGCACACACGCTG
cov pid 961 . . . 0 . . . 1040
1 G12662.t1 100.0% 100.0% A------GCCTTGCAAAACAGTC-----------------TACGACCCTCAGGACCCGGCCAAGCATCTCTACGATAT--
2 Genome 91.8% 40.9% GAAGCACGTCCGGAGGAAGAGTCGCCCATCGTTCGCTTTGTGCGGACGCCGGAAGGCGGT--GGTGTCGCTGTGGTGCGC
cov pid 1041 : . . . . 1 . . 1120
1 G12662.t1 100.0% 100.0% --------TGACGATGGTAAGCGAC------AGTTGCTC--ACGAGCTCGGCCCAGCTAA----TTGTGATAGAATCCAC
2 Genome 91.8% 40.9% GAAGTCGGGGGCGAAGGGAAGGAAATGCCGGGGCGGCTCAGATTAATCCGGTCCGGCAAGTGGTTTGTGGCAGA--CCAT
cov pid 1121 . . : 2 1200
1 G12662.t1 100.0% 100.0% GATCATCACACTTGCCG-----------------ACTGGTACCACTACCCC-------------GCACCTC-AAAACGA-
2 Genome 91.8% 40.9% G-TCGTTGTACTTGACGGAGGTAATTTTCTTCCCACTGGCCCCTTTCCCCCCTTTCAATGTACTCCACCTCTAGGACGAA
cov pid 1201 . . . . : . . . 1280
1 G12662.t1 100.0% 100.0% -CCGAACGA---AAGCGGCGGCGGAGAACTCAACGCTGATTAATGGACTCG------GGCGCTACGAAGGGGGCCC----
2 Genome 91.8% 40.9% GCCATGCCATCTACTCGGCAGCGGA-----------CGGTCTATTGACTCTCAGCTCGGCGCCCC--AGGCCGCCCTACC
cov pid 1281 . 3 . . . . : . 1360
1 G12662.t1 100.0% 100.0% -------ACTATCAGAACTCGCTGTGATCT---CTGTTCAACAC--GGCAAGCGCTA----TCGGTAGCTAATTCTGTTA
2 Genome 91.8% 40.9% ACTCCCTCCCATCAG--CTCGCTCTTCTCTTTACTACTGAACGCGGGGCAAGAGACAATCGTCGGTATCACGACYGATCT

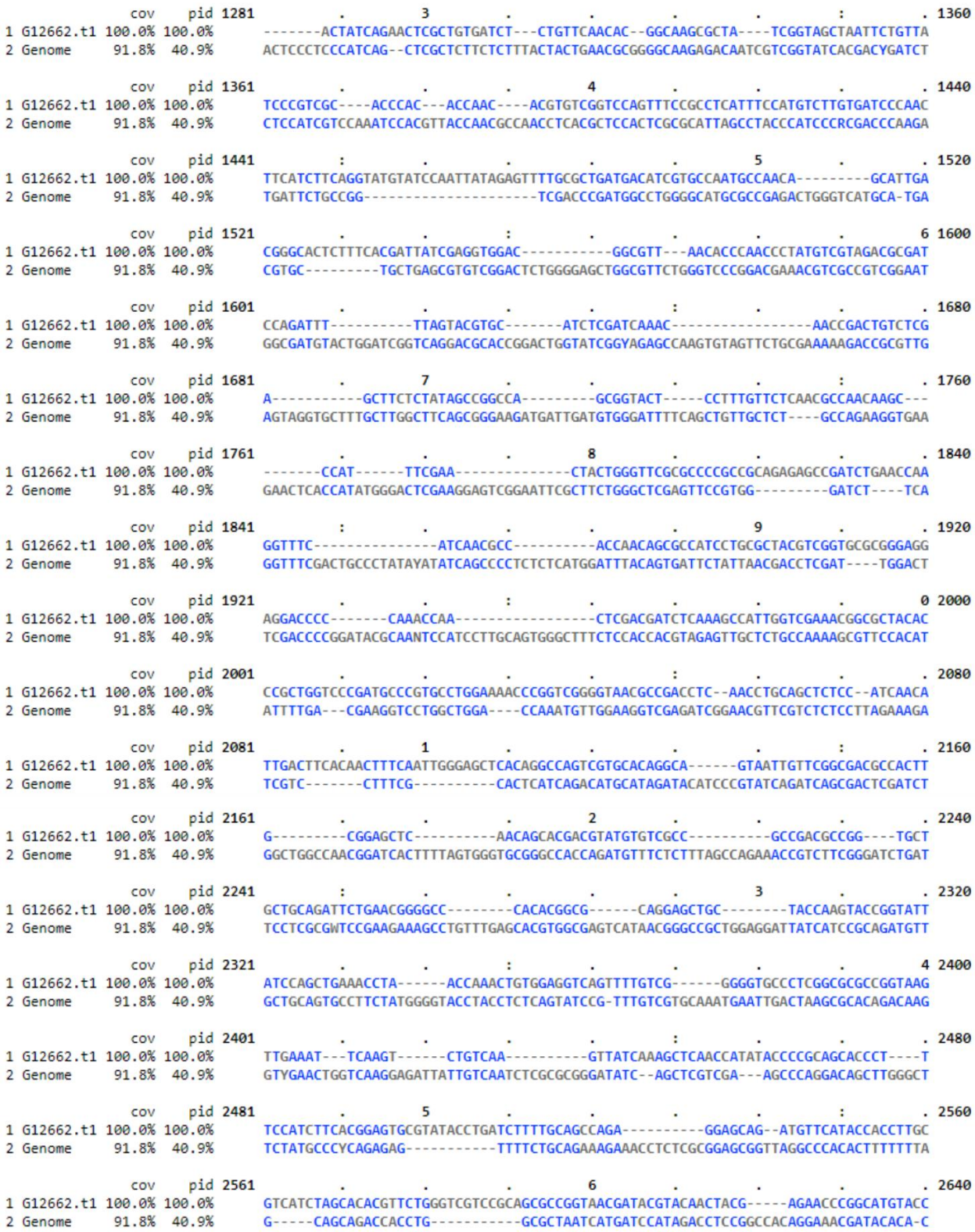

cov pid 1281 . 3 . . . . : . 1360
1 G12662.t1 100.0% 100.0% -------ACTATCAGAACTCGCTGTGATCT---CTGTTCAACAC--GGCAAGCGCTA----TCGGTAGCTAATTCTGTTA
2 Genome 91.8% 40.9% ACTCCCTCCCATCAG--CTCGCTCTTCTCTTTACTACTGAACGCGGGGCAAGAGACAATCGTCGGTATCACGACYGATCT
cov pid 1361 . . . 4 1440
1 G12662.t1 100.0% 100.0% TCCCGTCGC----ACCCAC---ACCAAC----ACGTGTCGGTCCAGTTTCCGCCTCATTTCCATGTCTTGTGATCCCAAC
2 Genome 91.8% 40.9% CTCCATCGTCCAAATCCACGTTACCAACGCCAACCTCACGCTCCACTCGCGCATTAGCCTACCCATCCCRCGACCCAAGA
cov pid 1441 : . . . . 5 . . 1520
1 G12662.t1 100.0% 100.0% TTCATCTTCAGGTATGTATCCAATTATAGAGTTTTGCGCTGATGACATCGTGCCAATGCCAACA---------GCATTGA
2 Genome 91.8% 40.9% TGATTCTGCCGG----------TCGACCCGATGGCCTGGGGCATGCGCCGAGACTGGGTCATGCA-TGA
cov pid 1521 . . : 6 1600
1 G12662.t1 100.0% 100.0% CGGGCACTCTTTCACGATTATCGAGGTGGAC----------GGCGTT---AACACCCAACCCTATGTCGTAGACGCGAT
2 Genome 91.8% 40.9% CGTGC---------TGCTGAGCGTGTCGGACTCTGGGGAGCTGGCGTTCTGGGTCCCGGACGAAACGTCGCCGTCGGAAT
cov pid 1601 . . . . : . . . 1680
1 G12662.t1 100.0% 100.0% CCAGATTT----------TTAGTACGTGC-------ATCTCGATCAAAC----------AACCGACTGTCTCG
2 Genome 91.8% 40.9% GGCGATGTACTGGATCGGTCAGGACGCACCGGACTGGTATCGGYAGAGCCAAGTGTAGTTCTGCGAAAAAGACCGCGTTG
cov pid 1681 . 7 . . . . : . 1760
1 G12662.t1 100.0% 100.0% A----------GCTTCTCTATAGCCGGCCA---------GCGGTACT-----CCTTTGTTCTCAACGCCAACAAGC---
2 Genome 91.8% 40.9% AGTAGGTGCTTTGCTTGGCTTCAGCGGGAAGATGATTGATGTGGGATTTTCAGCTGTTGCTCT----GCCAGAAGGTGAA
cov pid 1761 . . . 8 1840
1 G12662.t1 100.0% 100.0% -------CCAT------TTCGAA----------CTACTGGGTTCGCGCCCCGCCGCAGAGAGCCGATCTGAACCAA
2 Genome 91.8% 40.9% GAACTCACCATATGGGACTCGAAGGAGTCGGAATTCGCTTCTGGGCTCGAGTTCCGTGG---------GATCT----TCA
cov pid 1841 : . . . . 9 . . 1920
1 G12662.t1 100.0% 100.0% GGTTTC----------ATCAACGCC----------ACCAACAGCGCCATCCTGCGCTACGTCGGTGCGCGGGAGG
2 Genome 91.8% 40.9% GGTTTCGACTGCCCTATAYATATCAGCCCCTCTCTCATGGATTTACAGTGATTCTATTAACGACCTCGAT----TGGACT
cov pid 1921 . . : 0 2000
1 G12662.t1 100.0% 100.0% AGGACCCC-------CAAACCAA----------CTCGACGATCTCAAAGCCATTGGTCGAAACGGCGCTACAC
2 Genome 91.8% 40.9% TCGACCCCGGATACGCAANTCCATCCTTGCAGTGGGCTTTCTCCACCACGTAGAGTTGCTCTGCCAAAAGCGTTCCACAT
cov pid 2001 . . . . : . . . 2080
1 G12662.t1 100.0% 100.0% CCGCTGGTCCCGATGCCCGTGCCTGGAAAACCCGGTCGGGGTAACGCCGACCTC--AACCTGCAGCTCTCC--ATCAACA
2 Genome 91.8% 40.9% ATTTTGA---CGAAGGTCCTGGCTGGA----CCAAATGTTGGAAGGTCGAGATCGGAACGTTCGTCTCTCCTTAGAAAGA
cov pid 2081 . 1 . . . . : . 2160
1 G12662.t1 100.0% 100.0% TTGACTTCACAACTTTCAATTGGGAGCTCACAGGCCAGTCGTGCACAGGCA------GTAATTGTTCGGCGACGCCACTT
2 Genome 91.8% 40.9% TCGTC-------CTTTCG----------CACTCATCAGACATGCATAGATACATCCCGTATCAGATCAGCGACTCGATCT
cov pid 2161 . . . 2 2240
1 G12662.t1 100.0% 100.0% G---------CGGAGCTC----------AACAGCACGACGTATGTGTCGCC----------GCCGACGCCGG----TGCT
2 Genome 91.8% 40.9% GGCTGGCCAACGGATCACTTTTAGTGGGTGCGGGCCACCAGATGTTTCTCTTTAGCCAGAAACCGTCTTCGGGATCTGAT
cov pid 2241 : . . . . 3 . . 2320
1 G12662.t1 100.0% 100.0% GCTGCAGATTCTGAACGGGGCC--------CACACGGCG------CAGGAGCTGC--------TACCAAGTACCGGTATT
2 Genome 91.8% 40.9% TCCTCGCGWTCCGAAGAAAGCCTGTTTGAGCACGTGGCGAGTCATAACGGGCCGCTGGAGGATTATCATCCGCAGATGTT
cov pid 2321 . . : 4 2400
1 G12662.t1 100.0% 100.0% ATCCAGCTGAAACCTA------ACCAAACTGTGGAGGTCAGTTTTGTCG------GGGGTGCCCTCGGCGCGCCGGTAAG
2 Genome 91.8% 40.9% GCTGCAGTGCCTTCTATGGGGTACCTACCTCTCAGTATCCG-TTTGTCGTGCAAATGAATTGACTAAGCGCACAGACAAG
cov pid 2401 . . . . : . . . 2480
1 G12662.t1 100.0% 100.0% TTGAAAT---TCAAGT------CTGTCAA----------GTTATCAAAGCTCAACCATATACCCCGCAGCACCCT----T
2 Genome 91.8% 40.9% GTYGAACTGGTCAAGGAGATTATTGTCAATCTCGCGCGGGATATC--AGCTCGTCGA---AGCCCAGGACAGCTTGGGCT
cov pid 2481 . 5 . . . . : . 2560
1 G12662.t1 100.0% 100.0% TCCATCTTCACGGAGTGCGTATACCTGATCTTTTGCAGCCAGA----------GGAGCAG--ATGTTCATACCACCTTGC
2 Genome 91.8% 40.9% TCTATGCCCYCAGAGAG----------TTTTCTGCAGAAAGAAACCTCTCGCGGAGCGGTTAGGCCCACACTTTTTTTA
cov pid 2561 . . . 6 2640
1 G12662.t1 100.0% 100.0% GTCATCTAGCACACGTTCTGGGTCGTCCGCAGCGCCGGTAACGATACGTACAACTACG-----AGAACCCGGCATGTACC
2 Genome 91.8% 40.9% G-----CAGCAGACCACCTG----------GCGCTAATCATGATCCATAGACCTCCGGCCACAGGAAACGATACACA-C

Figure 38: Alignment of putative laccase sequence from *D. pusillus*-LMB4 draft genome and genome walking.

Line 1: putative laccase sequence G122662.t1 from *D. pusillus*-LMB4 draft genome; line 2: putative laccase sequence from *D. pusillus*-LMB4 genome walking. Consensus sequences are depicted by blue nucleotides.

5.4. Conclusions

The use of the putative laccase sequence proposed in chapter 4 allowed the sequencing of the first 2995 base pairs of *D. pusillus*-LMB4 genomic DNA. Although, this DNA fraction from *D. pusillus*-LMB4 was not enough to obtain a laccase codifying gene, analysis of this DNA sequence with protein and genomic databases showed correlation with other white rot fungi reported as ligninolytic species. This supports one more time the link between *D. pusillus*-LMB4 and delignification activity through lignin oxidation, and the existence of putative ligninolytic enzymes.

The first *de novo* genome assembly of *D. pusillus*-LMB4 allowed the identification of 13 putative laccase genes that could be involved in the delignification process. Those putative laccase enzymes could be responsible for the increased release of reducing sugars observed in SPS and ABTS oxidation on the zymogram of the partial purification fractions of EE-LMB4. The new putative laccase enzymes obtained from *D. pusillus* genome contain structural elements from other known laccase enzymes such as copper

binding domains. Moreover, also predicted protein structures show similarity with several laccase crystal structures.

Finally, the amplification of the putative laccase gene G12662.t1 from *D. pusillus*-LMB4 DNA confirmed the existence of at least one laccase enzyme that could play a role in *D. pusillus* delignification process, produce ABTS oxidation and boost the release of reducing sugars during SSP over EFB. Also, alignment of sequences obtained from genome walking and putative laccase gene, substantiate the fact that identified peptides in chapter 4 belong to a laccase protein sequence.

General conclusions

To the best of our knowledge, this is the first report of *Dictyopanus* genera as a ligninolytic organism. Moreover, it is the first enzymatic extract with laccase activity reported from *Dictyopanus pusillus* species. This enzymatic extract showed higher stability (thermal and pH) than the commercial laccase from *Trametes versicolor* (53739 Sigma-Aldrich), foretelling the use of *D. pusillus*-LMB4 ligninolytic enzymes as biotechnological tool in delignification processes.

Using lignocellulose from oil palm trees as substrate, it was possible to establish that *D. pusillus*-LMB4 enzymatic extract performs a delignification process as good as the commercial and purified laccase enzyme in terms of sugar release. As expected, enzyme production by *D. pusillus*-LMB4 is constitutive, which rendered complete purification and characterization of one laccase-like enzyme a very difficult task. Certainly, overexpression in a heterologous system would be required to consider using of a *D. pusillus*-LMB4 enzymes as biotechnological tools.

From the data collected from protein mass spectrometry and walking through the genome analyses, peptide and genetic sequences were obtained that showed high homology with basidiomycete ligninolytic enzymes or their corresponding genes, supporting the idea that *D. pusillus*-LMB4 possesses ligninolytic capacity. Whole genome sequencing followed by genome annotation allowed the identification of 14 putative laccase genes and the amplification of one laccase enzyme gene from *Dictyopanus pusillus*-LMB4 genome. This work comprises first reports of whole genome sequence and laccase sequence from the *Dictyopanus* genera.

Taking into consideration the interesting properties of *D. pusillus*-LMB4 enzymatic extract towards pretreatment of oil palm tree residues, enzymes identified from genome annotation provide a promising source of ligninolytic enzymes to be studied and refined by protein engineering for future work.

Perspectives

As pointed out, *D. pusillus*-LMB4 is a promising candidate for future research on ligninolytic basidiomycete fungi and their enzymes, either for a precise biotechnological goal or to enhance knowledge on fungal delignification processes. Although an enzymatic extract obtained from *D. pusillus*-LMB4 could be used as an enzymatic delignification pretreatment, it would be necessary to increase protein production for an efficient biotechnological industrial process. Of course, improving growth conditions would increase protein production but overexpression in a heterologous system such as *S. cerevisiae* would most probably allow higher protein yields. From the putative laccase enzymes identified through *D. pusillus*-LMB4 annotation and amplification, two DNA sequences would be useful to begin the overexpression of laccase enzymes. The overexpression will be improving the production of laccase enzymes from *D. pusillus*-LMB4. Also, the expression in a heterologous system will allow checking for increased stability (thermal and pH) of those laccase enzymes, as was probed in the crude enzymatic extract. Finally, the construction of laccase heterologous systems will allow future directed evolution studies of laccase enzymes from *D. pusillus*-LMB4.

Over the course of this study, laccase enzymes were sought within the annotated genome draft obtained from *D. pusillus*-LMB4. However, this data could also be a source of other ligninolytic and cellulolytic enzymes, including peroxidases and glycan hydroxylases, which could prove useful for various applications such as hazardous compound biodegradation, biosynthesis of bioactive molecules, or oxidoreduction biosensors. Just like the identified laccases, overexpression in a heterologous system in combination with protein engineering would probably be essential for an efficient usage. Moreover, the fact that *D. pusillus*-LMB4 was able to grow over EFB, which can be considered as a lipidic substrate, suggests that this fungus encodes lipases. Lipases are valuable enzymes in the industry, in particular as biocatalysts in biodiesel production processes, but also in the fragrance and flavor industry.

Overall, this study with all data collected on *D. pusillus*-LMB4, and especially the draft genome, will serve as reference for future academic researchers involved in fungi-lignocellulose relationships and can certainly open up new possibilities for various protein

engineering projects where properties of ligninolytic, cellulosic and lipolytic enzymes, among others, would be an asset.

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Appendix A: Supplementary data

Table A1: Peptides identified by MASCOT from purified *D. pusillus*-LMB4 enzymatic extract

Peptide	Laccase number match at NCBI
YTFEVDRR	0
QHFEVDRR	0
RVGETLMVGDDVTVTVLGVK	18
MRLIHSLIR	2
SQALLSHLLR	1
DNGLLSHILR	4
ATINEINNRDLR	10
LFSTSALVGKK	3
GKTIHSILLR	0
TGELLSHIIR	0
ALPPTFLAQAR	3
WPTKQPILR	3
RADPALYAAP	18
FLPVWSTSGR	6
DATILHEALR	0
VSTLIHISLR	16
MRLLSHILR	1
IPEIPDPSK	3
LPELPDGVR	0
RAGPVPAAADACPR	16
EVDVLVAIARR	0
LHGQMPNSAIIR	18
VALGETDSSGEKSDEGLVNYDR	5
FIDSGDPLLVGEQVRACDR	16
SIDLASGGARELASGDTALDPK	6
HSHHLWGIAQTGAVADQQGQIAGLGR	13
GNVGDACPAACQNCLILCAIK	14
ITEAGLPVALEIK	1
AGVPLAVFCLDTGR	6

IAQMVDPADPEAGKLQVNAAEITPVK	18
GDMASIVAVSCGEVNTGQFATK	11
QANKLDPLEVPV	5
KLYTDPITQR	0
NADSIAAFR	2
RNGEGLQYAVVPVTDSK	17
IAQVTGSFISAVKDACR	8
FIYVYVNK	2
LFHDPAAACAAIDPSIMR	6
LLTEGDSEVAR	1
NVVAERVLGLPR	3
KAIGGVCLNEGCIPAK	3
LVHDTLCVNKLYR	1
NPELTGIPR	0
ECLYNEEGDFVGFLMPR	2
ADGGLRMLSGNLGR	4
RVYINLAVFLLLLLASALWLIPR	4
ILMRAVTPDSGAVVFNDGK	14
DETPFTGDNHTTTELANAIR	15
ATVSVLLEPEAHAR	12
IGENICSNFITIDLAR	2
WNGGSSFYINQDK	11
GLDNSTLIPDNSTLK	10
SMSSGSAGV	5
MIVDSSPSPTSTPTAR	7
LTKTLENPNQLNTDEK	5
FIAGVWR	0
AGDVSMIFQDPR	11
VQDHSPDTSILCVFR	4
IPQALAQEYADQVMR	3
GLPTPSGIGNDPR	1
PENPHHGSSDRLALVGK	2
ATVSVLLEPEAHAR	12
HWTLCENTYHDR	10

QYASSSEFVMDLSR	1
TPGTEDLYTEVITR	0
LYSSCQSHETPSHELK	0
LGSVEKMNVHHVEVAK	1
ATVSVLLEPEAHAR	12
ITSLPDVPQTPHQILGK	6
LQNIICCTKLQK	0
VRPKSVGAMEGIDSDER	8
ALHEMGLRVPEDVAVAGFDDIEDGR	8
EGEADATTSTK	14
DTHRLGATIDVEHSHVR	2
TVIKDFKPDVVVGVGGFASGPVLR	12
WGSPYLSR	0
DPSRIPLALAQHLLR	14
NPHGFAGYK	6
KHSILGATPIAEYSYR	3
EPHPALEEVAEEMGLR	2
YNGSSLEEGFPQK	14
CMAISALVAVGPIADDLISK	12
RHSSPTSISNK	6
GWQLYALDMLGMGRSSRPPFK	9
IISGCPLPEACELYIVNR	5
GPKDGDLDR	0
AESAIVLEPGKEYLVESR	7
ELAAAAYDVGAR	4
GLPLPSHPSTGGMLR	15
LFNENYFAFEQALEEVVR	2
DALTPDAVALAESKR	14
QSLAALWGNAAPR	15
SVLISGHGNSCR	1
LTPGNPYMK	1
CCVSSCCSPCCQPTCCRTTTCR	10
QHGIRSVISNSDTPETR	3
KFTAPTDGLAVLTVSK	13

LVQETGKQDQAGFLMYVPIYQNGTNNDTLAER	0
CMAACAGK	0
GEAKSGFR	0
IIGRDRPVFILEGR	2
RHANASPCLALPR	6
HLATVATCNLNQWALDFTGNLR	5
VVADQQLIADTFLK	14
RPLDPGGAAAPTNTGTRR	16
TIMLTIIDKFGGGPVGLDTIAAAIGEER	9
AGIEGCR	0
GTRPLPLSDGCASLRQASEYNSDDR	16
ILDINGVPIKSVIEK	9
RLAQPPVEK	4
IAQSANGAR	6
ELYAEEGEWLLVHASNFRPIKR	1
RVAGLPLVEDPGADICTGPHLR	7
GVPHVLVMMCDGLGHGPLAAR	17
DLAAALKNLGAALGEVAGFVK	3
SIRAIFDTEGEEAFR	2
LSAWLDTEYVDMPYPYK	0
DTASAAAEEARSGEVAK	3
KIVGAILLVSLICLGFSDSVK	14
KLSTALALMGDPAVVYLDEPTTGMDPGAR	14
FAAGGDNYDCVYR	18
ADRAGDLAATPVWLMGAASR	9
QLLSSAEEWVK	2
WNGYSQNMKWIGPANYAELFTDPLFWK	2
NGLFDLSGKVAIVTGGNGGIGLGMAR	9
GPTVWLQPSFSKYMR	4
AAELGVRAFCVK	11
ARAAAGEPCSSR	11
GKPVVAYITDQSAR	2
FGLPPSKDVIR	2
AEAEPPAVPEGHPFAR	16

DPYTFSELEQWLDK	7
LSAVGYGEDKPIATNNTSAGR	15
DGCFCTK	0
DAPLGSSSPATGTVR	5
FRCVLAVAAPGQQTQTFSGSCEGR	14
NPPLAEALLSGDLVSLVLVCYR	14
FVIDASGYGR	11
FYATMFR	0
AHSKIELCASLGLATEEQLAALYR	8
GVLSASIVRIPGIYAGNR	11
IGIVIIGHADFSKGMR	6
ALAVHLKPVTAGCVRMLASTARPTICLAHLASK	0
FWYEKNLTTEEIADVESR	15
EGEWIQCGVDDEGPGISAELLPR	13
VVAKSGTLPNTGDNSNHEVGVVGAGLLSAALLAALGVSK	0
QKALADCKPEPNYNGGPWFAPSYK	18
KSGITSTADSGSYSDLNR	11
WYELEDANDIAVASLDAVLMRK	11
LYSKPSLIMCDKR	6
APNATAQPER	2
SYQSHTRNSLVQDPK	18
CHQRGIGVILDWTPAHFPK	2
ISGNFEAVLKK	0
SYAMTAEETNK	2
SKSFVQPVVEIPTVAKPR	13
VQEGIVKAPNQTEMVVR	3
VVRFYDGGVTIR	18
VYTRLLQDAGINDTSMTHLSCLCSK	4
HIAGSHFDY	0
LFLIAICVTIAVIILIIILAVTLQ	5
KTENSSPVTQHGWICQWCQELCK	0
DLYGLDV	0
ETMIANLIATQITLTLDWGLGSALGVVLLIAGFAMVGGVALLFRR	0
WYGRAVSLGHVK	11
ADAAEGK	0

DFLQNTASNTDCNCGHLSLSK	16
ALKCVCCR	0
VTAPEPGQAPSETSK	14
SKSILSIGLGCDAK	5
QPQPVEAGA	2
IICVTNHCDKYGNSK	4
ASNQPASVTDK	5
LELTGIVLIVVCGATASWITAR	15
ATMSASG	0

Figure A1: Laccase hypothetical sequence

EGEWIQCGVDDEGPGISAELLPRFWYEKNLTTEEIADVESRSYQSHTRNSLVQDPKGL
PLPSHPSTGGMLRGVPHVLVMMCDGLGHGPLAARIAQMVDPADPEAGKLQVNAAEIT
PVKGTRPLPLSDGCASLRQASEYNSDDREGEADATTSTKVVADQQLIADTFLKHSHHL
WGIAQTGAVADQQGQIAGLGRRNGEGLQYAVVPVTDSKKIVGAILLVSLICLGFSDSVK
NPPLAEALLSGDLVSLVLVCYRFIDSGDPLLVGEQVRACDRLHGQMPNSAIIRKLSTAL
ALMGDPAVVYLDEPTTGMDPGARFRCVLAVAAPGQQTQTFSGSCEGRDPSRIPLALA
QHLLRRADPALYAAPVTAPEPGQAPSETSKDETPFTGDNHTTTELANAIRRVGETLMV
GDDVTVTVLGVKVVRFYDGGVTIRQKALADCKPEPNYNGGPWFAPSYKYNGSSLEEG
FPQKDALTPDAVALAESKRLSAVGYGEDKPIATNNTSAGR

Figure A2: pJET1.2/blunt cloning vector

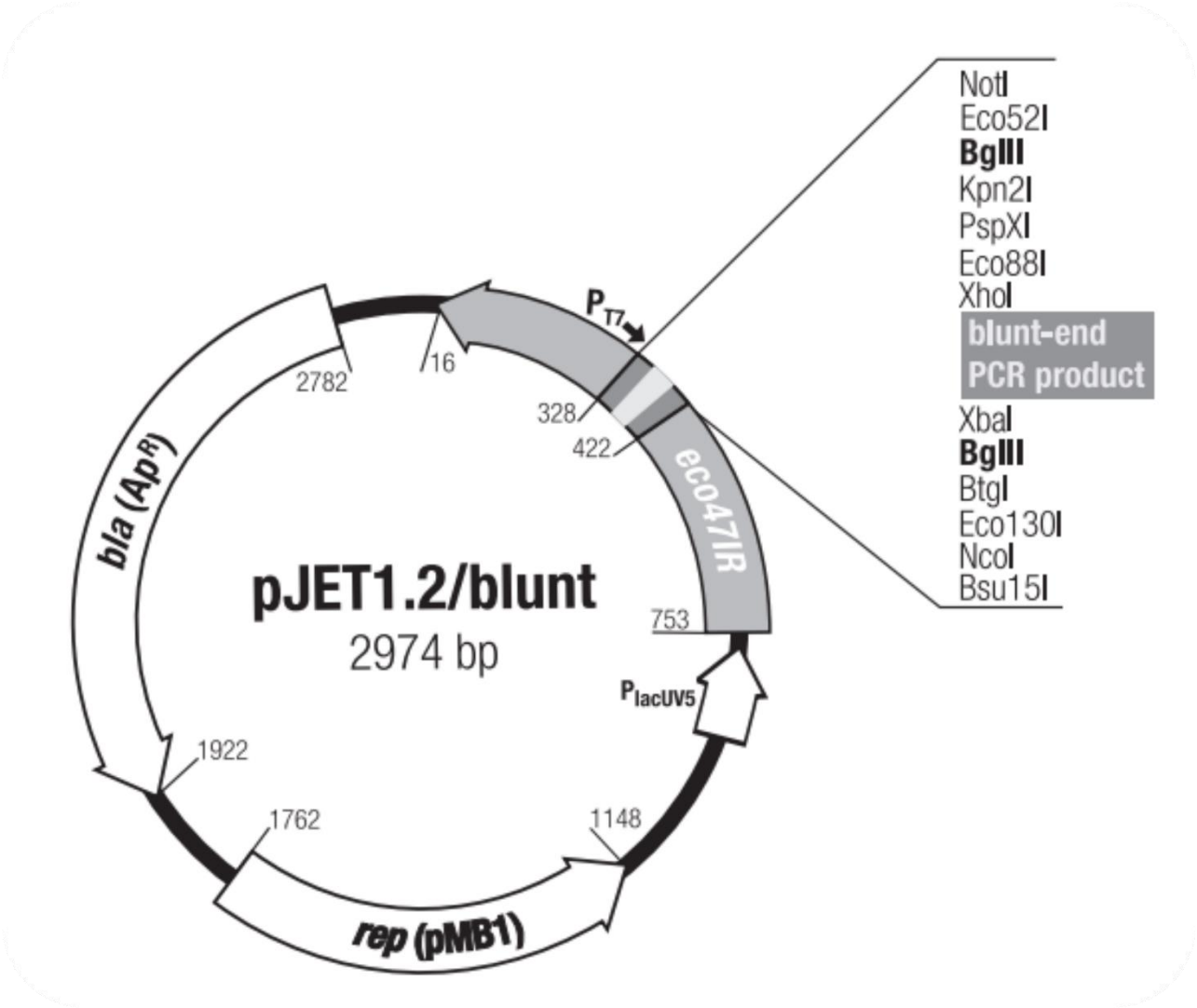

Figure A3: pD912-AKS expression vector

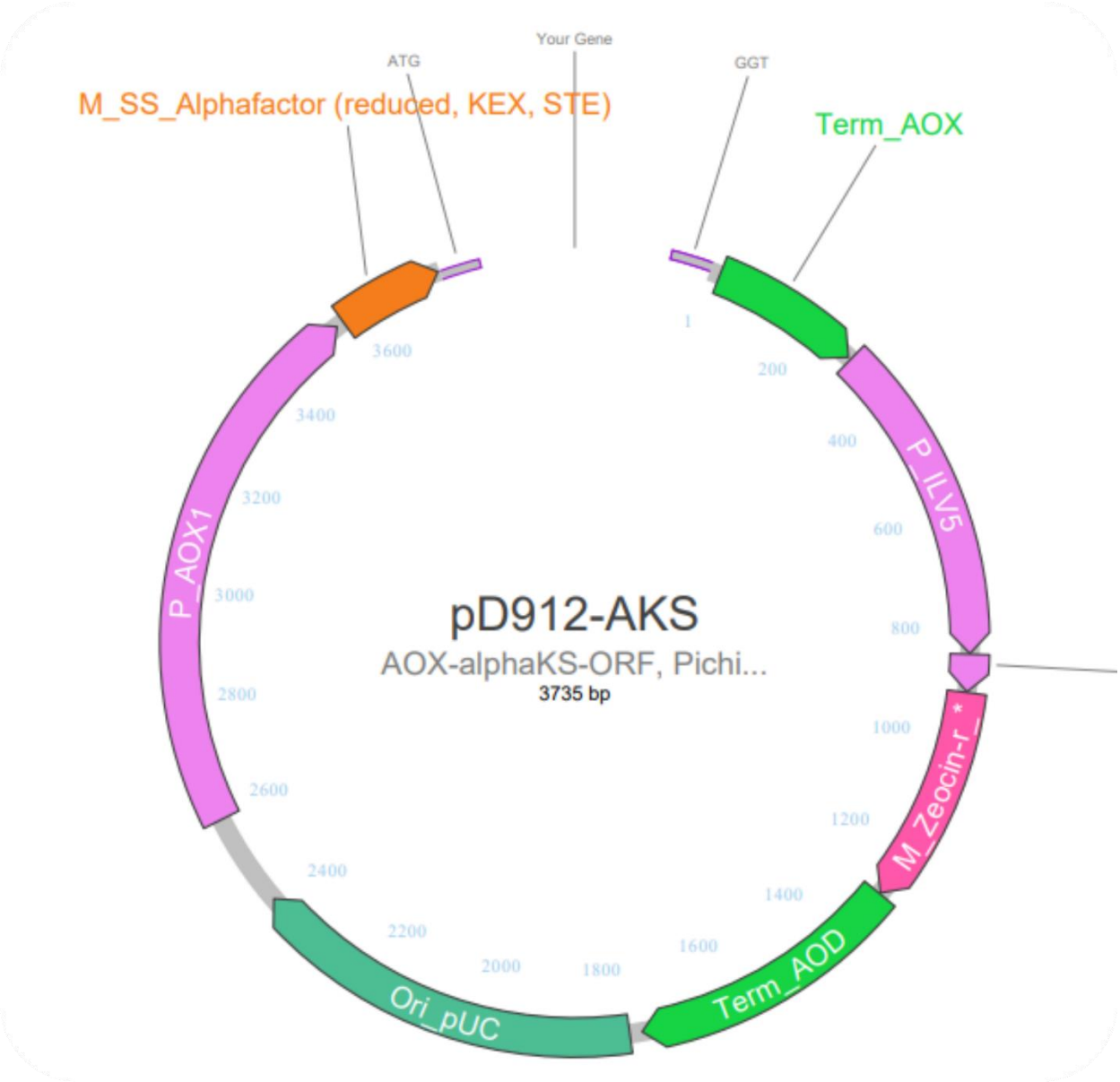

Figure A4: DNA sequence from *D. pusillus*-LMB4

5´- AATAGGGGCCATATATCTCACAATGAGACATCCAGCATCGAGACACCGTCA
TTGACGCGATTTTTCCCCGTCATTAATGTATATTGCCGCCACAGCTAAAGTCTGTAT
ATCCTTGTCGCTAGGGTTTTTTCTAATTCTGCATTAGAGCAATAAATCCTTCCGAAC
GTATCTTACCACGTCTGGACGGCTGAACCAAGAAATACTCCACCCACGACCGATCA

CGAACATCTTCTGGCGGCATTCCCAAGCCTCAAGCCGGTCGGTTCGCATTTCCATA
GCCCTCCTGATCTGATCGATATACCCAGCGACGATTTAATACTGTACACCATTACAT
CTGACGCTACCCTCCGCATATTCCTACCCGTCTTGGATTCACCGCAGCATCTACAG
CTCCACACTGCCCTGGATCTATTTTCTTCGATACCCTTCATCGTCTCCTCAAAACTA
TCTGCAAACTCCAGTGTCTTCTGGTGGGACCGAGAGATTGTCGGGGACTCGATAT
CCGCCATCTTAAAGACCGAAGCAGAATTAGACAACGTCCGCGTCCGGAGATTAAA
AGAAATCAAAGACGAAGGGTGGGATCTGTTTCTCCGCGTACTTGACGATGGTTCC
GCCGTCGTCACCGCCGTCGCTGTATAAATCTTTTCGTTTAGCCCGTATACTCATTC
ACTGACCCATCTCAGAATATAGATCGCAGACCGCCGACGTTGCTCCGACAGTTCA
CTCTGCAGAAGACTCTCCCTTCGACTTTTCCCAGTCCCCCGTCTCACATGTACCTG
CTGCCCAAGGCAGACCGCTCTTCCATCACTCTTATAACGACTTCACCTCTGCGGTC
ATTCGACCTGTCGCCCCTCCTGTTTCTCGACGCACATGCAGACGGCCTGCGTCTC
CGCGCACACACGCTGGAAGCACGTCCGGAGGAAGAGTCGCCCATCGTTCGCTTT
GTGCGGACGCCGGAAGGCGGTGGTGTCGCTGTGGTGCGCGAAGTCGGGGGCGA
AGGGAAGGAAATGCCGGGGCGGCTCAGATTAATCCGGTCCGGCAAGTGGTTTGT
GGCAGACCATGTCGTTGTACTTGACGGAGGTAATTTTCTTCCCACTGGCCCCTTTC
CCCCCTTTCAATGTACTCCACCTCTAGGACGAAGCCATGCCATCTACTCGGCAGCG
GACGGTCTATTGACTCTCAGCTCGGCGCCCCAGGCCGCCCTACCACTCCCTCCCA
TCAGCTCGCTCTTCTCTTTACTACTGAACGCGGGGCAAGAGACAATCGTCGGTATC
ACGACYGATCTCTCCATCGTCCAAATCCACGTTACCAACGCCAACCTCACGCTCCA
CTCGCGCATTAGCCTACCCATCCCRCGACCCAAGATGATTCTGCCGGTCGACCCG
ATGGCCTGGGGCATGCGCCGAGACTGGGTCATGCATGACGTGCTGCTGAGCGTG
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ATCAGCGACTCGATCTGGCTGGCCAACGGATCACTTTTAGTGGGTGCGGGCCACC
AGATGTTTCTCTTTAGCCAGAAACCGTCTTCGGGATCTGATTCCTCGCGWTCCGAA
GAAAGCCTGTTTGAGCACGTGGCGAGTCATAACGGGCCGCTGGAGGATTATCATC
CGCAGATGTTGCTGCAGTGCCTTCTATGGGGTACCTACCTCTCAGTATCCGTTTGT
CGTGCAAATGAATTGACTAAGCGCACAGACAAGGTYGAACTGGTCAAGGAGATTAT
TGTCAATCTCGCGCGGGATATCAGCTCGTCGAAGCCCAGGACAGCTTGGGCTTCT
ATGCCCYCAGAGAGTTTTCTGCAGAAAGAAACCTCTCGCGGAGCGGTTAGGCCCA
CACTTTTTTTAGCAGCAGACCACCTGGCGCTAATCATGATCCATAGACCTCCGGCC
ACAGGAAACGATACACACTTCTTTTCAATGGGCCGGAGCAGAAGAATGAGTGAGT
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TTCAGTCGAGCAGTTGTCGATCAGCTGATCAAAACTCTAGAGGACGATCCTCTTCC
TCACCTCACTCCCAATGAACAGGCCCATCTCTTGGTGCTCATTCAGACTACCCTTG
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TCGACGAACAACGGCGGGCCCTCGACGCGAACGGACTGCNNTTNNNNNTATCRAT
GCGCTCATTCTATATCCTCAACCACCGAGCGTCCGCCCCGGGGAGCCCTGACTCG
GGCAACGCGATACGCAGACAAACAGGGCGCCGGGAACGGCTCCGGTATCGGGAT
ATGGTCTGGGCCTTTCACAGCGAGAGTCAAGATTTGCTCTTGTCGGCGTCCCCCC
CCCCCGACATGAATGCATTTT – 3´

Figure A5: Putative laccase protein sequences

G253.t1

MRIVGVVAAVLSCLSLSLAAQNLTSAHSSSLSQRKHTGISSLLKGISAATGFVESSVGAI
STLTIVNKEIAPDGYSRSSVLAGGTFPGPLITGNKGEKFTINVQNDLTDTTMLRATSIVW
LTAFRLLFIDNRRFQHWHGFFQYHSPYEDGVAFVTQCPIIPESSFVYDFKTNQQAGTF
WYHSHLSTQYCDGLRGPLVVYDPNDPHENLYGPFDIYLLFRSILESAADIDDNGFGRY
SNGPASDLAVISVTQGKRYRFRIVSMSCDPNYVFSIDGHSMTIIEADSVNTDPLTVDSL
QIFSAQRYSVVVHANQTIGNYWLRANPNVGTTGFDGGINSAILRYVGAPASEPTSTPLS
INPFVETNLHSLGYSTVPGKPYPGGADININLNIAFGDNGKFTVNGNTFSPPTVPVILQIL
SGARTAQELLPAGNVYTLPPNKVVEISVPGTSNTGAPHPFHLHGHTFYVIRSAGSTEY

NYHNPLVRDVVSTGVAGDNVTFRFVTDNAGPWLLHCHIDWHLDKGLAIVFAENAPATS
AGLPSWSNLCPTYNAFTAAGGS

G3223.t1

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SLHAH

G5839.t1

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AILRYVGAPASEPTSTPLSINPFVETNL

G6228.t1

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DDVDPTTNATTATAALVETDLHPLVATAVPGTAVAGGADITMNLVISLDFTTFTFEINGA
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G6430.t1

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G8544.t1

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VLLQILSGTQNAQDLLPPGSIYSLPPNKVIEISIPNGGSAGVPVSTRLRLRIQNYSPAAMK
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G12662.t1

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G16121.t1

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G16512.t1

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G16540.t1

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YDSLTPDQL

G1684.t1

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GVFFSTENVYNEGSPFVTNCPFGPGESYTYTLPLGEQTGTFWYHSQLSVQYGDGLRG
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G2824.t1

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ENATVDAAYGPQSFVLDHMDVVDLVIQNGDSGKHPFHIHGHKYQIVERSTDYTSDDPS
VITTANQTNPLRRDTILIESGGSATLRFIADNPGVWFFHCHIEWHLEVGLAIQLIEAPLVA
QQYISNVPDSLAANCHAQGLPSSGNAAGFASTTDLEGLPLGPYLQNNGWHSKGIGAM
AGCVLTAVLGMLSVVWYSLGGRITEEEQEKEVREQLDAKAKRGKLFGLLKPKA

G3514.t1

METVPEPVPEWAEVNGLGGGAHEDDSTILLNEGRYAEVSVPRGSRTRLRLINAGTFAP
LRVSVDAHVLVLIAAYGTPLAMPYPQFCDILVHPAQKYSVFVSREQGEEEQGLSPQDA
APGCSPRHAILRYTFSSKQTSATALPLLTMKPGPPPGTDDAAWWDALPHFDEWDDLR
PASSVHHPSAQDQIATATLPFTFSIQRTLAQRCVPKTGASRWYISFSASSKDGQTFTLS
SPWTSSGFSGTAAARGQ

Appendix B: Paper draft

Simultaneous pretreatment and saccharification of empty fruit bunch from oil palm using enzymatic extracts from *Dictyopanus pusillus*

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Abstract

Lignocellulose could be used massively in the process of bioethanol production. The pretreatment of the plant biomass remains a critical requirement. While pretreatment processes primarily involve chemical and physical approaches, the biological breakdown of lignin using ligninolytic enzymes is quickly becoming an interesting eco-friendly alternative to classical processes. Ligninolytic enzymes are industrially relevant biocatalysts involved in the biodegradation of recalcitrant compounds such as dyes and lignin. As a result, bioprospection of wild fungi from naturally occurring lignin-rich sources remains a suitable method to uncover and isolate new species exhibiting laccase activity. In this study, species of white rot fungi were collected from Colombian forests. Strain isolation was performed based on laccase activities measured in crude enzymatic extracts from solid-state fermentation using a lignocellulose source from oil palm as matrix. Three strains showed laccase activity and were identified as *Dictyopanus pusillus* LMB4, *Pleurotus* sp. LMB2, and *Pleurotus* sp. LMB3. In addition to high temperature and low pH tolerance, an enzymatic extract from *Dictyopanus sp.* LMB4 showed the highest laccase activity (267±18 $U*L^{-1}$). Also, the simultaneous pretreatment of empty fruit bunch from oil palm with enzymatic extracts with ligninolytic activity from *D. pusillus* LMB4 and commercial cellulolytic cocktail from *Trichoderma reesei* reached 44.80 ± 5.21 $g.g^{-1}$. In order to find the enzymes responsible for the high lignolytic activity, we draft? sequenced *Dictyopanus* sp. LMB4 DNA by long-read SMRT sequencing. It has been possible to annotate with a high level of confidence 13 genes coding for laccases, which is more than other related species with available genomes. To the best of our knowledge, this represents the first report of an enzymatic extract exhibiting laccase activity in *Dictyopanus*, offering means to exploit this fungus in the delignification process of lignocellulosic by-products from oil palm.

Keywords: Lignocellulose, *Dictyopanus*, Laccase, Simultaneous pretreatment.

1. Introduction

The accumulation of agro-industry lignocellulosic postharvest by-products is a direct consequence of the global demand for crops employed in the food supply chain and bio-renewable fuel production. Following this trend, global palm oil production has increased 43% over the past 10 years to reach 52 million tons in 2015, primarily due to high biodiesel demand (FAO, 2016). As a result, the product-to-waste ratio for palm oil production remains significant (1:3), generating important lignocellulosic biomass accumulation (Sulaiman, Abdullah, Gerhauser, & Shariff, 2011). This represents a particularly pressing environmental issue for the largest producing countries such as Malaysia and Indonesia. One alternative to overcome the significant build-up of cellulosic biomass is the production of bioethanol by fermentation of syrups extracted from cellulose and hemicellulose hydrolysis. Lignocellulosic ethanol production is an eco-friendly alternative to current agro-industry by-products, in addition to offering an important source of renewable energy (Gupta et al., 2016).

Lignocellulose is a raw material composed of lignin, cellulose, and hemicellulose, forming a complex aromatic polymer that provides rigidity and strength to plant cell walls. While cellulose represents an inestimable carbon energy source on a global scale, releasing cellulose from lignocellulose by lignin removal represents a major challenge in many industrial processes, including the bioethanol and pulp and paper industries (Bhutto et al., 2017; Bilal, Asgher, Iqbal, Hu, & Zhang, 2017; Kumar & Sharma, 2017). To this day, delignification is either performed by chemical strategies using environmentally damaging acids or alkaline solutions, and/or through physical processes such as high temperature and pressure conditions (Chen & Fu, 2016). A biological delignification process using ligninolytic enzymes that can breakdown lignin through an oxidation mechanism would therefore offer a valuable alternative for the pretreatment of lignocellulose (Munk, Sitarz, Kalyani, Mikkelsen, & Meyer, 2015). Laccases (EC 1.10.3.2), manganese peroxidases (EC 1.11.1.13), and lignin peroxidases (EC 1.11.1.14) are the most promising ligninolytic catalysts for such biological pretreatment. These enzymes are primarily expressed and secreted from basidiomycete fungi, especially the Agaricomycetes class (Wong, 2009). Fungi are the main organisms associated to wood decay colonization due to their ability to secrete oxidoreductase enzymes and their affinity for phenolic polymers such as lignin. In a fungus, expression of ligninolytic enzymes is triggered by monosaccharides released by decomposing leaves and wood (Fritsche & Hofrichter, 2005). Studies on

fungi lignocellulose decomposition have demonstrated that species involved in wood decay produce a pool of many enzymes acting against the three main lignocellulose components (Abbas, Koc, Liu, & Tien, 2005; Martínez, Ruiz-Dueñas, Martínez, del Río, & Gutiérrez, 2009).

Enzymatic extracts from fungi exhibiting ligninolytic activities are thus currently positioned as one of the most promising biotechnological tools for the management of recalcitrant pollutants such as dyes, pesticides, phenolic compounds, and agro-industry residues. Nevertheless, fungus-based lignocellulosic pretreatment processes for industrial applications is still hampered by the difficulty to produce large amounts of highly active enzymes. However, these problems can be overcome by the use of recombinant organisms and/or screening of species with enhanced enzymatic ability (Alcalde, 2015; Upadhyay, Shrivastava, & Agrawal, 2016). It is known that fungi can adapt to a broad range of environmental conditions. Thus, it is conceivable that specific strains have developed the ability to produce higher titers of a given enzyme and/or to perform chemical reactions in harsh conditions.

Herein, we describe the isolation and initial characterization of wild Colombian basidiomycete crude enzymatic extracts exhibiting laccase activity. Our main goal was to identify a new fungal enzymatic extract capable of sustaining harsh experimental conditions for extended periods of time, such as higher temperatures and lower pH, while favoring an increase in the release of reducing sugars during simultaneous pretreatment and saccharification (SPS) processes of empty fruit bunch from oil palm trees. Laccase activity was the main enzymatic activity found in our extracts, which includes a highly active isolate from *Dictyopanus* sp LMB4. To the best our knowledge, this represents the first reported *Dictyopanus* isolate harboring laccase activity. The DNA of this isolate was sequenced to shed light on the enzymes responsible for this high lignolytic activity. The enzymes found in the present study will certainly serve as a foundation for protein engineering approaches aiming at catalytic and/or stability improvements for the development of laccase biocatalysts for delignification processes (Gasser, Hommes, Schäffer, & Corvini, 2012; Madhavan, Sindhu, Parameswaran, Sukumaran, & Pandey, 2017; Rodgers et al., 2010).

2. Materials and methods

2.1 Fungi isolation and growth conditions

Fruit bodies from basidiomycete fungi growing on decaying wood were collected in a tropical humid forest in Colombia, following previously published parameters to favor the presence of delignification enzymes (Blanchette, 1984; Worrall, Anagnost, & Zabel, 1997). Delignification begins with the release of monosaccharides from decaying wood, a process that induces ligninolytic enzyme production in fungi. Indeed, monosaccharides are recognized by membrane proteins of the fungi cell wall, further inducing the expression and secretion of ligninolytic enzymes that depolymerize lignin to allow molecular access to cellulose (Fritsche & Hofrichter, 2005). The main inclusion criteria were macroscopic properties belonging to the orders of *Agaricales*, *Russulales*, and *Polyporales* due to the possible ligninolytic activity of these organismes (Floudas et al., 2012; Peláez, Martínez, & Martínez, 1995). Collected samples were kept in wax paper bags to prevent deterioration. Isolation of the collected fungi was performed in wheat bran extract agar composed of 18 $g.L^{-1}$ agar, 10 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ peptone, 2 $g.L^{-1}$ yeast extract, 0.1 $g.L^{-1}$ KH_2PO_4, 0.1 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.085 $g.L^{-1}$ $MnSO_4$, 1000 mL wheat bran extract, 0.1 $g.L^{-1}$ chloramphenicol, 0.1 $g.L^{-1}$ and 600 $U.L^{-1}$ nystatin. Pilei were adhered to the top cover of Petri dishes, allowing spores to fall and, eventually, to germinate on the culture media. Top covers were rotated every 24 h for 3 days and those containing the pilei were replaced by new sterilized ones (Choi, Hyde, & Ho, 1999). Sub-cultures in the same media were incubated at 25°C to obtain axenic strains from these isolates. The presence of microscopic sexual basidiomycete properties was checked, including septate hyaline hyphae and clamps. Twelve ligninolytic fungi belonging to genera *Aleurodiscus*, *Dictyopanus, Hyphodontia, Mycoacia, Phellinus, Pleurotus, Stereum, Trametes* and *Tyromyces* were axenically isolated from 43 collected wild-type strains. Fungi collection was planned under the regulations of Colombia's Environmental Ministry. The research permit in biological biodiversity was obtained from the *Corporación Autónoma de Santander* (file number 153-12 REB) and with the agreement of the *Ministerio del Interior*, certifying the absence of ethnic groups in the area (application number 1648, August 14, 2012).

2.2 Molecular identification of selected isolates

Total genomic DNA was extracted from selected isolates following a standard phenol-chloroform protocol. Briefly, fungi were grown in wheat bran extract agar for 15 days and 0.5 g of mycelium was placed in a tube with a lysis solution (0.1 M $NaCl_2$, Tris-HCl pH 8, 5% SDS) and 0.5 mm diameter glass beads. Finally, the aqueous fraction was collected, and the fungus DNA was precipitated with isopropanol. The

DNA pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) (Płaza G. A., 2004). A pair of primers within the Internal Transcribed Spacer regions (ITS1/ITS4) was used to amplify ribosomal DNA by PCR (Gardes and Bruns 1993). PCR conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 51°C for 1 min, and finally 3 min at 72°C (Izzo & Mazzola, 2009). PCR products were loaded onto 1% agarose gels, separated by electrophoresis, stained with GelRed (Biotium, USA) and viewed by UV light. PCR products were sequenced by the Sanger method using the same PCR primers. Sequences were aligned using the NCBI nucleotide-BLAST (MEGA BLAST algorithm) to confirm the identity of selected isolates.

2.3 DNA extraction, sequencing and genome analysis of *Dictyopanus* LMB4

Mycelium from *D. pusillus* grown on Potato Dextrose Agar (PDA) was used to extract all the genomic DNA (gDNA) by high salt phenol chloroform cleanup protocol recommended by PacBio® systems. 0.5 g of mycelium was placed in a tube with a lysis solution (0.1 M NaCl2, Tris-HCl pH 8, 5% SDS) and 0.5 mm diameter glass beads until to see the mycelium broken and followed by centrifugation at 11.000 g for 10 minutes. The supernatant was mixed in the same proportion with a phenol-chloroform-isoamyl alcohol solution 25:24:1 and centrifugated at 11.000 g for 5 minutes, the new supernatant was mixed in the same proportion with a chloroform-isoamyl alcohol 24:1 and centrifugated at 14.000 g for 10 minutes. Finally, the aqueous fraction was collected, and the fungus protein was precipitated with absolute ethanol in a ratio 10:3 supernatant-ethanol followed by centrifugation at 11.000 g for 15 minutes, the new supernatant was mixed with ethanol in a ratio 10:17 supernatant-ethanol for precipitated the DNA by centrifugation at 11.000 g for 15 minutes. The DNA pellet was dissolved in DEPC-treated water DNases-free

The genomic DNA of *Dictyopanus* LMB4 was sequenced using five SMRT cells on a Pacific Biosciences RS II system at the Génome Québec Innovation Centre (McGill University, Montreal, Canada). The resulting sequencing reads were *de novo* assembled in contiguous sequences using Canu version 1.7 (Koren, Walenz et al. 2017). The heterozygous genome was then reduced using Redundans version 0.14a (Pryszcz and Gabaldón 2016). The genes encoding proteins have been predicted with WebAUGUSTUS (Hoff and Stanke 2013) using Laccaria bicolor as a training dataset. The resulting predicted gene sequences were annotated using the webserver of eggNOG-mapper (Huerta-Cepas, Forslund et al. 2017). The sequences corresponding to putative laccases (EC 1.10.3.2) were further analyzed using InterProScan

(Jones, Binns et al. 2014) to verify the presence of multicopper oxidase signatures PS00079 / PS00080 and Cu-oxidase PFAM domains PF00394, PF07731, and PF07732, as suggested (Moreno, Feng et al. 2017). The Laccase and Multicopper Oxidase Engineering Database (Sirim, Wagner et al. 2011) was also used to validate that the identified sequences were laccases. was also used to validate that the identified sequences were laccases. Finally, this Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QVIE00000000.

2.4 Fiber analysis of oil palm by-products

Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL) were determined by the Van Soest method using the FiberCap™ system (Foss Analytical AB, Denmark). Cellulose and hemicellulose percentages were estimated as the difference between ADF and ADL, and NDF and ADF respectively, while lignin concentrations corresponded to ADL percentages in dry weight of oil palm by-products. Additionally, values were used to estimate the total carbon concentration in fermentations. Assays were performed in duplicate.

2.5 *Basidiomycete* fungi screening through solid-state fermentation (SSF)

The main selection criterion of isolated wild-type fungi was ligninolytic activity, observed in the crude extracts from SSF using lignocellulosic material from oil palm by-products (Rodríguez Couto & Sanromán, 2005). SSF was performed in 250 ml flasks in sterile conditions. Each flask contained 12 ml of basal media in deionized water, comprising 0.2 $g.L^{-1}$ yeast extract, 0.76 $g.L^{-1}$ peptone, 0.3 $g.L^{-1}$ urea, 0.25 $g.L^{-1}$ $CuSO_4 \cdot 5H_2O$, 1.4 $g.L^{-1}$ $(NH_4)_2SO_4$, 2 $g.L^{-1}$ KH_2PO_4, 0.3 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.4 $g.L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.005 $g.L^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.0016 $g.L^{-1}$ $MnSO_4$, 0.0037 $g.L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.0037 $g.L^{-1}$ $CoCl_2 \cdot 6H_2O$, and 2.5 $g.L^{-1}$ of empty fruit bunch (EFB) chopped into chunks of approximately 2 cm. Each flask was inoculated with eight agar plugs cut from actively growing fungal mycelium grown on wheat bran extract agar. Each SSF batch isolation contained thirty flasks and fermentation was held without agitation at 25˚C for 30 days. Every three days, three flasks were used to collect crude enzymatic extracts.

2.6 Crude enzymatic extracts recovery

Crude enzymatic extracts were obtained by addition of 30 ml of 60 mM sterile phosphate buffer into the fermentation flask that was shaken for 24h at 150 rpm. Whole flask contents were then collected in 50 mL tubes, vortexed for 15 minutes at 1500 rpm and centrifuged twice at 9000 rpm for 15 minutes to remove

suspended solids. Supernatants were taken as crude enzymatic extracts (Lim, Lee, & Kang, 2013) and concentrated by lyophilization to evaluate the effects of pH and temperature on enzymatic activity and the SPS.

2.7 Reducing sugars quantification

Reducing sugars were quantified by the oxidation of 3,5-dinitrosalicylic acid to 3-amino,5-nitrosalicylic acid (DNS) by the reducing extremity of sugars released. The reaction was followed at 420 nm; a standard curve obtained with glucose was used to quantify the concentration of reducing sugars (Miller, 1959).

2.8 Ligninolytic and cellulase assays

Crude enzymatic extracts obtained from SSF were assayed for laccase, lignin peroxidase and manganese peroxidase activities. Laccase activity was followed by the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, USA) (Johannes & Majcherczyk, 2000). Reactions were initiated by mixing 40 µL of culture supernatant, 150 µL of 50 mM acetate buffer (pH 4.5) and 10 µL of 1.8 mM ABTS; activity of mixtures was estimated by reading absorbance at 420 nm. Manganese peroxidase activity was measured by the formation of Mn^{3+}-malonate complexes at pH 4.5 in 50 mM sodium malonate buffer containing 0.5 mM $MnSO_4$ (Järvinen, Taskila, Isomäki, & Ojamo, 2012). Reactions were performed by mixing 20 µl of culture supernatant, 100 µl of 20 mM citrate buffer at pH 4.5, 40 µl of sodium malonate buffer and initiated with 40 µl of fresh 0.8 mM H_2O_2. Readings at 270 nm were used to estimate the transformation of Mn^{+3} to Mn^{+2} as manganese peroxidase activity. Lignin peroxidase activity was measured by the transformation of 3,4-dimethoxybenzyl alcohol (VA) (Sigma-Aldrich, USA) to veratryl aldehyde (VAD), which exhibits a yellow color (Khindaria, Yamazaki, & Aust, 1995). Reactions were performed by mixing 20 µl of culture supernatant, 100 µl of 20 mM citrate buffer at pH 3, 40 µl of 10 mM VA and initiated with 40 µl of fresh 0.8 mM H_2O_2. Enzymatic activity was estimated at 310 nm. Enzyme activities were expressed in U per liter ($U.L^{-1}$). One unit of enzymatic activity was defined as the quantity of enzyme needed to transform 1 µmol of substrate per minute. Absorbance readings were performed with a ThermoFisher Multiskan™ GO Microplate Spectrophotometer.

The total cellulolytic activity was quantified by units of paper filter ($UPF.ml^{-1}$). In tubes, 500 µL of commercial cellulase solutions from *Trichoderma reesei* Sigma Aldrich C2730 Celluclast® (USA) were

incubated with 500 µL of 50 mM citrate buffer at pH 4.8, 50 and 5 mg of filter paper for 1 h, at 50 °C. The concentration of reducing sugars released was measured by the oxidation of 3,5-dinitrosalicylic acid (DNS), as described above (Ghose, 1987).

2.9 Effect of pH and temperature on laccase activity and stability

The effect of pH was examined for crude enzymatic extracts exhibiting the highest laccase activity. A pH range from 2 to 8 (50 mM hydrochloric acid buffer, pH 2; 50 mM citric buffer pH 3-4; 50 mM acetate buffer pH 4.5-5, and 50 mM phosphate buffer pH 6-8) was evaluated using ABTS as substrate. The effect of temperature on enzyme activity and stability was measured with crude enzymatic extracts in 50 mM acetate buffer pH 4.5 at 40°C, 50°C and 60°C for 7 h. Finally, comparison of crude enzymatic extracts with a control laccase from *Trametes versicolor,* 53739 Sigma-Aldrich (Canada) was performed in triplicate using pH and temperature conditions exhibiting the highest activity. All components (except enzymes) were sterilized separately and mixed under environmentally sterile conditions.

2.9 Simultaneous pretreatment and saccharification of EFB

The simultaneous pretreatment was performed in 50 ml tubes containing 1.5 g EFB, 16 ml of 50 mM acetate buffer at pH 4.5 and combining either the laccase enzyme from *D. pusillus* or the commercial laccase from *T. versicolor* (53739 Sigma-Aldrich-Canada) with the cellulase from *T. reesei* (Sigma Aldrich C2730 Celluclast®). For the reaction mixture both laccase and cellulase were added in a volume of 2 ml to reach a final concentration of 25 $U*L^{-1}$ and 50 UPF respectively. The tubes were incubated at 40°C for 72 hours. The saccharification process was evaluated by the production of reducing sugars, measured by a DNS assay. Assays were performed in triplicate and all components (except enzymes) were sterilized separately and mixed under environmentally sterile conditions.

Simultaneous pretreatment and saccharification of EFB was conducted with enzymatic extracts exhibiting laccase activity and cellulases according to a Plackett-Burman design to evaluate significant variables in the process. Five independent variables were evaluated: pH (3 to 5) using either 50 mM acetate buffer (pH 3 and 4) or 50 mM citrate buffer (pH 5), temperature (25, 35 and 45 °C), copper concentration (1, 3 and 5 mM), laccase (100, 200 and 300 $U.L^{-1}$) and cellulase (50, 100, 150 $UPF.mL^{1}$) activities. SPS was performed in 50 ml tubes with 1.5 g EFB and 20 ml of total volume, including 2 ml each of laccase enzymatic extract and cellulase concentrate. The mixture was incubated for 72 h and the concentration of

reducing sugars was measured in each tube. Results represent four independent experiments and were analyzed using Statgraphics Centurion XVII.

3 Results and Discussion

3.1. Fungi isolation

From all fruit bodies collected, fifteen axenic cultures were obtained, reflecting a 30% overall effectiveness of the isolation technique. This low percentage probably results from biota mycoparasitism associated to basidiomycetes, mainly by *Trichoderma* species. These fungi possess fungicide and antagonistic activity against basidiomycete cell walls, in addition to releasing enzymes such as chitinases and glucanases (Colavolpe, Mejía, & Albertó, 2014; Schubert, Fink, & Schwarze, 2008). Moreover, basidiomycete recovery from collected samples can also suffer from competition with ascomycete fungi. Competition between these two fungi heavily relies on nutrient accessibility, growth factors favoring ascomycetes due to their faster growing pace in complete culture media, or even in the presence of simple nutrient sources observed in advanced stages of wood decay (Boddy, 2000). Based on macroscopic properties, twelve fungi isolates were identified. Isolated strains belong to the order i) *Hymenochaetale: Hyphodontia* (2 isolates), *Phellinus* (1 isolate); ii) *Polyporales: Aleurodiscus* (1 isolate), *Mycoacia* (2 isolates) *Stereum* (1 isolate) *Trametes* (1 isolate), and *Tyromyces* (1 isolate) and *iii) Agaricales*: *Dictyopanus* (1 isolate), *Pleurotus* (2 isolates). The last order members are part of the *Agaricomycetes* class, which has been evolutionarily recognized in the production of oxidoreductases and hydrolases involved in wood decay (Eastwood, 2014; Floudas et al., 2015; Kim et al., 2016).

3.2. Screening of isolates

Enzymatic extracts were screened for laccase, manganese peroxidase and lignin peroxidase activity, enzymes known to participate in the delignification process. From the crude enzymatic extracts obtained by SSF, only five isolates showed laccase activity in our screening assay. Surprisingly, we were unable to measure any peroxidase activity other than through the ABTS assay. We believe that our peroxidase assays were not sensitive enough to quantify the potential peroxidase activities on SS supernatant. However, secretome profile analyses of basidiomycete fungi reveal that laccase is the prevalent ligninolytic activity characterized in fermentation assays using lignocellulose as substrate, in

addition to exhibiting the highest activity relative to peroxidases (Fernández-Fueyo et al., 2016; Mali, Kuuskeri, Shah, & Lundell, 2017). Regardless, the use of lignocellulosic by-products from oil palm as substrate to screen fungi isolates allowed the direct evaluation of laccase enzyme production, which could potentially serve as pretreatment of oil palm by-products. Isolates having ligninolytic activity were identified as *Dictyopanus* sp. LMB4 (22.3 $U.L^{-1}$), *Pleurotus* sp. LMB2 (69.5 $U.L^{-1}$), and *Pleurotus* sp. LMB3 (57.2 $U.L^{-1}$). Laccase activity of the *Hyphodontia* and *Trametes* isolates was considered too low to warrant further characterization. For the three most active isolates, the highest laccase activity was detected after 20 days of fermentation (Fig. 1). Using these three isolates, laccase activity conditions were optimized by increasing the copper concentration and carbon-to-nitrogen ratio (C/N) (Baldrian & Gabriel, 2002; Li, Wang, Liu, Li, & Yao, 2011). As a result, the isolate exhibiting the highest laccase activity under these newly optimized conditions was *Dictyopanus* sp. LMB4 (267.6 $U.L^{-1}$ after 28 days of fermentation). To the best of our knowledge, this represents the first observation significant laccase activity in a crude enzymatic extract from a fungus from the *Dictyopanus* genus. Furthermore, this activity is similar to a previously reported *Trametes sp*. laccase activity evaluated under comparable fermentation conditions using lignocellulosic by-products from oil palm (218.6 $U.L^{-1}$) (Singh, Sulaiman, Hashim, Peng, & Singh, 2013). The maximal laccase activities of the *Pleurotus* isolates were significantly lower than the one observed in *Dictyopanus* sp. LMB4, with 98 $U.L^{-1}$ for *Pleurotus* sp. LMB2, and 66.9 $U.L^{-1}$ for *Pleurotus* sp. LMB3 (Fig. 2).

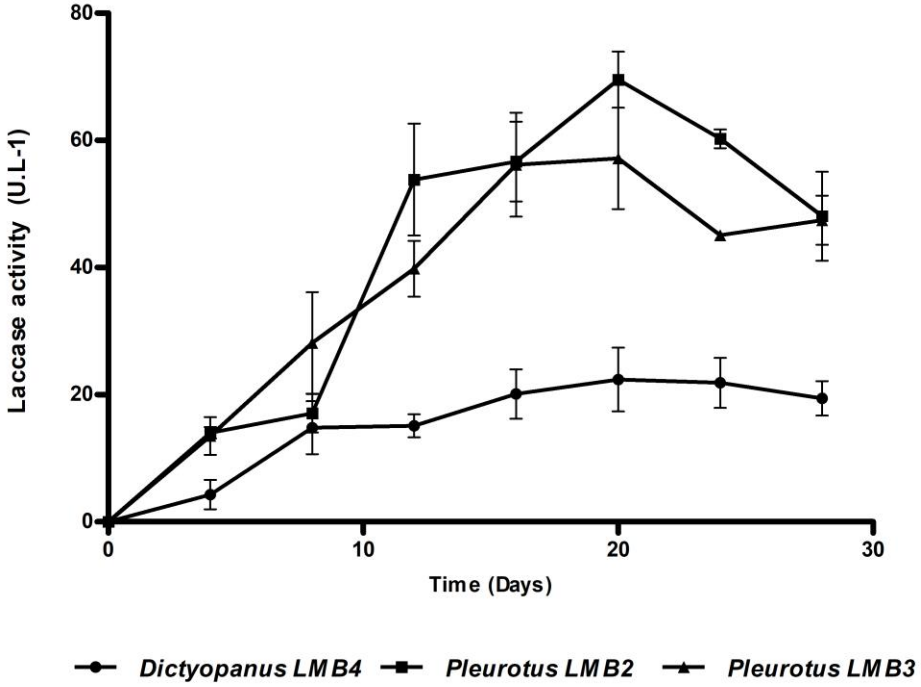

Figure 1. Laccase activity of SSF isolates. Enzyme activity was measured from culture supernatants with a 1.9 C/N ratio without copper. Laccase activity was only detected in five isolates, with the highest activities observed for *Pleurotus* and *Dictyopanus* genera.

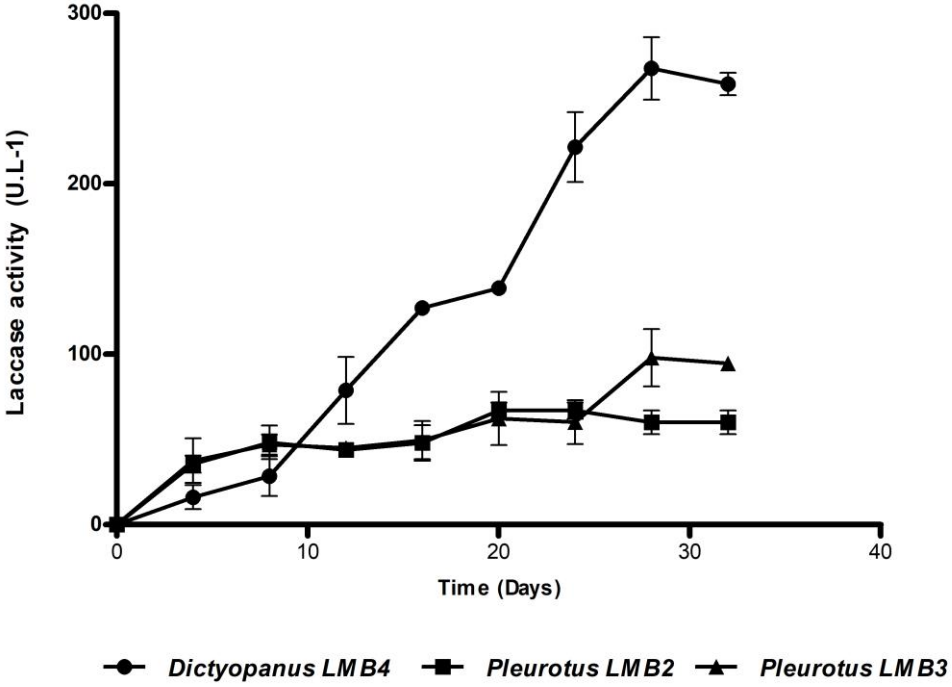

Figure 2. Laccase activity of selected isolates under optimized assay conditions. Enzyme activity was measured from supernatants obtained through SSF with a 19 C/N ratio and 5 mM copper. Isolates from *Dictyopanus* and *Pleurotus* genera were evaluated. *Dictyopanus* LMB4 demonstrated the highest laccase activity (267 ± 18 $U.L^{-1}$), a 12-fold increase from the first screening.

Upon optimization of the growth conditions, the crude enzymatic activity of *Dictyopanus* sp. LMB4 increased 6- and 12-fold after 20- and 28-day incubations, respectively, highlighting the importance of copper and carbon source accessibility for proper enzyme expression. Similar observations were reported on other laccase extracts. Indeed, a 4-fold increase in laccase activity was observed for a *Ganoderma lucidum* isolate when glucose was added to the fermentation medium (Hailei, Ping, Yuhua, & Yufeng, 2015). The authors concluded that accessibility to an easy absorption carbon source such as glucose improved mycelial growth, which is proportional to the amount of laccase secreted by the fungi. Similarly, laccase activity was improved when copper was added to submerged fermentations of *Colorios versicolor*, due to the fact that copper ions are strong stimulators of laccase expression (Kajita et al., 2004). The breakdown of lignocellulosic from EFB by white rot fungi (Kamcharoen, Champreda, Eurwilaichitr, & Boonsawang, 2014; Piñeros-Castro & Velásquez-Lozano, 2014), and EFB pulp (Martín-Sampedro, Rodríguez, Ferrer,

García-Fuentevilla, & Eugenio, 2012) has been previously reported, but the use of enzymatic extracts in the pretreatment of EFB had not been reported yet. Finally, our laccase activity per gram of substrate for *D. pusillus* using oil palm by-products is 31.5 $U.g^{-1}$ after 12 days of SSF, which is higher than the reported 7.5 $U.g^{-1}$ obtained from *Pycnoporus sanguineus* in similar SSF conditions (Vikineswary et al., 2006).

3.3 Molecular identification of selected isolates

Using extracted DNA from all three isolates showing laccase activity and the ITS1/ITS4 primers, we obtained PCR products yielding 0.7 kb fragments, correlating with previous fungi identification reports (Gardes & Bruns, 1993). Sequencing of the respective rDNA confirmed the identity of the three isolates displaying laccase activity through taxonomic identification. The *Dictyopanus* sp. LMB4 isolate showed 99 % identity with *Dictyopanus pusillus,* while the two other isolates, identified as *Pleurotus* sp. LMB2 and *Pleurotus* sp. LMB3, respectively, shared 94 and 93 % identity with the *Pleurotus* genus. These results support the usefulness of taxonomic classification during fungi sample collection and isolation selection. The *Dictyopanus* genus belongs to the *Agaricomycetes* class, and its genus is known to include species capable of bioluminescence, which have been suggested to be linked to delignification processes through the use of secondary compounds produced during lignin degradation (Bechara, 2015). *Dictyopanus* isolates were also reported as an alternative for the pretreatment of remazol brilliant blue R (Machado, Matheus, & Bononi, 2005) and bamboo in ethanol production (Suhara, Kodama, Kamei, Maekawa, & Meguro, 2012), further supporting the potential use of this fungus in large-scale biomass degradation.

3.4 Effect of pH and temperature on laccase activity and stability

Characterization of crude enzymatic extract isolates showed that pH values between 3 and 5 provided the highest laccase activity for *D. pusillus* LMB4, with a maximum activity at pH 3 (Fig. 3a). This pH range corresponds to other laccase preferences in fungi (Baldrian, 2006). Moreover, thermal stability of the *D. pusillus* LMB4 crude enzymatic extract was found to be quite robust, with reduced activity only observed at 60°C (46% activity loss after 6 hours of incubation). This behavior is quite different from that observed with the *T. versicolor* commercial laccase under the same experimental conditions, showing 28% and 78% activity loss after a 6h incubation at 50°C and 60°C, respectively (Fig. 4). Thus, *D. pusillus* LMB4 appears to harbor laccases with enhanced thermostability and higher tolerance to lower pH values. However, long incubation of this crude enzymatic extract at low pH resulted in an important activity loss

(Fig. 3b). Previous studies have shown that a laccase from *Physisporinus rivulosus* remained stable at 50°C with optimal activity at pH 3.5 (Hildén et al., 2013). Similarly, a laccase from *Trametes trogii* was shown to sustain temperatures up to 75°C, although only for short 5-min incubations (Yan, Chen, Niu, Chen, & Chagan, 2015). Our results suggest that the laccase activity from the *D. pusillus* LMB4 extract has higher tolerance to acidic and thermally induced perturbations than previously identified fungal laccases.

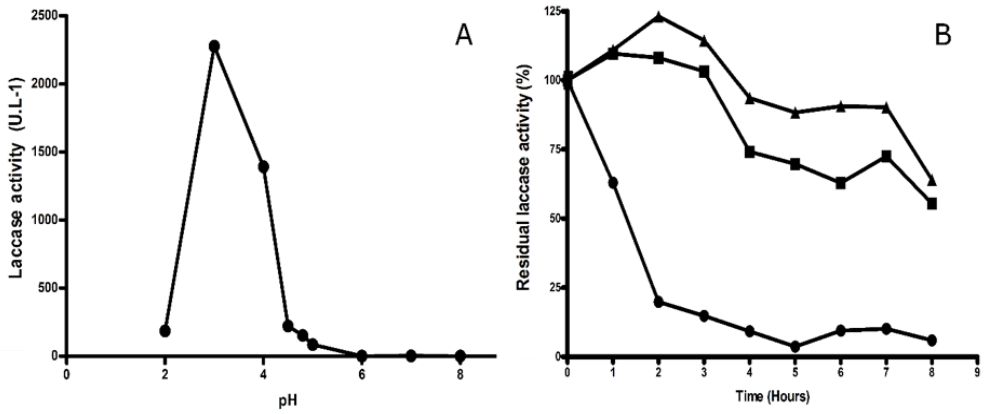

Figure 3. Evaluation of pH tolerance for a *D. pusillus* LMB4 extract exhibiting laccase activity. A) Laccase activity from a crude *D. pusillus* LMB4 enzymatic extract at different pH values. B) pH stability assay performed with the crude enzymatic extract from *D. pusillus* LMB4. Laccase activity was evaluated at 40°C under different pH conditions, circles pH3, squares pH 4 and triangles pH 5.

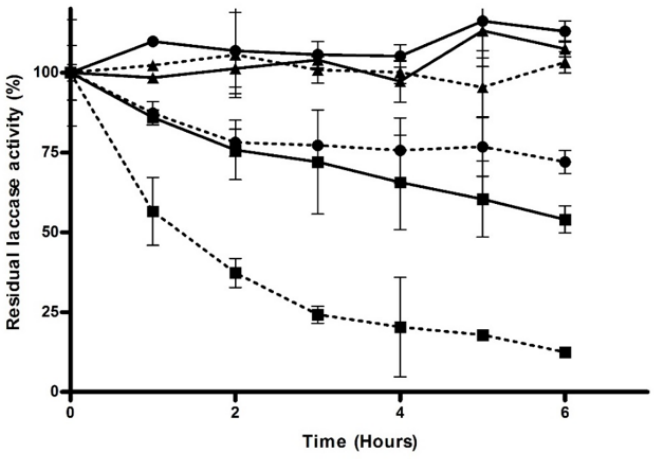

Figure 4. Thermal stability evaluation of a *D. pusillus* LMB4 extract exhibiting laccase activity. Laccase enzymatic activity was evaluated after different temperature incubations, 40°C (triangles), 50°C (circles), and 60°C (squares). Solid lines represent the crude enzymatic extract from *D. pusillus* LMB4 while dashed lines represent the commercial laccase from *T. versicolor*, 53739.

3.5 Investigation of *D. pusillus* LMB4 laccases

Given the striking lignolytic activity of *D. pusillus* LMB4, its DNA was sequenced by long-reads SMRT sequencing, thus allowing annotation and analysis of the encoded laccases in its genome. It was possible, after *de novo* assembly, to find a genome with a level of heterozygosity estimated at 13.53%. The reduction in homozygous genome allowed to find an assembly of XXXX bp distributed in 3463 contigs (N50 = 23741 bp). A total of 16866 CDSs (after the splicing of the 95174 annotated introns) was predicted to be encoded in the genome of *D. pusillus* LMB4.We were able to annotate confidently 13 CDSs as putative complete laccases, To each putative protein sequences founded were checked with the protein-BLAST tool at NCBI server to find a correlation with other laccase enzymes reported at DBP, and the sequences were alignment between them to identified new consensus regions. Also, the four conserved copper-binding motifs: Cu1 (HWHGFFQ), Cu2 (HSHLSTQ), Cu3 (HPFHLHG), and Cu4 (HCHIDFHL) (Kumar, Phale et al. 2003), were searched into the putative protein sequences obtained (Table 1).

Locus tag	Length a.a.	Identity %	Query cove %	Multicopper oxidase signatures		Cu-oxidase Pfam domains			Copper-binding motifs				Genbank accesion	Organism
				PS00079	PS00080	PF00394	PF07731	PF00394	Cu 1	Cu 2	Cu 3	Cu 4		
G253.t1	553	57,35		X	x	x	x	x	x	x	x		AAR03582.1	*Volvariella volvacea*
G3223.t1	535	57,31		X	x	x	x	x	x	x	x		KDR80952.1	*Galerina marginata*
G5839.t1	146	70,42				x							CBV46340.1	*Meripilus giganteus*
G6228.t1	508	57,71			x	x	x	x	x		x	x	XP_001874989.1	*Laccaria bicolor*
G6430.t1	576	50,28		X	x	x	x	x	x				CBV46340.1	*Meripilus giganteus*
G8544.t1	696	59,11		X	x	x	x	x	x	x			XP_001874989.1	*Laccaria bicolor*
G12653.t1	545	63,85		X	x	x	x	x	x	x	x		XP_007334124.1	*Agaricus bisporus*
G12662.t1	529	57,48		X	x	x	x	x	x	x	x		KDR80952.1	*Galerina marginata*

G16121.t1	580	54,84	X	x	x	x	x	x	x	x		XP_001874989.1	*Laccaria bicolor*
G16512.t1	558	60,79	X		x	x	x	x	x	x		XP_001874989.1	*Laccaria bicolor*
G16540.t1	540	60,44	X	x	x	x	x	x	x	x	x	AFD97050.1	*Coprinus comatus*
G503.t1	733	38,96			x	x	x	x				XP_007301460.1	*Stereum hirsutum*
G1684.t1	531	53,5	X	x	x	x	x			x		AGO04563.1	*Lentinula edodes*
G2824.t1	638	70,92	X	x	x	x	x	x	x	x		KDR68425.1	*Galerina marginata*
G3514.t1	249	29,07		x								XP_007301460.1	*Stereum hirsutum*

3.6 Simultaneous pretreatment and saccharification of EFB

Fiber analysis of palm empty fruit bunch revealed that they are composed of 77.53% NDF, 77.53%, 58.32% ADF and 17.15% ADL (see methods for details). These values indicate that the EFB composition of lignocellulosic polymer used for the SSF was 40.79% cellulose, 19.21% hemicellulose, 17.15% lignin, and 22.47% impregnated oil and ashes. These results are in accordance with typical reported EFB composition, with cellulose being the main component, followed by hemicellulose and lignin (Chang, 2014).

Reducing sugar release was observed when the cellulolytic enzymatic extract from *T. reesei* was used alone (20.84 ± 0.7 $g.g^{-1}$). Higher reducing sugar release from EFB was observed when the cellulolytic enzymatic extract from *T. reesei* was used with the commercial laccase enzyme from *T. versicolor* (46.47 ± 5.9 $g.g^{-1}$) or the enzymatic extract from *D. pusillus* (44.80 ± 5.21 $g.g^{-1}$), confirming that ligninolytic enzymes such as laccases favor cellulose hydrolysis, as previously shown (Davidi et al., 2016; Mukhopadhyay, Kuila, Tuli, & Banerjee, 2011). These results suggest that a mix of cellulolytic and ligninolytic enzymes enhance the release of reducing sugars. However, production of reducing sugars was not significantly different when the commercial laccase from *T. versicolor* or enzymatic extracts from *D. pusillus* were mixed with the cellulolytic enzymatic extract from *T. reesei* (Fig. 5).

To identify the dominant variables affecting the reducing sugar release, we compared the effect of pH, temperature, copper concentration, and the concentration of laccase ($U.L^{-1}$) and cellulase (UPF) by a Plackett-Burman analysis (P value < 0.05 with a confidence level of 95%). In both cases (SPS with laccase from *T. versicolor* and *D. pusillus*) the pH is the dominant variable, followed in second place by temperature and copper concentration. Where the high pH values and temperatures up to 45˚C promotes the increment of sugar release by SPS. These results agree with what was observed in the stability experiments, where the activity of the enzymatic extract is compromised at pH lower than 4 (Fig.3B). And it has been proven that the laccase activity at basic pH in a biotechnological process is a desirable characteristic, due that the process at pH low values increase degradation of the enzymes (Margot et al., 2013).

It is worth to mention that for SPS with the commercial laccase, the cellulase is the third most important factor (Fig.6A), while on the other hand, for the enzymatic extract of *D. pusillus* it is not (Fig.6B). The requirement of a cellulase activity in the case of the commercial laccase to compete with the results obtained from the *D. pusillus* extracts see Fig.6A; perhaps due that the production of ligninolytic enzymes use to be accompanied by cellulolytic enzymes production in basidiomycete fungi during wood decay process (Zhang et al., 2016; Presley & Schilling, 2017) Also some author had been demonstrated the efficiency of enzymatic extracts from basidiomycete due to the production of ligninolytic and cellulolytic enzymes in SSF using wheat straw as substrate (Xu, et al., 2018). Highlight the importance of *D. pusillus* as an efficient, accessible and cheap source of relevant biotechnological assets in the field of delignification processes.

The highest reducing sugar concentration for the enzymatic extract with laccase activity from *D. pusillus* was 65.87 $g.g^{-1}$ at a pH value of 4.5, 45°C and with a 2:1 ratio of laccase to cellulase enzymatic units. For the commercial laccase from *T. versicolor*, reducing sugar production reached 64.13 $g.g^{-1}$ in the same conditions. These results confirm that the enzymatic extract from *D. pusillus* exhibits similar ligninolytic efficiency than the purified commercial laccase from *T. versicolor.* We want to point out that the EFB is a good lignocellulose source to obtain reducing sugars, due that during the palm oil extraction, the palm oil bunches have a previous *"sterilization"* process (to obtain the oil palm fruits from the bunch), which could be considered as a pretreatment. Such first pretreatment might improve the later delignification process performed by enzymes.

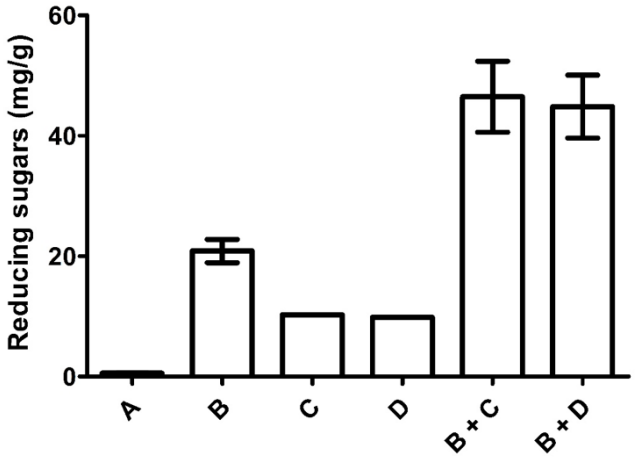

Figure 9. Comparative production of reducing sugars from EFB using enzymatic extracts alone and mixes between them. A) without any enzymatic extract; B) cellulolytic extract from *T. reesei*; C) commercial laccase from *T. versicolor*; D), enzymatic extract with laccase activity from *D. pusillus*.

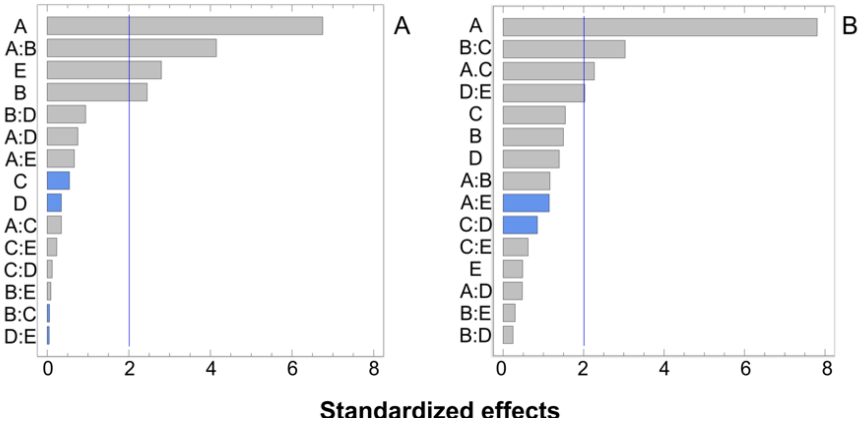

Figure 6. Pareto charts from Plackett-Burman multilevel analysis. A) Cellulolytic extract from *T. reesei* mixed with commercial laccase from *T. versicolor.* B) Cellulolytic extract from *T. reesei* mixed with enzymatic extract from *D. pusillus.* Parameters: A, pH; B, temperature; C, copper concentration; D, $U.L^{-1}$ of laccase and E, UPF of cellulase. **Note:** The blue line represents the statistically significant threshold of 95% confidence. And grey and blue bars highlight positive and negative effects respectively.

4. Conclusion

This study demonstrates that a crude enzymatic extract from a wild Colombian source of *D. pusillus* LMB4 exhibits significant laccase activity (267 ± 18 $U.L^{-1}$). This crude enzymatic extract was probed for the successful pretreatment of low-cost lignocellulosic raw materials (oil palm by-products), suggesting that an upscaling of this process could help with the delignification of starting materials in cellulosic bioethanol production. An increase in copper and glucose concentration during solid-state fermentation proved beneficial, resulting in a 12-fold increase in laccase activity and suggesting that ligninolytic enzyme expression can further be induced to improve enzyme production in *D. pusillus* LMB4. The genome sequencing of *D. pusillus* LMB4 revealed 13 laccasses that explain the high

lignolytic activity. The SPS of EFB also showed that the enzymatic extract from *D. pusillus* exhibits good ligninolytic capacity at basic pH values, in addition to demonstrating higher pH and thermal stability than the purified commercial laccase from *T. versicolor*. These properties depict the enzymatic extract with laccase activity from *D. pusillus* as a very good candidate for future protein engineering applications, aiming to provide a suitable biotechnological tool for lignocellulose pretreatment such as for cellulosic bioethanol production.

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